Regulation of the translational inhibitor 4E-BP1 by phosphorylation

by Anne-Claude Gingras

Department of Biochemistry
McGill University
Montréal, Québec, Canada,
August 2001

A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

À mes parents et à Bri,

cette thèse je dédie.

ABSTRACT

In most instances, translation is regulated at the initiation phase, when a ribosome is recruited to the 5' end of an mRNA. The eIF4E-binding proteins (4E-BPs) interdict cap-dependent translation initiation by binding to the translation factor eIF4E, and preventing recruitment of the translation machinery to mRNA. The 4E-BPs do not inhibit cap-independent translation, such as that driven by picornavirus IRESes. The 4E-BPs inhibit translation in a reversible manner. Hypophosphorylated 4E-BPs interact avidly with eIF4E, whereas 4E-BP hyperphosphorylation, elicited by stimulation of cells with hormones, cytokines, or growth factors, results in an abrogation of eIF4E binding activity. Here I show that upon infection of cells with encephalomyocarditis virus (EMCV) or poliovirus, 4E-BP1 becomes dephosphorylated, and binds more strongly to eIF4E. This dephosphorylation could play a role in the shutoff induced by EMCV. Conversely, infection with adenovirus results in hyperphosphorylation of 4E-BP1 and 4E-BP2 early in infection, at a time when viral protein synthesis proceeds via a cap-dependent mechanism. I also showed that the pathway leading to 4E-BP1 phosphorylation is dependent upon the activity of PI3 kinase, Akt/PKB and FRAP/mTOR. I then analyzed the phosphorylation of individual phosphorylation sites. Thr37 and Thr46 are phosphorylated by FRAP/mTOR immunoprecipitates. *In vivo*, a high stoichiometry of phosphorylation on these sites is observed in conditions of serum deprivation, or following treatment of cells with PI3K or FRAP/mTOR inhibitors. Phosphorylation on these sites is, however, required for the subsequent phosphorylation of the serum-sensitive sites, which I identified as Ser65 and Thr70. Phosphorylation of 4E-BP1 can be further ordered: phosphorylation of Thr37 and Thr46 precedes that of Thr70, which occurs prior to Ser65 phosphorylation. Phosphorylation of a combination of sites is required to mediate release from eIF4E, as phosphorylation of either Thr37 and Thr46 in combination, or phosphorylation of Ser65 and Thr70 together, is insufficient to mediate release. Taken together, these results have uncovered a complex mechanism for the regulation of 4E-BP1 binding to eIF4E by phosphorylation.

RÉSUMÉ

Dans la plupart des situations, la traduction est régulée à la phase d'initiation, qui consiste à recruter un ribosome à l'extrémité 5' (ou extrémité en coiffe) d'un ARNm. Des protéines (appelées 4E-BPs), qui interagissent avec le facteur d'initition de la traduction eIF4E, inhibent la traduction dépendante de la coiffe, mais non la traduction indépendante de la coiffe, comme celle des ARNms des picornavirus. L'inhibition de la traduction par les 4E-BPs est réversible. Dans leur état hypophosphorylé, les 4E-BPs lient fortement eIF4E, alors que les formes hyperphosphorylées sont incapables d'interagir avec eIF4E. J'ai démontré qu'à la suite d'infections avec des picornavirus (poliovirus et encephalomyocarditis virus), 4E-BP1 est déphosphorylé et lie avidement eIF4E. Dans le cas d'infections par EMCV, cette déphosphorylation corrèle temporellement avec l'inhibition de la synthèse protéique de l'hôte causée par le virus. La déphosphorylation de 4E-BP1 n'est pas observée suite à l'infection par adénovirus: au contraire, 4E-BP1 et 4E-BP2 deviennent hyperphosphorylés tôt lors de l'infection. Il a été reporté que la phosphorylation de 4E-BP1 est augmentée suite à une stimulation des cellules avec des hormones, des cytokines, ou des facteurs de croissance. J'ai démontré que la phosphorylation de 4E-BP1 dépend de l'activité des kinases PI3K, Akt/PKB et FRAP/mTOR. L'analyse de la phosphorylation de 4E-BP1 sur différent sites m'a permis de révéler une régulation complexe de la phosphorylation de cette protéine. Deux sites, Thr37 and Thr46, sont phosphorylés par une protéine FRAP/mTOR immunoprécipitée. Ces sites sont phosphorylés dans les cellules à de très hauts niveaux, même en l'absence de facteurs de croissance. De plus, la phosphorylation de ces sites n'est que peu affectée par le traitement avec des inhibiteurs de PI3K ou de FRAP/mTOR. La phosphorylation de Thr37 et Thr46 est toutefois cruciale pour la régulation de l'activité de 4E-BP1, puisqu'elle est requise pour la phosphorylation d'un autre groupe de sites (Ser65 et Thr70), qui, eux, sont sensibles à la présence de facteurs de croissance et dépendent de l'activité de PI3K et de FRAP/mTOR. En fait, la phosphorylation de 4E-BP1 est très hiérarchique: la phosphorylation de Thr37 et de Thr46 précède celle de Thr70 qui, elle-même, est prérequise pour la phosphorylation de Ser65. Des études *in vitro* nous ont aussi permis de conclure que plusieurs de ces sites doivent être phosphorylés afin de permettre la dérépression de eIF4E.

PREFACE

This thesis consists of several published papers for which I am either first author or co-first author. The results presented in chapter 5 have been submitted for publication.

Chapter 2
Gingras, A.-C., Svitkin, Y., Belsham, G. J., Pause, A. and Sonenberg, N. (1996) *Proc. Natl. Acad. Sci. U S A.* 93: 5578-5583.
Gingras, A.-C. and Sonenberg, N. (1997) *Virology.* 237: 182-186.

Chapter 3
Gingras, A.-C., Kennedy, S. G., O'Leary, M. A., Sonenberg, N. and Hay, N. (1998) *Genes Dev.* 12: 502-513.

Chapter 4
Gingras, A.-C., Gygi, S. P., Raught, B., Polakiewicz, R. D., Abraham, R. T., Hoekstra, M. F., Aebersold, R. and Sonenberg, N. (1999) *Genes Dev.* 13: 1422-1437.

Chapter 5
Gingras, A.-C., Raught, B., Gygi, S. P., Niedzwiecka, A., Miron, M., Burley, S. K., Polakiewicz, R. D., Wyslouch-Cieszynska, A., Aebersold, R. and Sonenberg, N. (2001) *submitted*

I wish to acknowledge the work of my co-authors for each of the chapters, and thank all of them for their remarkable collaboration, support and assistance. I have done my best to summarize the role of each co-author in the work presented, but in several instances, it has been difficult to draw (succinctly) a margin. In addition to the co-authors, numerous kind souls have provided reagents for these studies. Their contribution is acknowledged at the end of each chapter.

Chapter 2.

Yuri Svitkin performed the encephalomyocarditis infection of the KrebsII ascites; he also pulse-labeled the ascites with [^{35}S]methionine and performed the *in vitro* translation shown in Fig. 2.2.

Arnim Pause cloned 4E-BP1 into an expression vector for bacterial expression. Graham Belsham guided me at the beginning of this project.

Chapter 3.

I am co-first author with Scott Kennedy on this manuscript. Scott Kennedy, Maura O'Leary and Nissim Hay generated the stable cell lines overexpressing myristylated Akt used in these study. In addition, Scott and Maura performed several of the transient transfections depicted in Figures 1, 2, 3 and 7. Scott also performed the *in vitro* kinase assay for Akt. Nissim Hay, the senior author, wrote large portions of the introduction and discussion of this paper.

Chapter 4.

Steven P. Gygi determined by mass spectrometry the sequence of the phosphopeptides of 4E-BP1. Brian Raught and I performed together some of the large scale labelings necessary for the identification of the phosphopeptides. Brian also helped with some of the other *in vivo* labelings, and the preparation of some of the tryptic maps. Roberto D. Polakiewicz and his group at New England Biolabs generated and purified the phosphospecific antibody to Thr37/Thr46 (I tested the antibody's activity under different conditions). Merl F. Hoekstra and his group supplied me with constructs of his-tagged mouse 4E-BP1 mutants and with a baculovirus-expressed, purified flag-FRAP/mTOR. Robert T. Abraham provided the polyclonal antibody to FRAP/mTOR. Brian helped with the writing of the manuscript.

Chapter 5.

I am co-first author with Brian Raught on this paper. Brian and I performed the large scale [^{32}P] labeling and several of the other experiments described together. Brian performed most of the transfections, as well as some of the 2D gels and phosphopeptide maps. Steven P. Gygi determined by mass spectrometry the sequence of the phosphopeptides. Anna Niedzwiecka

performed the fluorescence spectroscopy measurement of affinity, and Aleksandra Wyslouch-Cieszynska synthesized the phosphopeptides used in this study. Mathieu Miron helped with the cloning of the 4E-BP1 Ser65Ala for mammalian expression. Roberto D. Polakiewicz and his group at New England Biolabs generated and purified the phosphospecific antibodies to Ser65 and Thr70 (I tested the antibodies' reactivity). Brian helped with writing of the manuscript.

Chapters 1 and 6

Parts of Chapters 1 and 6 are reproduced from several reviews which I co-authored with Brian Raught, Steven Burley and Nahum Sonenberg. They were published as follows:

Gingras, A.-C., Raught, B. and Sonenberg, N. (1999) *Annu. Rev. Biochem.* 68: 913-963.

Gingras, A. C., Raught, B. and Sonenberg, N. (2001) *Genes Dev.* 15: 807-826.

Gingras, A.-C., Raught, B. and Sonenberg, N. (2001) *In Prog. Mol. Subcell. Biol.* R. E. Rhoads, eds. Springer-Verlag, Berlin. 143.

Raught, B. and Gingras, A.-C. (1999) *Int. J. Biochem. Cell Biol.* 31: 43-57.

Raught, B., Gingras, A.-C. and Sonenberg, N. (2000) *In Translational control of gene expression.* N. Sonenberg, J. W. B. Hershey and M. B. Mathews, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 245-294.

Raught, B., Gingras, A.-C. and Sonenberg, N. (2001) *Proc. Natl. Acad. Sci. U S A.* 98: 7037-7044.

Sonenberg, N., Burley, S. K. and Gingras, A.-C. (1998) *Nat. Struct. Biol.* 5: 172-174.

Sonenberg, N. and Gingras, A.-C. (1998) *Curr. Opin. Cell. Biol.* 10: 268-275.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

ACKNOWLEDGMENTS

I wish to first thank all of my collaborators, both in the Sonenberg laboratory and outside which made this work not only possible, but also "fun" to do. From the Sonenberg lab, in chronological order, Arnim Pause, Graham Belsham, Yuri Svitkin, Denis Rousseau, Alessandra Gradi, Hiroaki Imataka, Eran Rom, Kyoko Kohara, Francis Poulin, Mathieu Miron, Shigenobu Morino, Stéphane Pyronnet and Brian Raught (I'll come back to you later) have all been great friends and taught me a lot! Many thanks also to members of the Branton lab, especially to Steve Whalen, who taught me all I know about phosphorylation, and who is one of the greatest persons I've ever met. I would like to also thank several other McGill and Montreal scientists for advice, in particular Sylvain Meloche, with whom we had a fruitful collaboration. I am indebted to George Thomas and Sabina von Manteuffel for their role in delineating the signaling pathway to 4E-BP1, and to Aleksandra Wyslouch-Cieszynska (a.k.a Ola) and Anna Niedzwiecka for help with phosphopeptide synthesis and affinity measurements. The work on Akt/PKB would not have been possible without the leadership of Nissim Hay, and the help of Scott Kennedy. Thanks to Eric Brown, Merl Hoekstra, Jie Chen, Bob Abraham, David Sabatini, Andrew Peterson and all of the FRAP/mTOR community for helpful discussions and various reagents. Thanks to all the scientists who provided reagents for our studies, or whom I had the pleasure to assist. Thanks to the structural people for allowing me to live an exciting moment in eIF4E history: Stephen Burley, and Joe Marcotrigiano (crystal structure), and Gerhard Wagner, Hiroshi Matsuo, and Mark Fletcher (NMR). Many, many thanks to Roby Polakiewicz for valuable phosphospecific antibodies, and even more valuable advice. Finally, the mass spectrometry determination of all the phosphopeptide sequences was made possible only through a fruitful collaboration with the group of Ruedi Aebersold, and especially with Steve Gygi. (I liked working with Ruedi so much, I'll do my postdoc with him.)

A special thanks to Colin Lister for everything, in particular for keeping the lab running. Is there any way I could convince you to come work in Seattle? Thanks also to Fabienne and Chantal for technical assistance, and to sweet Meena for pampering me. I also want to thank all the friends I made in the lab and in the department over the numerous years (You know who you are). Thanks for your support, your friendship... and your beer.

Thanks to Nahum for his amazing patience (e.g. reading my first paper 26 times before we sent it), the words of wisdom, and for the rides home (they beat LaRonde any day). Thanks also for teaching me the rudiments of English, and (finally) letting me finish my Ph.D. Furthermore, I am grateful for the trust you had in me, and for helping me to become (I hope) a better scientist. I will never forget you (I'm sure you won't let me anyway!).

I wouldn't be able to say in a few words how much I am indebted to Brian, and how lucky I am to have you both as a collaborator and as a "sweetie-pie". Thanks for not strangling me during writing sprees, and for reading and improving everything I wrote over the past few years (including this thesis). Also thanks for being such a dedicated and passionate scientist: you are great to work with. Finalement, je tiens à remercier toute ma famille, et en particulier mes parents Nicole et Paul qui m'ont inculqué une soif de connaissance qui m'a prédisposée à la science. Merci !

CHAPTER 1

GENERAL INTRODUCTION

1.1 Overview of translational control

Regulation of protein synthesis in eukaryotes plays a critical role in development, differentiation, cell cycle progression, cell growth, and apoptosis (1). Translational control allows for a more rapid response than transcriptional modulation because no mRNA synthesis, processing or transport is required, and can be used to coordinate gene expression in systems that lack transcriptional regulation, such as reticulocytes or platelets (1, 2). Translational control plays a particularly important role in early developmental processes, when localized translation is utilized to establish polarity (3), and localized translation in neurons may be critical for learning and memory (e.g. 4). Following transcription, processing and nucleocytoplasmic export, mRNAs are competent for translation. However, two transcripts present in identical quantities may be translated at very different rates. This phenomenon is due, in part, to the fact that the ribosome does not bind to mRNA directly, but must be recruited to mRNA by the concerted action of a large number of eukaryotic translation initiation factors (eIFs). This recruitment step, also referred to as the initiation phase, is a complex process that culminates in the positioning of a charged ribosome (that is, an 80S ribosome loaded with an initiator tRNA in its P site) at an initiation codon (5). As discussed further below, the ribosome recruitment process is rate-limiting for translation in many cases, and is subject to exquisite regulation.

In this introduction, I will first briefly review the mRNA structural elements and the general process of translation initiation. A description of the structure-function relationships of the initiation factors involved in ribosome recruitment is also provided, followed by a comparison of several models of ribosomal recruitment in eukaryotes. Examples of translational control at the level of ribosome binding will be provided, followed by a discussion on the regulation of the activity of the translation factors involved in this process. Importantly, the structure, function and regulation of a family of

inhibitory proteins regulating this translation step will be reviewed in detail. Regulation by phosphorylation of one of these proteins, 4E-BP1, is the topic of this thesis.

1.2 Anatomy of an mRNA

In addition to the coding sequence, eukaryotic mRNAs possess noncoding sequences in the 5' and 3' untranslated regions that determine translational efficiency.

1.2.1 Cap Structure

The structure m^7GpppN (or the "cap", where m is a methyl group and N any nucleotide) is present at the 5' end of all nuclear transcribed mRNAs (6). The cap facilitates translation of most cellular mRNAs, through specific interaction with the initiation factor eIF4E (see section 1.4). Uncapped mRNAs generally direct protein synthesis at lower efficiency than their capped counterparts in *in vitro* translation systems (7, 8). In addition to its role in translation, the cap is also important for other cellular processes, including pre-mRNA splicing, nucleocytoplasmic transport of small nuclear RNAs (and possibly mRNA), and mRNA stability (reviewed in 9). Certain viral RNAs, including those of picornaviruses, caliciviruses, and some plant viruses, lack a cap, and are translated by alternative mechanisms (see section 1.5).

1.2.2 Poly(A) Tail

The majority of eukaryotic mRNAs possess a 3' poly(A) tail, which ranges from 50 bases in length in yeast to more than 200 bases in length in higher eukaryotes (10). The poly(A) tail plays an important role in determining both mRNA stability (11) and translational efficiency. The translational role of the poly(A) tail is mediated through the poly(A) binding protein PABP. mRNAs that do not possess a poly(A) tail include most histone mRNAs and some viral RNAs (reovirus, rotavirus, and others; 12). The poly(A) tail synergizes with the cap in translation (reviewed in 13).

1.2.3 cis-Acting Elements

Various cis-acting elements in the untranslated regions (UTRs) of an mRNA can modulate translational efficiency, including (a) the sequence flanking the initiation codon (start site "context"; 14), (b) secondary structure in the 5' UTR (15, 16, 17; see section 1.6), (c) upstream open reading frames (18), (d) binding sites for specific regulatory proteins (19, 20), (e) oligopyrimidine tracts at the extreme 5' terminus (so-called 5' TOP mRNAs; see section 1.6 and 21), and (f) IRESes (see section 1.5.2 and 22, 23, 24).

1.3 General mechanism of translation initiation

Initiation commences with the dissociation of a preexisting 80S ribosome, possibly promoted by eIF6 (see, however, evidence to the contrary; 25) , which binds to the 60S ribosomal subunit, and eIF3 and eIF1A, which bind to the 40S ribosomal subunit (Fig. 1.1, step 1). Joining of an eIF2-GTP-Met-tRNAi ternary complex to the 40S subunit forms a 43S preinitiation complex (step 2). The 43S complex is competent to bind to mRNA, but it requires a set of factors termed the eIF4 group, as well as the hydrolysis of ATP, to assemble on the mRNA 5' end (step 3). The mechanism of action and regulation of the eIF4 polypeptides will be discussed in detail in section 1.4. The 40S subunit, with its associated cohort of initiation factors, is thought to traverse the mRNA 5' UTR in a linear and processive fashion in a 5'-to-3' direction (in a process termed scanning; see section 1.5.1.1), until it encounters an initiation codon in a favorable sequence context (14, 23). A 48S initiation complex is then formed at the start codon (step 4). Scanning from the 5' end is not the only mechanism by which a ribosome can access mRNA and proceed to an initiation codon; alternative initiation mechanisms will be discussed in section 1.5. Once an initiation complex reaches a start codon, and a codon/anticodon interaction established, the associated initiation factors are released in a process mediated by a GTPase-activating protein (GAP), eIF5, which promotes GTP hydrolysis by eIF2

(step 5). Dissociation of the initiation factors allows for joining of the 60S subunit (a step catalyzed by eIF5B) and the beginning of polypeptide elongation (step 6).

The rate-limiting step in translation initiation under most circumstances is binding of the 43S preinitiation complex to mRNA (Figure 1.1, step 3; for details see 1, 26). This step is a primary target for translational control.

1.4 eIF4 initiation factors: structure and function

The eIF4 initiation factors (eIF4F, eIF4A, and eIF4B) are responsible for recruiting a ribosome to the mRNA. eIF4F is a complex of three polypeptides: (a) eIF4A, an RNA-dependent ATPase and RNA helicase; (b) eIF4E, a 24-kDa cap-binding protein; and (c) eIF4G, a large polypeptide containing binding sites for eIF4E, eIF4A, eIF3, PABP, and the kinase MnkI. eIF4F interacts with both the cap (through eIF4E) and the ribosome-associated eIF3 (through eIF4G). Thus, eIF4F executes the pivotal function of bridging the mRNA and the ribosome. The other molecular interactions mediated through eIF4G are critical in translation and/or serve in translational regulation. The role of the various eIF4 factors in mediating ribosome recruitment is depicted in Fig. 1.2, and is summarized below.

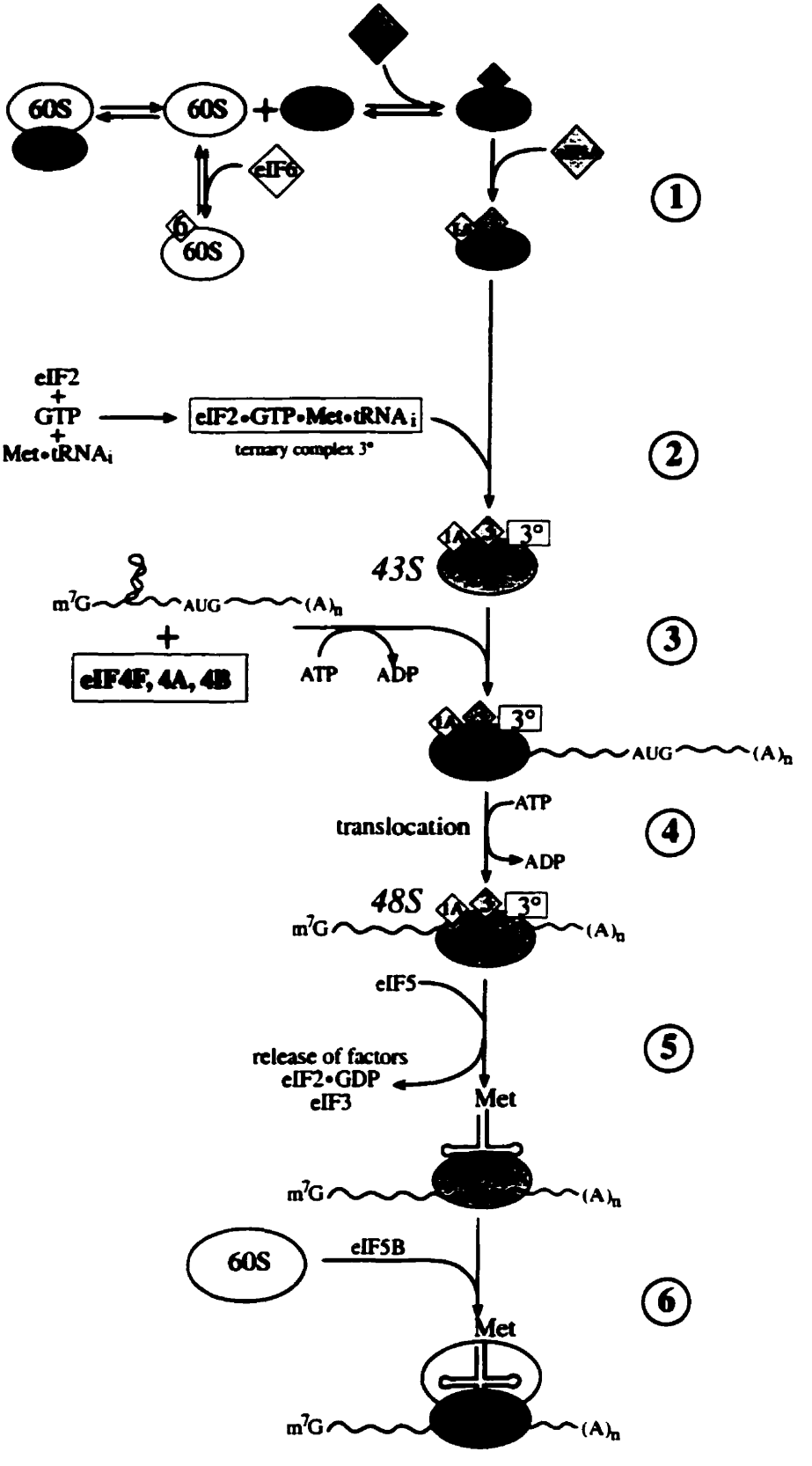


Figure 1.1 Mechanism of translation initiation. Adapted from Gingras et al. Annu Rev. Biochem. (1999). See text for details.

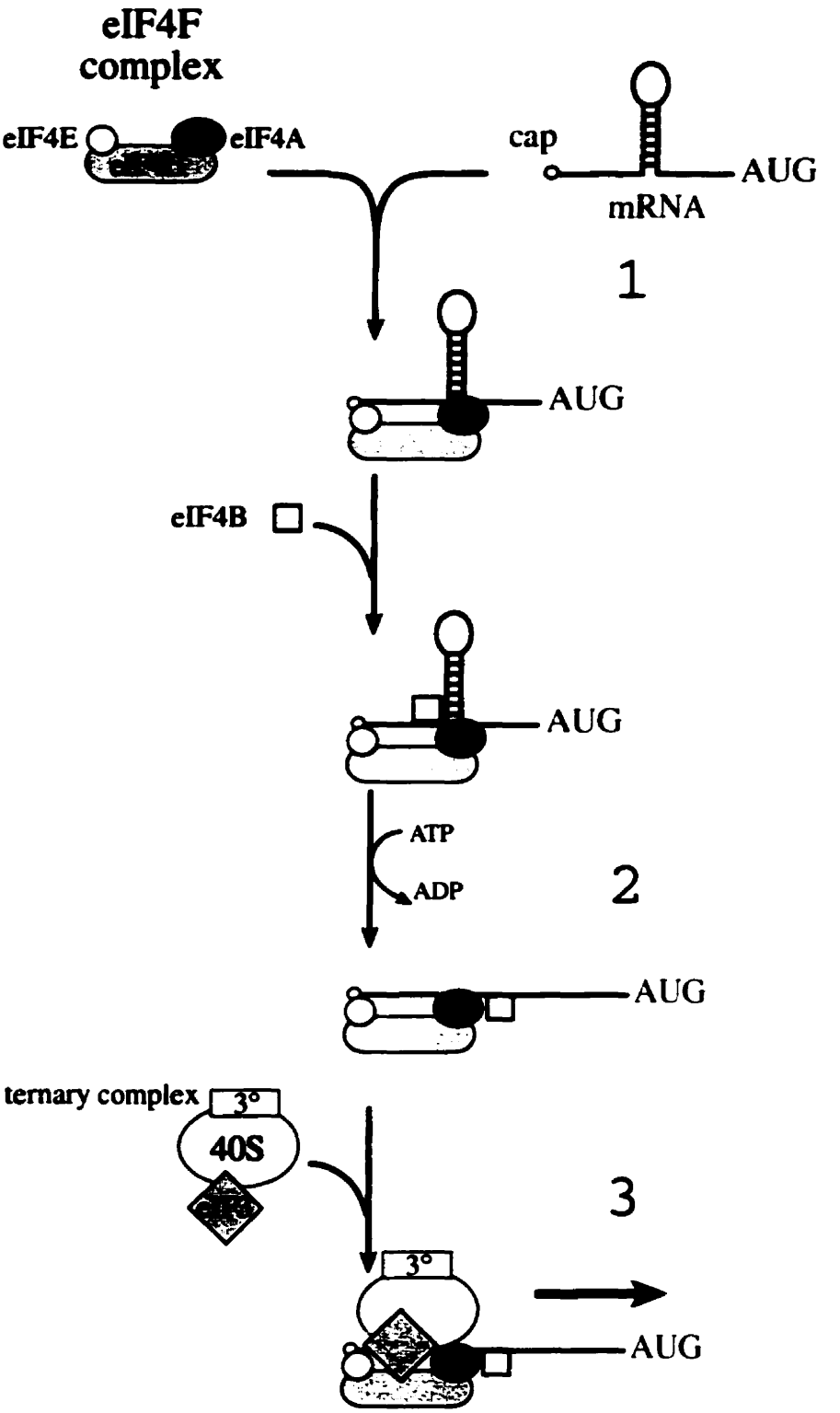


Figure 1.2. Ribosome recruitment. Through eIF4E, eIF4F binds to the mRNA 5' cap structure. eIF4A, along with the co-factor eIF4B, unwinds cap-proximal mRNA secondary structure in an ATP-dependent manner. The 40S ribosomal subunit bound to eIF3 (and to the ternary complex consisting of eIF2, GTP and Met-tRNAi) is recruited to the single-stranded mRNA, through eIF4G.

1.4.1 eIF4E

eIF4E was identified by its ability to cross-link to the cap (27) and was subsequently purified by affinity chromatography on a cap-containing matrix (28). eIF4E mediates cap recognition in initiation and is essential for cap-dependent translation. Depletion of eIF4E (and associated proteins) from cell-free extracts drastically reduces translation of capped mRNA; full activity is restored by addition of bacterially expressed or purified native eIF4E (29). eIF4E is conserved across species: eIF4E proteins from plants, *Drosophila*, and *Aplysia*, among others, share striking homology to mammalian eIF4E. *Saccharomyces cerevisae* and human eIF4E are 32% identical, and mammalian eIF4E can rescue the lethality caused by disruption of the yeast eIF4E gene (30).

The three-dimensional structure of mouse eIF4E was solved by X-ray crystallography, and that of yeast eIF4E by NMR (Fig. 1.3A and B; 31, 32). Both proteins possess the same overall organization, resembling a cupped hand (or baseball glove) and consisting of a single α/β domain composed of an eight-stranded, antiparallel curved β-sheet, backed on its convex surface by three long α-helices. The modes of cap binding utilized by yeast and mouse eIF4E are almost identical, and all of the residues involved in cap-binding are conserved from yeast to mammals. m^7GDP occupies a narrow slot on the concave surface of eIF4E, where m^7G binding is provided by π–π stacking interactions between the base and the indole side chains of two tryptophans (Trp56 and Trp102 in mouse, Trp58 and Trp104 in yeast eIF4E) (31, 32; Fig. 1.3C). For simplicity, subsequent numbering will be given only for mammalian (human/mouse) eIF4E. This binding is coordinated by hydrogen bonds involving Glu103 (the carboxylate oxygen atoms act as acceptors for N-1 and N-2 hydrogens of the guanine ring) and the backbone amide of Trp102 (which is a hydrogen bond donor for O-6 of the guanine ring). A van der Waals contact between the N-7 methyl group of the guanine ring and Trp166 further coordinates these interactions. Several direct and water-mediated hydrogen bonds between amino

acids and phosphate groups of the cap also contribute to the binding. Positively charged residues in the cap-binding slot (Arg112, Arg157, and Arg162) coordinate the negatively charged oxygen atoms of the phosphate moieties in m^7GDP.

A human structural eIF4E homolog of unknown function, termed 4EHP (30% identity to eIF4E at the amino acid level) also interacts with the cap, despite the fact that one of the tryptophans (Trp56) involved in cap binding in mouse eIF4E is replaced by a tyrosine in 4EHP (33). 4EHP thus binds to the cap via sandwiching of the m^7G ring between a tyrosine (Tyr78) and a tryptophan (Trp124), indicating that aromatic residues other than tryptophan can participate in cap binding (34). This mode of cap binding (π–π electron stacking between aromatic side chains) is also found in a structurally unrelated protein, VP39 methyltransferase, a bifunctional protein from vaccinia virus that methylates the 2' hydroxyl of the ribose in the cap and also acts as a processivity factor for the viral poly(A) polymerase (35, 36). In this case, the side chains that sandwich the m^7G ring are a tyrosine and a phenylalanine; Fig. 1.3D (37). Thus, the mechanism of cap binding appears to be highly conserved amongst proteins of different origins and functions (34) and could be utilized, for example, for recognition of the cap by nuclear cap-binding proteins (38) and/or by the decapping enzyme (39).

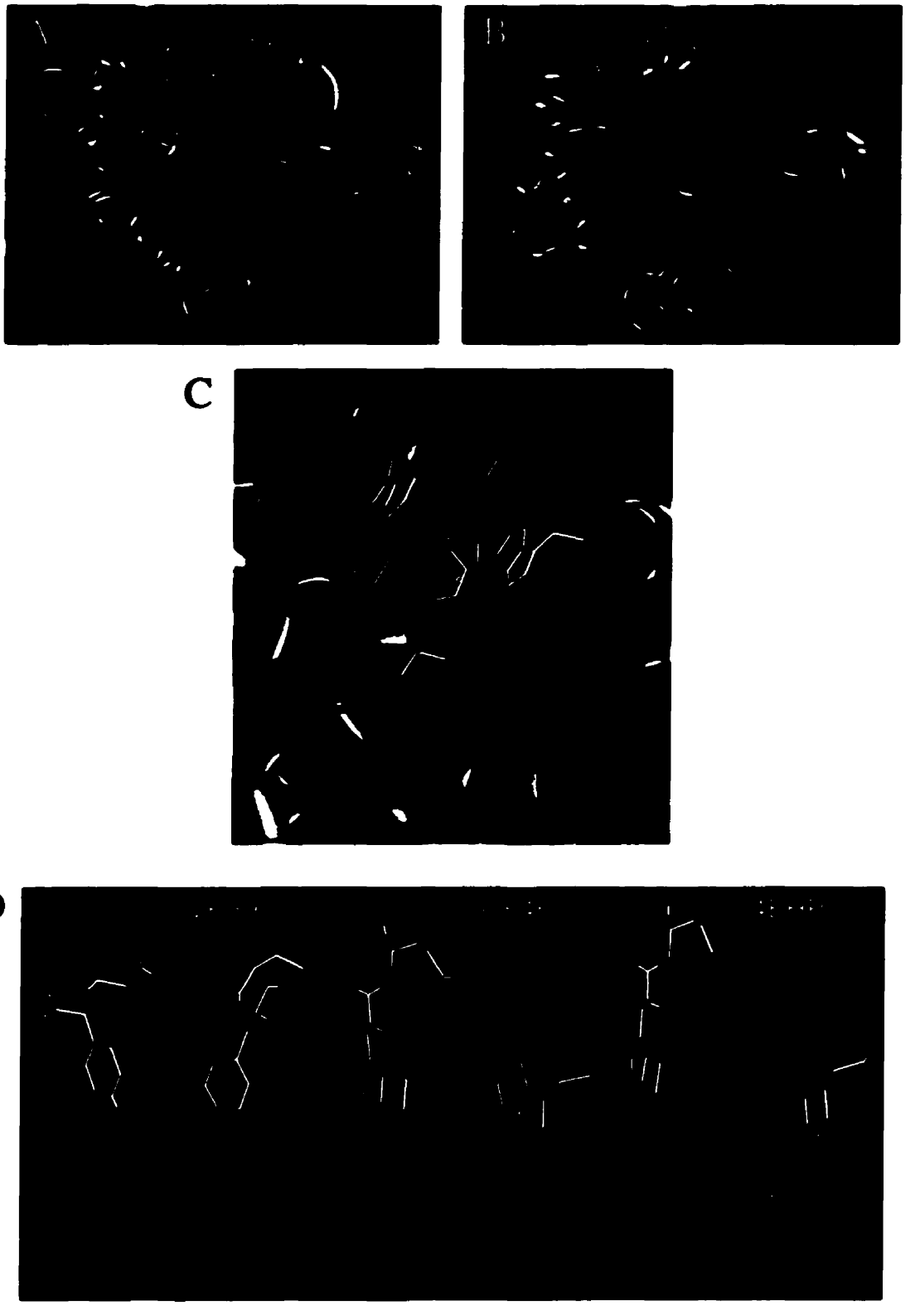


Figure 1.3. Structure of eIF4E and cap recognition. (A-C) Three-dimensional structure of eIF4E (blue) bound to m7GDP cap analog (yellow). (A) Ribbon diagram of the mouse eIF4E structure determined by X-ray crystallography; (B) ribbon diagram of the yeast eIF4E structure determined by NMR; (C) magnified view of the cap-binding area of mouse eIF4E. The amino acid side chains involved in the interaction are highlighted. (D) Comparison of the cap recognition by VP39, eIF4E and 4E-HP.

1.4.2 eIF4G

The physical "bridging" of ribosomes to mRNA in mammalian cells is coordinated primarily by the large molecular scaffolding proteins, eIF4GI and eIF4GII, which are 46% identical and possess predicted molecular masses of 171 kDa and 176 kDa, respectively (40).

The use of viral proteases, which cleave the mammalian eIF4GI protein into three fragments (amino-terminal, middle, and carboxy-terminal; 41), together with biochemical and functional analyses of these segments, has been helpful in clarifying the roles that each of the domains of the eIF4GI protein plays in the formation of a functional translation initiation complex. The amino-terminal fragment interacts directly with eIF4E (41, 42; see below). This region also interacts with the poly(A) binding protein (43), an interaction which facilitates mRNA translation, in systems which are poly(A) dependent (reviewed in 13). Thus eIF4G is responsible for binding to proteins that interact with the 5' and the 3' UTRs (13). The middle domain contains binding sites for eIF3 and eIF4A (41, 44), and possesses RNA-binding activity, due to an RRM (RNA-recognition-motif)-like domain (45). The carboxy-terminal fragment of eIF4GI contains a second, independent binding site for eIF4A (41, 44), and interacts with an eIF4E-kinase termed Mnk1 (MAP-kinase-interacting kinase-1; also called MAP kinase signal-integrating kinase 1). The C-terminal domain of eIF4G does not appear to be strictly required for translation, as the middle domain of eIF4G plus the eIF4E binding site alone are sufficient to mediate ribosome binding and translation of capped mRNAs (46).

eIF4GII is a functional homologue of eIF4GI such that all of the features described above for eIF4GI are conserved in eIF4GII (43, 47, 48). In mammals, a more distant structural homolog of eIF4G with a predicted molecular mass of 102 kDa was recently cloned, and referred to as p97, DAP-5 (death-associated protein-5), or NAT1 (novel apolipoprotein B

mRNA editing enzyme target no. 1). p97 is homologous only to the carboxy-terminal two-thirds of eIF4GI, and does not contain a region corresponding to the amino-terminal one third of eIF4G, which includes the eIF4E- and PABP-binding sites. As expected, p97 binds both eIF3 and eIF4A, but not eIF4E or PABP (43, 44, 49, 50). p97 binds to eIF4A, but only through the domain homologous to the middle domain of eIF4GI (49). Rather than acting as a true translation factor, p97 inhibits translation of both capped mRNAs, and of mRNA translating via an IRES (43, 44, 49, 50), although it cannot be excluded that p97 stimulates the translation of some specific mRNAs.

The best characterized polypeptide-binding site on eIF4G is that for eIF4E. It maps within the N-terminal portion of the protein, at position 572-578 in human eIF4GI (42, 43). Deletion and site-directed mutagenesis studies demonstrated that the consensus eIF4E binding site is YXXXXLΦ, where Φ is usually L, but may also be M or F (42, 51). This motif is present in eIF4G proteins from yeast to human (42, 52, 53). It is also found in a family of translational repressors, the 4E-binding proteins (4E-BPs), which compete with eIF4G for binding to eIF4E (see section 1.7.5). The structure of a cocrystal of eIF4E and an eIF4GII peptide encompassing the eIF4E-binding domain has been solved (54). The eIF4G fragment binds to the dorsal, convex surface of eIF4E, directly behind the cap-binding slot by interacting with several evolutionarily conserved residues through hydrogen bonds, salt bridges, and van der Waals contacts (Fig. 1.6C; 54). The cocrystal structure shows that Trp73 in eIF4E interacts with the eIF4G peptide. Mutation of this tryptophan to alanine abolishes the ability of human (48, 54) and yeast (55) eIF4E to interact with eIF4G. Consistent with an important role in mediating the interaction with eIF4G, Trp73 in eIF4E is essential for growth in yeast (55).

1.4.3 eIF4A

eIF4A is a 46kDa polypeptide (in mammals) exhibiting RNA-dependent ATPase and bidirectional RNA helicase activities (56-60). eIF4A is member the DEAD-box protein family, named after one of the motifs shared by all family members (61). There are two functional mammalian isoforms: eIF4AI and eIF4AII, which are highly homologous and functionally equivalent (62-64). The helicase activity of eIF4AI in isolation is relatively weak (in *in vitro* assays), but is strongly stimulated by eIF4B (see below; 58). Although eIF4A and eIF4B interact biochemically (i.e. eIF4B stimulates eIF4A ATPase and helicase activities; 65) and genetically (i.e. a multicopy suppressor of eIF4B complements an eIF4A mutation in yeast; 66), no stable physical interaction between the two proteins has been detected. The helicase activity of eIF4A is further stimulated upon association with the other subunits of the eIF4F complex: The eIF4F complex, in combination with eIF4B, is approximately 20-fold more active in an *in vitro* helicase assay than eIF4A in combination with eIF4B (58). Based on this observation, and considering the targeting of eIF4F to the mRNA 5' end through the interaction of eIF4G with eIF4E, it was proposed that the eIF4F complex is the physiological RNA helicase (58). Also, because eIF4A bound to eIF4G can be readily exchanged with free eIF4A, it has been suggested that eIF4A can be recycled through the eIF4F complex to effect RNA unwinding (64, 67). This recycling could explain (a) the requirement for free eIF4A in addition to eIF4F for efficient ribosome binding (2); (b) the presence in cells of excess (three- to sixfold) eIF4A relative to other initiation factors (68) and (c) the dramatic inhibitory effect on translation of dominant-negative eIF4A mutants (67), best explained as an inability of the mutant protein to recycle in and out of eIF4F.

An unresolved question, however, is how the helicase activity of eIF4A facilitates ribosome binding. The most accepted model regarding 43S preinitiation complex binding is that eIF4F assists in delivering eIF4A to the 5' end of the mRNA, which is

then appropriately positioned to unwind secondary structure in the mRNA to provide a single-stranded RNA region as a landing pad for the ribosome (5, 23). This hypothesis is consistent with the observations that the translation of mRNA with an extensive 5' secondary structure is generally poor (15, 69) and that the requirement for the 5' cap in translation and ribosome binding is directly proportional to the degree of secondary structure in the mRNA 5' UTR (70-72). Although this model is attractive because it is consistent with most available data, there is no direct evidence that eIF4A serves to unwind the mRNA secondary structure *in vitro* or in the cell. Possible alternative roles for eIF4A could be in rearranging secondary structure in the rRNA, or in dissociating other proteins from the mRNA (59, 60).

1.4.4 eIF4B

The function of eIF4B (a 69 kDa polypeptide in mammals; 73) in translation initiation is not well understood. eIF4B stimulates the helicase activity of eIF4A and facilitates the binding of the ribosome to the mRNA in a reconstituted ribosome-binding assay (74, 75). However, in contrast to eIF4A, which is an essential factor, eIF4B appears to play only a facilitating role because 48S ribosomal initiation complexes can be formed in its absence (76) and a yeast strain disrupted in the eIF4B gene, TIF3, is viable (65, 66). Mammalian eIF4B homodimerizes and also interacts with the p170 subunit of eIF3 (77, 78). In addition, eIF4B is an avid RNA-binding protein (77, 79, 80), that contains two separate RNA-binding domains (80, 81). An eIF4B-related protein (termed eIF4H) was discovered based on its stimulatory activity in a reconstituted *in vitro* translation assay (82). eIF4H exhibits 39% identity to mammalian eIF4B, and purified eIF4H can substitute for eIF4B to some extent in a reconstituted translation assay (82). The exact function of eIF4B and eIF4H in translation initiation remains unclear.

1.5 Mechanisms of ribosome binding

Prokaryotic ribosome binding and selection of the proper initiation site is mediated via the Shine-Dalgarno sequence, GGAGG, present in the mRNA and responsible for a base-pairing interaction with the sequence CCUCC, located in the 3' end of the 16S rRNA. Eukaryotes mRNAs do not possess the Shine-Dalgarno recognition sequence. Instead, ribosome binding and selection of the proper initiation codon in eukaryotes is effected by one or more of the mechanisms discussed below, and involves protein-protein and protein-RNA interactions, rather than the typical prokaryotic RNA-RNA interaction. The first distinction between the following eukaryotes models is whether translation requires the presence of the cap structure (cap-dependent versus cap-independent), and/or eIF4E (Fig. 1.4). The second distinction is whether other eIF4 translation initiation factors, such as eIF4A, eIF4B and eIF4G (and in particular, the middle domain of eIF4G, responsible for the binding of eIF4A and eIF3) are necessary.

1.5.1 Cap-dependent translation initiation

1.5.1.1 Scanning Model

The scanning model postulates that the ribosome must first be targeted to the mRNA via the interaction of eIF4E with the cap structure (see section 1.4.1). Once the 43S preinitiation complex is bound to mRNA, it must reach the initiation codon. The mechanism by which the ribosome traverses the 5' UTR is ill-defined, but it is thought that in most cases the 43S complex migrates processively along the mRNA, inspecting each base, until it encounters an initiation codon flanked by a favorable sequence context (14). Whether the 43S ribosome complex migrates in a 5'-to-3' direction by an active process requiring energy (ATP hydrolysis) or by some other mechanism, such as diffusion, is not known. Although ATP hydrolysis has been implicated in scanning (71), this energy could be harnessed directly for the active movement of the ribosome, or indirectly for disruption of the secondary structure to facilitate ribosome diffusion along

the mRNA. Notwithstanding these uncertainties, the scanning model is consistent with a large body of genetic (e.g. 17, 83) and biochemical (e.g. 84) data. (For a comprehensive review see 23). A key tenet of the scanning model is that the interaction of the 40S ribosomes with mRNA requires a free 5' end on the transcript, which is threaded through the ribosome akin to threading the eye of a needle (14). A pivotal experiment on which scanning is predicated demonstrated that, although prokaryotic ribosomes could bind to circular RNA, eukaryotic ribosomes could not (reviewed in 14), pointing to the inability of eukaryotic ribosomes to bind mRNA internally.

1.5.1.2 Ribosome shunting

A deviation from the linear scanning model is a phenomenon that has been variably referred to as ribosome shunting, jumping, discontinuous scanning, or repositioning. Ribosomes utilizing this mechanism bind in a cap-mediated fashion, commence scanning, but then bypass a stretch of the 5' UTR to resume scanning at a downstream position. This mechanism was first described for the 35S mRNA of the cauliflower mosaic virus (85) and then extended to other viral transcripts, including the adenovirus major late mRNAs (86) and Sendai virus mRNAs (87). The nature of the signals that effect the jumping and the trans-acting factors involved in the shunt mechanism are unknown.

1.5.2 Cap-independent translation

1.5.2.1 Internal ribosome entry – the picornavirus model

A second alternative mechanism for the recruitment of eukaryotic ribosomes to mRNAs is via direct binding of the 40S subunit to an internal RNA structure, bypassing the cap and the 5' end altogether. This mode of translation initiation was reported first for two picornaviruses, poliovirus and encephalomyocarditis virus (EMCV; 88, 89). Picornavirus mRNAs do not possess a 5' cap and thus translate by a cap-independent mechanism. In addition, they possess long 5' UTRs of approximately 600 to 1200 nucleotides, which

exhibit significant secondary structure and contain numerous unused AUGs (22, 90, 91), two features that are generally inhibitory for translation. Translation of most picornavirus RNAs is, however, very efficient, even when cap-dependent protein synthesis is blocked in the host cell (22, 91). An internal sequence from the picornavirus RNA was characterized that could recruit ribosomes in a 5' end-independent manner and could confer cap-independent translation to a reporter transcript. This structure was termed the IRES (for Internal Ribosome Entry Site) or RLP (for Ribosome Landing Pad); the former acronym is generally used today (reviewed in 22, 91). Internal ribosome binding is not restricted to uncapped viral RNA: Much attention has been given recently to the identification, characterization and regulation of putative IRESes found in capped mRNAs of cellular origin (reviewed in 24). Ribosome binding to picornavirus IRESes (and probably to the cellular IRESes) requires the complete set of initiation factors necessary for 5' end cap-dependent translation, except eIF4E (and the N-terminal region of eIF4G).

1.5.2.2 Internal Ribosome Binding – the hepatitis C virus model

There is a precedent for a much simpler and prokaryote-like mode of ribosome binding which is used by the hepatitis C virus (HCV) and related pestivirus mRNAs (92, 93). HCV IRES (about 300 nucleotides in length) exhibits the surprising property of direct binding to salt-washed ribosomes in the absence of any translation initiation factors. Furthermore, a functional initiation complex can be assembled at the start site in the absence of eIF4E, eIF4G, eIF4A and eIF4B, and without the requirement for ATP hydrolysis (eIF2 and eIF3 are required, however, for precise selection of the initiation codon and subunit joining; reviewed in 23). How the HCV IRES recognizes and bind specifically to ribosomes has not been completely elucidated: RNA-RNA interactions as well as RNA-protein interactions could be involved (23).

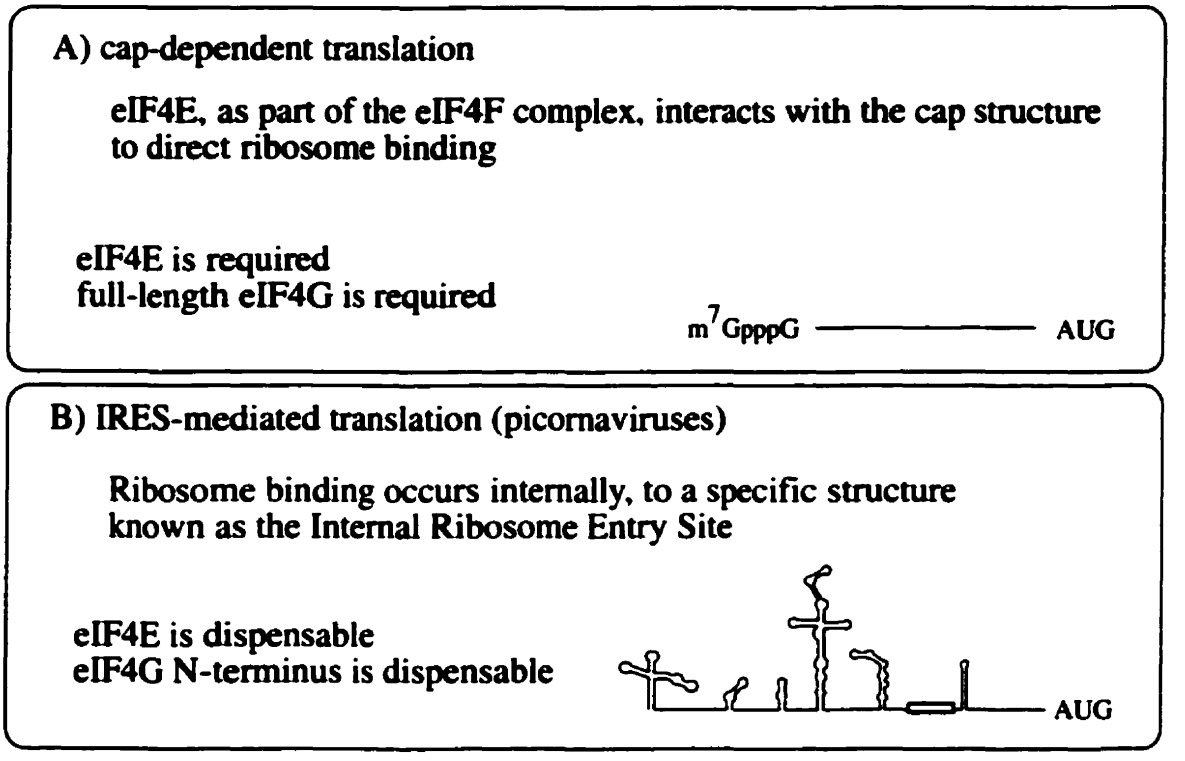


Figure 1.4 Comparison of the cap-dependent and IRES-driven mechanisms of initiation. See text for details.

1.6 Global control of protein synthesis versus regulation of translation of individual mRNAs

Treatment of cells with mitogens, hormones or growth factors generally leads to an increase in translation. Conversely, nutrient deprivation or environmental stresses such as heat shock, osmotic shock or UV irradiation generally reduce protein synthetic rates (reviewed in 1, 40, 94, 95). These changes in protein synthetic rates are often correlated with changes in the level or activity eIF4F, resulting in differences in the rate of ribosomal recruitment to mRNA (see section 1.7). That is, growing or stimulated cells contain high levels of eIF4F, whereas in quiescent or stressed cells low eIF4F levels are detected.

While global changes in protein synthesis following hormone or growth factor stimulation are relatively small (up to two-fold), a disproportionate and dramatic increase in the translation rates of a relatively small number of mRNAs is observed. How might different mRNA populations be regulated to different extents in response to a given stimuli or stress? As adumbrated more than 25 years ago by Lodish (96), this is most likely due, in part, to the discriminatory activity of the general translation apparatus. One possible mechanism for this "translational discrimination", or "mRNA competition", is a significant disparity in the requirement of individual mRNAs for eIF4F helicase activity. Since 40S ribosomal subunit binding appears to require a region of single-stranded mRNA, cap-proximal 5' UTR secondary structure may be expected to reduce translational efficiency by rendering the mRNA less accessible to a ribosome, and/or by impeding the scanning process. When eIF4F helicase activity is low, translation of mRNAs which do not possess significant 5'UTR secondary structure would be expected to be affected to a lesser degree than mRNAs possessing more extensive 5'UTR secondary structure. Indeed, eIF4E overexpression preferentially enhances the translation of mRNAs with structured 5'UTRs (97), dominant-negative eIF4A mutant

proteins preferentially inhibit translation of these poorly translated mRNAs *in vivo* (98), and eIF4B inactivation in yeast preferentially inhibits translation of reporter mRNAs possessing long and structured 5'UTRs (99).

Many specific examples of mRNAs which are regulated in this fashion have been identified. For instance, while insulin stimulation increases global translation rates by approximately two-fold, the increase in the translation rate of the ornithine decarboxylase (ODC) mRNA, which possesses a relatively long, and highly structured 5'UTR, is ~20-30 fold (100). The ODC mRNA translational block can be relieved by removing 5'UTR structured elements, or by overexpression of eIF4E (100, 101). Many mRNAs coding for oncogenes or other proteins important for growth or proliferation control possess large, putatively highly structured 5'UTRs (102), and are thus predicted to be targets for translational discrimination. Consistent with this prediction, overexpression of eIF4E or eIF4G in rodent cells induces cellular transformation (see chapter 6; also reviewed in 103), and overexpression of eIF4E, eIF4G and eIF4A has been noted in several different tumor types (104; chapter 6).

Not all regulated mRNAs are controlled by the discriminatory activity of the general translation apparatus, however. Several different specific 5'- or 3'UTR sequence elements have also been demonstrated to modulate translational efficiency (reviewed in 105). For instance, the 5' terminal oligopyrimidine tract (5'TOP) is a cap-proximal structural element (consisting of an uninterrupted stretch of 4-14 pyrimidines) that confers very stringent translation regulation. In conditions of nutrient deprivation, lack of growth factors, contact inhibition, or after initiation of a differentiation program, the translation of 5'TOP-containing mRNAs is potently repressed (21, 106). Stimulation of the cells (with hormones, cytokines, or growth factors, for example) overcomes the translational repression.

The advent of cDNA array and DNA chip technologies has allowed for the determination of the number and nature of translationally regulated mRNAs in different cell types, and in response to distinct kinds of stimuli or stress (reviewed in 24). When applied to a sucrose density gradient, mRNAs which are actively translated sediment with heavy polysomes (mRNAs associated with several ribosomes), whereas mRNAs which are poorly translated (or not translated at all) are found in lighter fractions. By comparing these two mRNA populations in cells subjected to a stress or stimulus, those transcripts which are translationally activated (or inhibited) can be identified. For example, ~1% of mRNAs (of the 1200 genes screened) in fibroblasts were found to shift to polysomes in response to serum-stimulation (107), while nearly 10% of the trancripts (472 genes screened) were observed to be under translational control following activation of resting T cells (108). Following poliovirus infection (a condition in which little or no intact eIF4F is present) less than 3% of mRNAs (200 out of 7000) remain associated with heavy polysomes (109).

Taken together, these data reinforce the view that in response to many types of stimuli or stress, global changes in protein synthesis are relatively modest, but the translation rates of a small population of mRNAs is dramatically modulated. This phenomenon likely involves several mechanisms, two of which are: 1) translational discrimination, due to differences in the requirement for eIF4F helicase activity, and 2) specific sequence elements, such as the 5'TOP, which confer potent translational inhibition in starved or resting conditions.

1.7 Regulation of ribosomal recruitment in mammalian cells

eIF4F complex formation and activity are regulated via several different modes in mammalian cells. Interaction with repressor peptides, phosphorylation of its constituent

proteins, proteolysis of the eIF4G subunit, and transcriptional regulation (see Chapter 2; reviewed in 22, 40) may all participate in the modulation of eIF4F activity in different situations. In this way, a complex network of signal transduction pathways cooperatively regulate translation initiation rates. Here, I will briefly discuss the phosphorylation of the eIF4 factors (eIF4E, eIF4G and eIF4B; eIF4A being unphosphorylated in mammalian cells). I will then describe in more detail the mechanism of regulation of ribosomal recruitment via the interaction with a family of repressor proteins, the 4E-BPs.

1.7.1 Phosphorylation of eIF4E

The phosphorylation of mammalian eIF4E in response to all stimuli so far examined occurs primarily on a single residue, Ser209 (numbering for the murine protein), with minor phosphorylation detected in certain cases on threonine residues (most likely Thr210; 110, 111, 112). The phosphorylation state of eIF4E, in general, correlates with translation rates and the growth status of the cell (reviewed in 94, 113). eIF4E phosphorylation is modulated in response to a variety of extracellular stimuli: treatment of cells in culture with hormones, growth factors, cytokines or mitogens results in a net increase in eIF4E phosphorylation (see Table I and 40, 53, 94). eIF4E is hypophosphorylated during mitosis (114, 115), a cell cycle phase during which translation rates of most (but not all) mRNAs are low (116-118). Also, eIF4E becomes dephosphorylated after heat-shock (119), concomitant with a decrease in global translation rates. Infection with adenovirus (115), influenza virus (120), or encephalomyocarditis virus (121) are accompanied by a decrease in eIF4E phosphorylation (see below for cases where there is a lack of correlation between eIF4E phosphorylation and translational rates).

Table I. Effect of various stimuli on the phosphorylation of eIF4E and 4E-BP1

stimulus	cell type	eIF4E	4E-BP1	references
Actinomycin D			+[b]	(122)
Adenovirus infection (early)	HeLa, 293	-[a]	+	(123, 124)
Amino acid addition	numerous cell lines / animal models		+	(reviewed in 125)
Angiotensin II	vascular smooth muscle	+	+	(126, 127)
Anisomycin	Swiss 3T3; 293		+	(128)
Anti-CD3	mature $CD4^+$ or $CD8^+$ thymocytes	+		(129)
Arsenite	293; CHO.K1	+	-	(130)
Cholecystokinin	rat pancreas	+	+	(131, 132)
Concanavalin A	peripheral blood mononuclear cells	+		(133)
DAMGO[c]	CHO overexpressing μ-receptor		+	(134)
Epidermal growth factor	mammary epithelial cells	+		(135)
	P19	+		(136)
	Swiss 3T3, 3T3-L1, PC12		+	(137-139)
Gastrin	AR4-2J tumor cells		+	(140)
GMCSF + SLF	hematopoietic MO7e	+	+	(141)
Heat shock	reuber hepatoma		+	(142)
Hepatocyte growth factor	epithelial human MKN74		+	(143)
High glucose	isolated rat pancreatic islets	+		(144)
Insulin	NIH3T3	+		(100, 145, 146)
	3T3-L1	+	+	(137, 138, 147, 148)
	CHO	+		(149, 150)
	skeletal muscle		+	(151, 152)
	Swiss 3T3; 32D; 293; CHO-IR		+	(123, 128, 153, 154)
	epithelial cells; cardiac myocytes		+	(155, 156)
Insulin-like growth factor I	rat aortic smooth muscle		+	(157)
Insulin-like growth factor II	Swiss 3T3-L1		+	(137, 148)
Interleukin 1β	CHO.K1	+		(130)
Interleukin 3	myeloid progenitor		+	(153)
Leucine addition	numerous cell lines / animal models		+	(reviewed in 125)
Lipopolysaccharide	B lymphocytes	+		(158)
	peritoneal macrophages; Raw264.7		+	(159)
L-pyrroline-5-carboxylic acid	rabbit reticulocyte lysate	+		(160)
Nerve growth factor	PC12	+	+	(139, 161)
Platelet-derived growth factor	NIH 3T3	+		(100, 145, 146)
	lung fibroblasts	+		(162)
	Swiss 3T3-L1; aortic SM		+	(137, 152, 157)
PHA + phorbol ester	human T cells	+		(163)
Phenylephrine	Rat1a expressing α1A adrenergic rec.		+	(164)
Phorbol ester	NIH 3T3, CHO, PBL, B cells	+		(100, 133, 145, 146, 149, 150, 158)
	3T3-L1, leukaemic T cells, retic. lysate	+		(133, 147, 160)
	Swiss 3T3		+	(128)
Prostaglandin F2α	vascular smooth muscle	+	+	(165)
Serum	NIH 3T3	+	+	(100, 145, 146, 166)
	Swiss 3T3	+		(167)
	CHO	+		(150)
	3T3-L1		+	(137, 152)
Tumor necrosis factor α	U937, HeLa, ME180, BAEC, FS, HUVEC	+		(130, 168)

[a] (-) no change in phosphorylation

[b] (+) increase in phosphorylation; blank space indicates not determined

[c] Abbreviations used: DAMGO, [D-Ala^2,N-Me-Phe^4,Gly^5-ol]-enkephalin; GMCSF, granulocyte-macrophage colony stimulating factor; SLF, Steel factor; PHA, phytohemagglutinin.

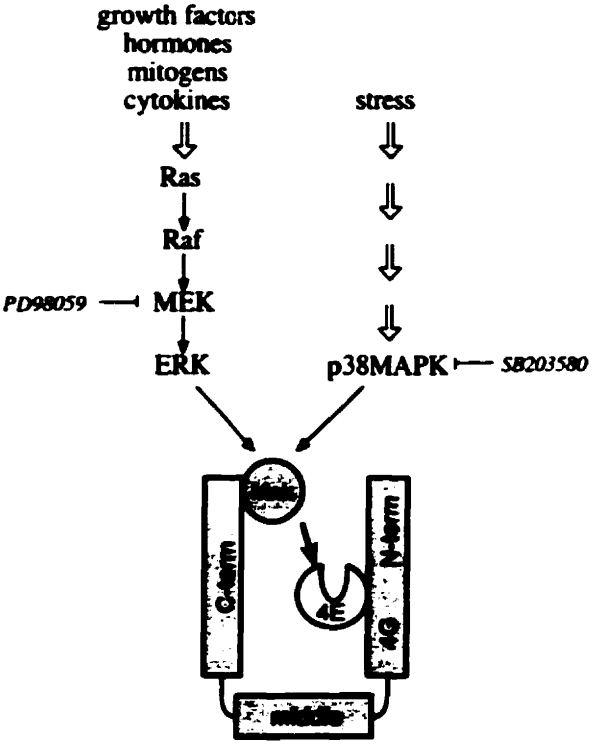
1.7.1.1 Signaling pathway to eIF4E phosphorylation

A putative role for the ras/raf/ERK MAPK pathway (reviewed in 169, 170) in eIF4E phosphorylation was suggested by the observation that eIF4E phosphorylation is increased in ras- or src-transformed cells (146, 171). The ERK signaling cascade is activated by extracellular stimuli, and is specifically inhibited by PD98059 (reviewed in 169, 170, 172). Phosphorylation of eIF4E is induced by serum or insulin, and is prevented to a large extent by PD98059 treatment (145, 149). However, the ERKs cannot directly phosphorylate eIF4E *in vitro*, arguing against a direct role for the MAPKs in eIF4E phosphorylation *in vivo* (149).

Certain stresses, such as anisomycin or arsenite treatment, increase eIF4E phosphorylation, even though translation rates actually decrease in response to these drugs (145). The p38 subfamily of MAPKs, like the JNK family, is activated in response to many types of environmental stress, including hyperosmolarity, heat shock, UV irradiation, and exposure to LPS, arsenite, or anisomycin (169, 170). p38 MAPK activity (but not JNK activity) is specifically prevented by the pharmacological compound SB203580 (173). In agreement with a role for p38 MAPK in mediating eIF4E phosphorylation induced by stress, induction of eIF4E phosphorylation by anisomycin is prevented in cells pre-incubated with SB203580 (130, 145).

The mitogen-stimulated pathway acting through the ERKs and the stress-activated pathway acting through the p38 MAPKs converge at a common eIF4E kinase termed Mnk1 (174, 175; MAP kinase interacting kinase 1 or MAP kinase signal integrating kinase 1). Mnk1 was isolated via interaction screening as a substrate for both ERK1 and p38 MAPK, and activation of either the ERK or p38 MAPKs (but not the JNK kinases) stimulates Mnk1 kinase activity (174, 175). Mnk1 efficiently phosphorylates eIF4E Ser209 *in vitro* (175) and *in vivo*, following stimulation of either the ERK or p38 MAPK

A



B

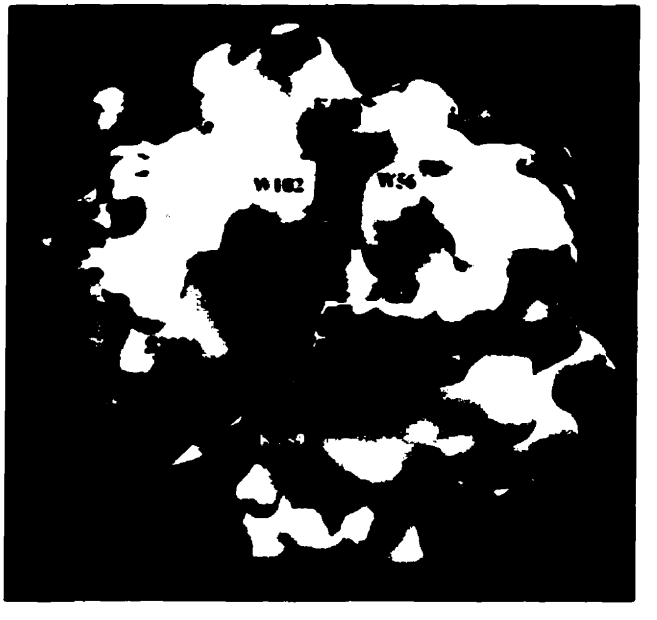


Figure 1.5. eIF4E phosphorylation. (A) Signaling pathway to eIF4E phosphorylation. Growth factors, mitogens, cytokines or other extracellular stimuli activate the ERK pathway. Stress activates the p38 MAPK pathway. Both of these pathways can activate Mnk1, an eIF4G-associated kinase. Mnk1 phosphorylates eIF4E on Ser209. (B) A GRASP surface model of mouse eIF4E. The surface of eIF4E is color-coded based on the charge of the amino acids (blue = basic; red = acidic). The cap is bound in a narrow slot between Trp56 and Trp102. The position of Ser209 and Lys 159 is shown. A possible path for the mRNA is indicated by the yellow arrow.

C

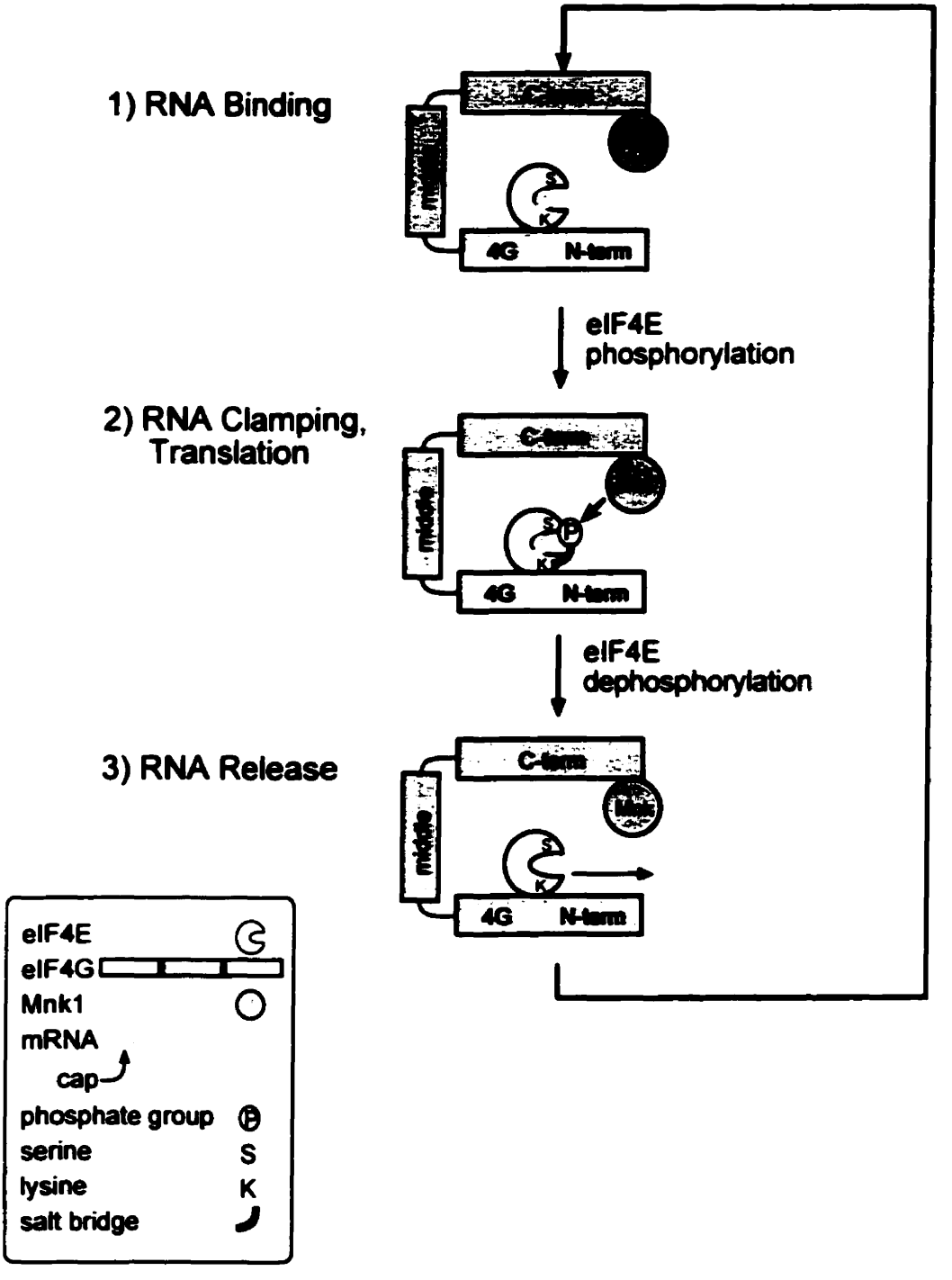


Figure 1.5. (C) eIF4E clamping cycle model. Step 1: eIF4E and Mnk1 interact with the eIF4G proteins. Capped mRNA is bound by unphosphorylated eIF4E. Step 2: eIF4E bound to mRNA is phosphorylated by Mnk1. Phosphoserine 209 and a nearby lysine residue (Lys159) form a salt bridge to cover and "clamp" the mRNA in place. Translation ensues. Step 3: eIF4E is dephosphorylated by an unknown phosphatase, effecting mRNA release. Unphosphorylated eIF4E is competent to bind to another mRNA.

cascades (48, 176). Mnk1 does not interact directly with eIF4E. Rather, Mnk1 binds to eIF4G family proteins (48, 176). Thus, the eIF4Gs recruit Mnk1 to its substrate (Fig. 1.5). The interaction between eIF4E and eIF4G is required for eIF4E phosphorylation *in vivo*, because a mutant eIF4E protein which cannot interact with eIF4G is not efficiently phosphorylated in mammalian cells (48).

1.7.1.2 Function of eIF4E phosphorylation

The function of eIF4E phosphorylation is not well understood. Unphosphorylated recombinant eIF4E can stimulate translation *in vitro* (e.g. 29), and can bind to mRNA or mRNA cap analogs (e.g. 177, 178). Thus, phosphorylation is not strictly required for eIF4E function. However, the crystal structure of eIF4E (31) suggests that the phosphorylated form of eIF4E could have a stronger affinity for capped mRNA. Ser209 is located below the cap-binding slot and lies on the proposed trajectory of the mRNA chain (Fig. 1.5; 31). Facing Ser209 on the opposite side of the putative mRNA path is Lys159, which could potentially form a retractable salt bridge over the mRNA with a phosphorylated Ser209. This bridge could act as a retractable clamp to stabilize the mRNA in the cap-binding slot. Thus, phosphorylation may enhance the binding of eIF4E to capped mRNAs and longer cap analogs (dinucleotides or more); however, it is not obvious from the structure that binding to shorter cap analogs (e.g. m^7GDP) should be enhanced. Consistent with this model, phosphorylation of eIF4E has been described to enhance its affinity for mRNA (179).

The fact that eIF4E is phosphorylated by an eIF4G-bound kinase raises the interesting possibility of an eIF4E phosphorylation – mRNA binding cycle. It may be envisioned that upon formation of the eIF4F - mRNA complex, eIF4E is phosphorylated by Mnk1 to "clamp" the bound mRNA in place. It is also conceivable that the mRNA must then be "unclamped" to catalyze subsequent rounds of initiation, a task presumably accomplished

by an eIF4E phosphatase (Fig. 1.5c). Indeed, it was proposed, based on comparisons between [^{32}P] incorporation and ratio of isoforms detected by isoelectric focusing, that agents which increase the phosphorylation of eIF4E also lead to an increase in the turnover rate of phosphate (e.g. 158). eIF4E phosphorylation may thus provide an additional level of control in translation initiation. It could also function to strengthen the eIF4F-mRNA interaction, or enable more efficient re-initiation (13).

1.7.2 Phosphorylation of eIF4GI

The eIF4Gs have been known to be phosphoproteins for some time (110, 120, 135, 147, 180-183), however, the intracellular signaling pathways regulating their phosphorylation, the location of the phosphorylation sites within the proteins, and the functional consequences of eIF4G phosphorylation have only just begun to be understood. Recently, two distinct sets of phosphorylation sites have been identified in the eIF4GI protein. One set is located in the carboxy-terminus, with the majority of the sites contained in a poorly conserved putative "hinge" region (aa 1035-1190) residing between the middle and carboxy-terminal eIF4A binding domains (184). The second, less characterized set resides in the amino-terminus (with phosphorylation on at least one serine, Ser274). The phosphorylation status of the carboxy-terminal phosphorylated region changes in response to serum or mitogen treatment, with phosphorylation on serines 1108, 1148 and 1192 increasing drastically (184). PI3K and FRAP/mTOR signaling (see section 1.8) modulates the phosphorylation of these residues (184). However, these serines are not phosphorylated directly by FRAP/mTOR, S6K1 or S6K2 in an *in vitro* kinase assay (184). Instead, eIF4GI amino-terminal sequences appear to confer serum- and mitogen-responsiveness (as well as kinase inhibitor sensitivity) to the carboxy-terminal region; truncation mutant proteins lacking the amino-terminus are constitutively phosphorylated in the "serum-stimulated" state, even in serum-starved cells, and acquire resistance to kinase inhibitor treatment. Thus, the PI3K - FRAP/mTOR

pathway(s) appears to regulate the accessibility of the carboxy-terminal region to other (rapamycin- and wortmannin-insensitive) kinases (184). A similar phenomenon has been noted for S6K1, in that autoinhibition and rapamycin-sensitivity are conferred by specific amino-terminal sequences (185, 186). Removal of this region results in a rapamycin-insensitive kinase (185, 186).

How then might phosphorylation modulate eIF4GI activity? The C-terminal phosphorylated region does not overlap with the binding site of any known eIF4G binding partners, and the interactions between eIF4GI and several known binding partners (eIF3, eIF4A and Mnk1) do not appear to be altered by phosphorylation (B. Raught, A.-C. Gingras, S. Morino and S. Pyronnet, unpubl.). Secondary structure predictions suggest that the phosphorylated region is relatively unstructured (B. Raught and A.-C. Gingras, unpubl.). This region also contains a caspase cleavage site (187), suggesting that it is solvent exposed. Thus, the phosphorylated region may act as a flexible "hinge" between the middle and C-terminal domains. In the absence of evidence for changes in protein-protein interactions, it was suggested that phosphorylation alters intramolecular interactions to cause short- or long-range changes in eIF4GI structure (184).

1.7.3 Phosphorylation of eIF4GII and p97

eIF4GII is also a phosphoprotein, but it does not appear to be phosphorylated to a significant extent in the domain corresponding to the eIF4GI C-terminal phosphorylated region (184). The eIF4GI C-terminal phosphorylated region shares a very low degree of homology with the corresponding region of eIF4GII. Further, eIF4GII phosphorylation does not appear to be responsive to serum or mitogen treatment in 293 cells, suggesting that the two eIF4G proteins may have evolved to respond differently to distinct intracellular signaling pathways. p97 is also a phosphoprotein in 293 cells. Like

eIF4GII, the region of p97 corresponding to the eIF4GI C-terminal phosphorylated region is not phosphorylated, and the phosphorylation status of p97 does not appear to be modulated by serum or mitogen treatment (184).

1.7.4 eIF4B phosphorylation

Mammalian eIF4B is a phosphoprotein (188-190) and treatment of cells with serum, insulin, or phorbol esters results in eIF4B hyperphosphorylation (147, 191, 192). However, the biological significance of eIF4B phosphorylation is unknown. In fact, recombinant eIF4B expressed in *E. coli* is active both in an *in vitro* helicase assay (58) and in a ribosome toe-printing assay (46, 76). eIF4B can be phosphorylated *in vitro* with several different kinases, including S6K1 (147, 192, 193). Intriguingly, two-dimensional tryptic phosphopeptide mapping has revealed that eIF4B possesses at least one serum-stimulated phosphorylation site (serine 422) which is sensitive to rapamycin, wortmannin and LY294002 (193). Ser422 is also the site phosphorylated by S6K1 *in vitro*. Furthermore, overexpression of rapamycin-resistant S6K1 proteins confers rapamycin resistance to Ser422 phosphorylation *in vivo* (106). eIF4B is only the second substrate to be ascribed to S6K1 (the other being the ribosomal S6 protein; 106) Thus, PI3K and FRAP/mTOR (section 1.8) also appear to signal to eIF4B. The phosphorylation site is located at the beginning of an arginine-rich region, involved in RNA binding, but the effect of the phosphorylation of the activity of eIF4B has not been assessed.

1.7.5 4E-BP discovery, structure and function

A paramount mechanism of regulation of eIF4E activity is through its interaction with a family of repressor proteins termed the eIF4E-binding proteins (4E-BPs). A Far-Western assay was initially used to identify 4E-BP1 and 4E-BP2 (also known as PHAS-I and II; reviewed in 194) as binding partners for eIF4E (195). 4E-BP3 was subsequently cloned based on its homology to the other 4E-BPs (196). The 4E-BPs are small (approximately

10 to 12 kDa), heat-stable proteins. 4E-BP1 and 4E-BP2 share 56% identity, and 4E-BP1 and 4E-BP3 share 40% identity. Conservation is greatest (at least 74% identity) in the middle region of these molecules (residues 35-85 in 4E-BP1), which contains the eIF4E-binding site. Apparent 4E-BP orthologs have been found in numerous organisms, including several vertebrates (*Homo sapiens, Bos taurus, Sus scrofa, Rattus norvegicus, Mus musculus, Gallus gallus, Xenopus laevis, Oryzias latipes, Danio rerio, Cyprinus carpio*, etc.), tunicates (*Halocynthia roretzi*), flatworms (*Schistosoma mansoni, Echinococcus granulosus*), insects (*Drosophila melanogaster, Bombyx mori*), and cellular slime mold (*Dictyostelium discoideum*). In *S. cerevisiae*, a likely 4E-BP functional analog has been cloned. However, this translational inhibitor, known as Caf20p or p20, does not share homology with the 4E-BPs from other species (197), apart from the presence of a conserved sequence motif conferring eIF4E binding (Fig. 1.6E). To date, no plant EST related in sequences to yeast Caf20 or to mammalian 4E-BPs has been identified.

4E-BPs inhibit cap-dependent translation, both in cell-free translation assays and when overexpressed in cells (195, 196), whereas cap-independent translation is not affected (195, 196). Binding of the 4E-BPs to eIF4E does not appear to alter the affinity of eIF4E for the cap, because an eIF4E/4E-BP1 complex readily binds to a matrix-bound cap (195). Furthermore, in an electrophoretic mobility shift assay with capped RNA as a probe, the eIF4E-RNA complex is supershifted upon addition of 4E-BP1 (A.-C.G.). Instead, 4E-BPs appear to inhibit the activity of eIF4E by preventing its association with eIF4G, thus suppressing the formation of the eIF4F complex (198). 4E-BPs and eIF4G compete for binding to eIF4E because both eIF4G and the 4E-BPs possess a conserved amino acid motif (containing the "core" sequence YXXXXLΦ, in which X is any amino acid and Φ is an aliphatic residue, most often L, but sometimes M or F), necessary for interaction with eIF4E as described in section 1.4 (42). Deletion of this sequence in the

4E-BPs or mutation of either the tyrosine or the leucine to alanine abrogates eIF4E binding (42, 196) and relieves translational inhibition (196). In addition, a 20aa peptide derived from the eIF4E binding site of the mammalian 4E-BPs or eIF4Gs significantly inhibits cap-dependent translation in an *in vitro* translation assay (54, 199).

NMR and crystallographic data have provided the structural basis for the importance of this motif in mediating binding to eIF4E (Fig. 1.6). An area of the convex surface of eIF4E exhibits a remarkable evolutionary conservation amongst all eIF4E proteins (31). An NMR study in which eIF4E was titrated with 4E-BP protein indicated that this conserved area is the 4E-BP binding site (32). This was later demonstrated directly by crystallographic analysis of eIF4E bound to peptides derived from either 4E-BP1 (amino acids 51-66 of human 4E-BP1) or eIF4GII (54). When bound to eIF4E, both peptides exhibit an L-shaped extended-chain/alpha-helical conformation, and bind to the convex dorsal surface of eIF4E, opposite to the cap-binding slot (Fig. 1.6B; 54). An alignment of the sequences encompassing the eIF4E-binding sites of the mammalian 4E-BP and eIF4G proteins is shown in Fig. 1.6E (the invariant tyrosine is assigned position 0). Consistent with the high degree of conservation of the eIF4E-binding site between mammalian 4E-BPs and eIF4Gs, the cocrystal structure of an eIF4GII peptide with eIF4E shows a mode of binding similar to the 4E-BP peptide (Fig. 1.6C). Binding of 4E-BP1 to eIF4E involves residues previously demonstrated by mutagenesis studies to be critical for interaction (Tyr54 and Leu59 in human 4E-BP1; positions 0 and +5 in Fig. 1.6E), as well as other conserved residues that establish additional hydrogen bonds, van der Waals contacts, or salt bridges with eIF4E (54). Fig. 1.6D is a magnified view of the 4E-BP1 peptide bound to eIF4E. Residues involved in intermolecular contacts are highlighted. The conserved residues at positions -3 (Arg/Lys), 0 (Tyr), +2 (Arg), and +5 (Leu) establish contacts with eIF4E. The aliphatic portions of the side chains of -2 (Ile in 4E-BPs, Lys in eIF4G), +6 (Met in 4E-BP1, Leu in the other 4E-BPs and eIF4G), and the

nonconsensus positions +8 and +9 (all the substitutions observed among mammalian eIF4G/4E-BPs could support these interactions) also contact eIF4E. Nonconserved amino acids at positions -1, +1, +3, +7, and +10 make no close intermolecular contacts. Intramolecular interactions within the 4E-BP1 peptide stabilize the L-shaped structure (54). The cocrystal structure (Fig. 1.6) demonstrates that the amino acids in murine eIF4E involved in intermolecular contacts with 4E-BP1 include His37, Val69, Trp73, Leu131, Leu135, Glu132, Ile138, Glu140, and Asp147 (54). NMR spectroscopy demonstrated that residues 32-50 and 62-79 in yeast eIF4E were the most affected by 4E-BP2 addition (54), indicating that they are involved in 4E-BP binding. Thus, NMR and crystallographic studies identified an overlapping set of residues on eIF4E that constitute the binding site for 4E-BPs. Consistent with this information, mutation of Trp73 to alanine in mouse eIF4E abolishes 4E-BP1 and eIF4G binding (48; A.-C.G, unpublished data).

In the absence of eIF4E, the 4E-BPs are largely unstructured in solution, as determined by NMR and circular-dichroism studies (54, 199, 200). How then might this peptide recognize and bind to eIF4E? Specificity appears to be conferred via an "induced fit" mechanism, whereby eIF4E binding fixes a small region of the 4E-BP into the energetically favorable alpha-helical conformation (54). These results are consistent with the NMR studies which demonstrated that only a very small portion of 4E-BP1 becomes ordered in the presence of mouse or yeast eIF4E, whereas most of the protein remains disordered (199). A similar induced-fit is observed with the eIF4GII peptide, which is also unstructured in solution, and acquires an alpha-helical conformation upon eIF4E binding (54).

E

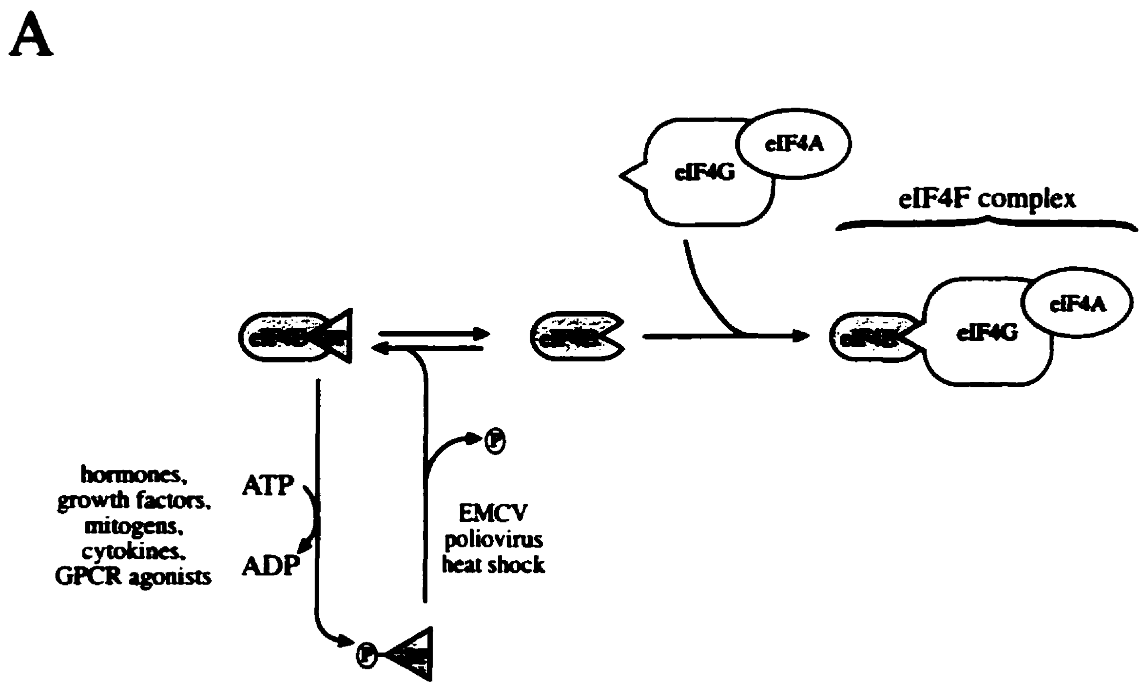
position	-2	0	2	4	6	8	10
heIF4GI	K K	R	Y	D R	E	F	L L G F Q F
heIF4GII	K K	Q	Y	D R	E	F	L L D F Q F
h4E-BP1	R I	I	Y	D R	K	F	L M E C R N
h4E-BP2	R I	I	Y	D R	K	F	L L D R R N
h4E-BP3	R I	I	Y	D R	F	F	L L E C K N
deIF4G	K K	Q	Y	D R	E	Q	L L Q L R E
d4E-BP	K L	I	Y	E R	A	F	M K N L R G
Tif4631p	K Y	T	Y	G P	T	F	L L Q F K D
Tif4632p	K Y	T	Y	G P	T	F	L L Q F K D
Caf20p	M I	K	Y	T I	D	E	L F Q L K P
consensus			Y				L Φ

Figure 1.6. The cocrystal structure of the 4E-BP1 and eIF4GII peptides bound to eIF4E. (A) The human 4E-BP1 peptide (orange) and the m7GDP cap analog (green) bind to opposing regions of eIF4E (blue); (B) The 4E-BP1 peptide binds to the dorsal convex surface of eIF4E and adopts an extended L-shaped conformation; (C) The eIF4GII peptide (red) also adopts an extended L-shaped conformation when bound to the same region of eIF4E; (D) A magnified view of the residues involved in mediating 4E-BP1 (orange) binding to eIF4E (blue); (E) Sequence alignment of the eIF4E-binding sites of several 4E-BPs, eIF4Gs, and yeast Caf20; h = human, d = drosophila.

Structural changes in the eIF4G proteins induced by eIF4E binding may not be limited to the small region cocrystallized with eIF4E: a study performed with yeast eIF4G revealed that following eIF4E binding, a 100 amino acid peptide acquires secondary structure and becomes resistant to proteolytic cleavage (201). This could explain the finding that upon binding to eIF4E, eIF4G becomes hypersensitive to cleavage by picornavirus proteases (202, 203). In sum, a highly efficient mechanism for the regulation of eIF4F formation has evolved in mammals, whereby the inhibitory 4E-BPs act as molecular mimics of the eIF4E binding motif present in the eIF4G proteins.

1.7.6 Regulation of 4E-BP1 Phosphorylation

Phosphorylation of specific serine and threonine residues modulates the affinity of the 4E-BPs for eIF4E (e.g. 195, 204, 205). While hypophosphorylated 4E-BPs bind efficiently to eIF4E, phosphorylation of a critical set of residues abrogates this interaction (e.g. 195, 204, 205). 4E-BP phosphorylation levels are modulated by many types of extracellular stimuli (Table I). In this regard, 4E-BP1 was first described ~20 years before its cDNA was cloned as a protein which is highly phosphorylated after insulin stimulation of rat adipocytes (137, 206-208). This protein was later biochemically purified and cloned, and termed PHAS-I (Phosphorylated Heat and Acid Stable protein-Insulin responsive; 209). The function of PHAS-I was ascertained when it was found to be the rat ortholog of human 4E-BP1 (195, 204). Hormones (insulin, angiotensin II, etc.), growth factors (EGF, PDGF, NGF, IGFI, IGFII, etc.), cytokines (IL-3, GMCSF in combination with steel factor, etc.), mitogens (TPA), G-protein coupled receptor ligands (gastrin, DAMGO), and adenovirus infection (210; chapter 2) induce hyperphosphorylation of 4E-BP1 (see Table I), accompanied (when assessed) by a resultant decrease in its interaction with eIF4E and an increase in cap-dependent translation rates (reviewed in; 40).



B

```
                                                    *
1   MSGGSSCSQTPS--RAIPATRRVVLGDGVQLPPGDYSTTP   4E-BP1
1   MSSSAGSGHQPSQSRAIP-TRTVAISDAAQLPH-DYCTTP   4E-BP2
1   MSTSTSC--------PIP-------GGRDQLPD-CYSTTP   4E-BP3

           *                  *    *
39  GGTLFSTTPGGTRIIYDRKFLMECRNSPVTKTPPRDLPTI   4E-BP1
39  GGTLFSTTPGGTRIIYDRKFLLDRRNSPMAQTPPCHLPNI   4E-BP2
25  GGTLYATTPGGTRIIYDRKFLLECKNSPIARTPPCCLPQI   4E-BP3

        *                              *
79  PGVTSPSS--DEPPMEASQSHLRNSPEDKRAGGEESQFEMDI   4E-BP1
79  PGVTSPGTLIEDSKVEVNNLNNLNNHDRKHAVGDDAQFEMDI   4E-BP2
65  PGVTTPPT------APLSKLEELKEQETEEEIPDDAQFEMDI   4E-BP3
```

Figure 1.7 The binding of the 4E-BPs to eIF4E is regulated by phosphorylation. (A) The 4E-BPs and eIF4Gs compete for a common binding site on eIF4E. Various stimuli increase the phosphorylation state of the 4E-BPs. Hyperphosphorylated 4E-BPs have a relatively low affinity for eIF4E. Conversely, a decrease in 4E-BP phosphorylation increases the affinity of the 4E-BPs for eIF4E. Free eIF4E interacts with eIF4G to form the translationally active eIF4F complex. GPCR: G protein coupled receptor. (B) Alignment of the mammalian 4E-BPs through the eIF4E binding site. The conserved eIF4E binding motif is boxed in blue. Phosphorylated residues in 4E-BP1 are highlighted in yellow, and indicated with a star.

Conversely, serum starvation (e.g. 128), amino acid deprivation (chapter 6; also see 125), picornavirus infection (211; chapter 2), and certain environmental stresses such as heat shock (in certain cell types; 212) or osmotic shock (213) lead to a decrease in 4E-BP1 phosphorylation, an increase in its affinity for eIF4E, and an inhibition of cap-dependent translation.

Six (serine/threonine) phosphorylation sites have been identified in the mammalian 4E-BP1 protein (Fig. 1.7; 205, 214). Five of the six sites are followed by a proline residue, and one is followed by a glutamine. Two phosphorylated residues, Thr37 and Thr46, lie on the amino terminal side of the eIF4E binding motif (located at aa 54-60), and four phosphorylated residues have been identified on the carboxy-terminal side of the eIF4E binding motif, Ser65, Thr70, Ser83 and Ser112. Phosphorylated residues may vary somewhat according to cell type and/or species. For example, phosphorylated threonines 37 and 46, Ser65, Thr70 and Ser83 account for all of the major phosphopeptides observed by two-dimensional phosphopeptide mapping of 4E-BP1 from human embryonic kidney 293 cells (215, 216; see chapters 3-5), although some minor phosphopeptides remain unidentified. However, in rat epididymal adipocytes Ser112 (Ser 111 in the rat protein) was reported to be a major insulin-stimulated residue (214). In other studies, Ser112 phosphorylation of rat 4E-BP1 was not detected (205, 217). The significance of cell type and/or species-specific differences in 4E-BP1 phosphorylation is not understood. Differences in the sensitivity of the different phosphorylation sites to stimuli, or inhibition by pharmacological inhibitors will be discussed in chapters 3, 4 and 5.

1.7.7 Phosphorylation of the other 4E-BPs

Alignment of the human 4E-BPs reveals that all of the phosphorylated residues in 4E-BP1 are conserved in 4E-BP2 and 4E-BP3, except for Ser112 (Fig. 1.7). However, the phosphorylation pattern of 4E-BP2 is less complex than that for 4E-BP1: while 4E-BP1

isolated from 293 cells migrates as six species in the two-dimensional gel electrophoresis system (216; chapter 5), 4E-BP2 isolated from 293 cells migrates as only 4 isoforms (218). This difference in the number of phosphorylated residues may affect the binding properties of 4E-BP2: While 4E-BP1 is rapidly released from eIF4E in response to adenovirus infection, 4E-BP2 is released much more slowly (219). Thus, the kinetics of eIF4E liberation after the application of a given stimulus could vary significantly in different tissues containing dissimilar 4E-BP1/4E-BP2 ratios (e.g. 220). Two-dimensional gel analysis and tryptic phosphopeptide mapping indicates that 4E-BP2 is phosphorylated on both Thr37 and Thr46 (218). These data suggest that at least one 4E-BP2 phosphorylation site (or other post-translational modification) remains to be identified. 4E-BP3 is also a phosphoprotein (196), but the identities of the phosphorylated residues in this 4E-BP have not been established.

1.8 Signal Transduction Pathway Mediating 4E-BP1 Phosphorylation

1.8.1 Role of the ERK pathway

Early reports indicated that the ERKs were responsible for the phosphorylation of 4E-BP1, based on *in vitro* phosphorylation, using either fractionated cell extract from activated cells, or purified ERK (e.g. 204, 221; chapters 4-5). Indeed, recombinant ERK2 readily phosphorylates Ser65, and prolonged incubation with ERK2 leads to phosphorylation of several additional sites (e.g. 205, 222). However, in most cell types, there is no relation between ERK activation and 4E-BP1 phosphorylation. ERK is not necessary for 4E-BP1 phosphorylation, as complete 4E-BP1 phosphorylation can be observed in conditions where ERK activation is not detected, for example, in human embryonic kidney (HEK) 293 cells and in the mouse fibroblast Swiss 3T3 cell line stimulated with insulin (128). Similarly, inhibition of ERK activation by PD98059, a pharmacological inhibitor of MEK (the kinase activating ERK) is without effect on 4E-BP1 phosphorylation (e.g. chapter 5 and ref. 127). Not only is ERK not required for

mediating 4E-BP1 phosphorylation, but it is also insufficient. Mutants in the PDGF (platelet-derived growth factor) receptor, which are fully functional in signaling to the ERKs, but defective in activation of the PI3 kinase pathway, are completely unable to relay a signal to 4E-BP1 phosphorylation following treatment of cells with PDGF (128). Similarly, treatment of cells with the pharmalogical inhibitors SQ20006, rapamycin, LY294002 and wortmannin (see below), which are without effect on the activation of ERKs, prevent (to a large extent) the hyperphosphorylation of 4E-BP1 (128, 138; chapters 4-5, 166). Taken together, these data indicated that the signaling pathway leading to 4E-BP1 phosphorylation is, in most cell types, different from that leading to the activation of the ERKs. Furthermore, the sensitivity to the drugs SQ20006, rapamycin, LY294002 and wortmannin, as well as the dependence upon PI3 kinase signaling suggested to us that the signaling pathway is similar to that leading to the activation of the ribosomal S6 protein kinase 1 (S6K1; see below). Indeed, the time course of activation of S6K1 closely parallels that of the phosphorylation of 4E-BP1 following serum-stimulation of HEK 293 cells (128).

1.8.2 S6K1.

The S6Ks (S6K1 and the recently cloned S6K2) are serine/threonine kinases, members of the AGC superfamily of kinases (homology to the kinase A, G and C families). S6K1 is itself regulated by phosphorylation on several serine and threonine residues, in a multi-step manner (reviewed in 106). Phosphorylation of S6K1 results in the activation of the enzyme, and acidic substitutions of the phosphorylated residues results in constitutive activation (the protein acquires resistance to rapamycin and wortmannin, two drugs which normally prevent S6K1 activation).

Because the activation pattern of S6K1 closely parallels that of 4E-BP1 phosphorylation, it was postulated that S6K1 could be upstream of 4E-BP1 (128). However, subsequent

studies have shown that S6K1 lies on a pathway that is parallel to, rather than upstream of, 4E-BP1, and both proteins apparently share a common upstream activator (223). This conclusion is based on the demonstration that a rapamycin-resistant form of S6K1 (all the phosphorylation sites are mutated to acidic residues) is unable to confer rapamycin resistance to 4E-BP1 (223). Instead, overexpression of the rapamycin-resistant S6K1, or any other S6K1 protein, decreased the phosphorylation of 4E-BP1 in the presence or absence of rapamycin (223). These data suggest that overexpression of S6K1 titrates out an upstream kinase regulating 4E-BP1 phosphorylation.

1.8.3 Components of the signaling pathway to 4E-BP1

The results with the pharmacological inhibitors wortmannin and LY294002, as well as the data obtained with the mutants of the PDGF receptor, pointed to a role for phosphoinositide-3 kinase (PI3K) in mediating 4E-BP1 phosphorylation. Furthermore, the sensitivity of 4E-BP1 to rapamycin indicated that the activity of the kinase FRAP/mTOR is necessary to relay signals to 4E-BP1. (The role of different members of this pathway in mediating 4E-BP1 phosphorylation will be presented in chapters 3-5). The function of these proteins is described below, with more emphasis on FRAP/mTOR, the most proximal kinase to 4E-BP1.

1.8.4 PI3K, and phosphatase and tensin homolog deleted from chromosome 10 (PTEN)

While phosphoinositides (or phosphatidylinositols; PtdIns) represent a small percentage of the total phospholipids, they play a crucial role in signaling, and act as second messengers for several cell processes. The PI3Ks constitute a large family of lipid kinases that phosphorylate inositides on the third position of the inositol ring. PI3Ks have been assigned to several families based on their primary structural features, mode of regulation, and lipid specificity (this discussion applies to class I PI3Ks; for a thorough review, refer to 224). The prototype of the class I PI3Ks is a dimer consisting of a

catalytic subunit of 110kDa (p110α), and a regulatory subunit of 85kDa (p85α). The N-terminal region of the catalytic p110 interacts with p85, and the C-terminal portion of the protein contains the catalytic domain. Between these domains are located a region which binds to Ras, and a "PIK domain" found in other phosphoinositide kinases. The regulatory p85 protein does not exhibit known enzymatic activity, and is composed of several protein-binding modules, such as a src-homology 3 (SH3) domain, two proline-rich fragments, two src-homology 2 (SH2) domains, and a binding site for the p110 subunit. The activity of PI3K is regulated by Ras proteins, at least for certain cellular responses (reviewed in 224). Furthermore, p85 regulates the activity and subcellular localization of PI3K through binding of its SH2 domains to phosphotyrosyl (pTyr) residues found, for example, in activated tyrosine-kinase receptors. Additional interactions between the proline-rich motifs of p85 and the SH3 motifs of src, lck, lyn, fyn, and abl were also reported (224). The interplay between membrane localization, binding to the several signaling molecules, and activation by Ras remains to be fully characterized.

PI3Ks have been implicated in the regulation of a variety of cellular processes, including cell survival, cell motility, proliferation, and differentiation. In response to extracellular stimuli, the regulatory PI3K subunit is recruited to the membrane, bringing the catalytic subunit close to its lipid substrates. Phosphorylated lipids act as second messengers to recruit and activate downstream targets, including the serine/threonine kinase Akt/PKB (protein kinase B; section 1.8.5). PTEN (phosphatase and tensin homolog deleted from chromosome 10; a.k.a. MMAC, mutated in multiple advanced cancers; or TEP, TGFβ-regulated and epithelial cell enriched phosphatase) is a lipid phosphatase that dephosphorylates PI3K lipid products and thus negatively regulates PI3K signaling (reviewed in 225, 226).

In addition to their well-characterized lipid kinase activity, PI3Ks exhibit some protein kinase activity. For example, p110α can phosphorylate its regulatory subunit, as well as the insulin receptor substrate-1 (IRS-1), at least *in vitro*, and possibly *in vivo* (reviewed in 224). The physiological consequences of these phosphorylation events remain, however, poorly understood.

Stimulus-induced 4E-BP1 phosphorylation is blocked by the inhibitors wortmannin (100 nM) or LY294002 (5 μM; e.g. chapters 3-5 and refs 128, 132). Wortmannin irreversibly inhibits the lipid and protein kinase activity of class I PI3Ks (IC50 in the range of 1-10nm *in vitro*) by covalently binding to a residue required for catalytic activity, Lys802 (227). LY294002 is a reversible inhibitor of class I PI3Ks (IC50 of ~1μM; 228). These two unrelated inhibitors were originally thought to be specific for PI3Ks (229). However, certain PI4Ks and members of a related kinase family, the phosphoinositide-kinases related kinases (PIKKs; see below), are also inhibited by these drugs, albeit at higher concentrations (230-234). Therefore, care should be taken regarding the interpretation of data generated using these pharmacological inhibitors (see chapter 3).

Additional evidence for a role for PI3K in 4E-BP1 phosphorylation came from studies with mutants in the platelet-derived growth factor (PDGF) receptor and in insulin receptor substrate-1 (IRS-1). Activation of PDGF receptor mutants that are unable to recruit PI3K fail to induce 4E-BP1 phosphorylation (128). Conversely, IRS-1 mutants lacking all tyrosine residues except those that activate PI3K retain the ability to induce 4E-BP1 phosphorylation (153). In chapter 3, the role of PI3K in signaling to 4E-BP1 is further addressed.

1.8.5 Akt/PKB

The serine/threonine kinase c-Akt proteins (a family of three proteins in mammals, also known as protein kinase B; PKB) are downstream effectors of PI3K (235). c-Akt/PKB was initially identified as the cellular counterpart of v-Akt, the transforming oncoprotein of the AKT8 retrovirus (236). v-akt results from a recombination event in which the viral gag protein is fused to the Akt/PKB sequences, inducing constitutive membrane localization of the protein. Regulated membrane localization is critical for the activity of c-Akt/PKB: these proteins possess at their N-terminus a pleckstrin homology (PH) domain that specifically binds PI3K-generated lipid products (reviewed in 237). After lipid binding, Akt/PKB is targeted to membranes, where it is phosphorylated (at Thr308 and Ser473) and activated by the phosphoinositide-dependent kinase 1 (PDK1, thought to be constitutively membrane localized) and a kinase activity termed PDK2 (237, 238)

The importance of both membrane localization and phosphorylation for Akt/PKB activity is emphasized by expression of mutant proteins: Artificial membrane targeting of Akt/PKB (accomplished, for example, by the fusion of an N-terminal myristylation signal, or by the fusion with the gag protein) results in constitutive kinase activation (and an acquired resistance to PI3K inhibition; 239, 240, 241). More recently, acidic substitutions of the two phosphorylated residues (mimicking phosphorylation) was also shown to result in Akt/PKB activation (242). Furthermore, Akt/PKB proteins bearing alanine replacement of these two phosphorylated residues (non phosphorylatable Akt/PKB) exhibits dominant-negative activity (243, 244).

Akt/PKB has been implicated in a variety of cellular processes, including cell growth and proliferation, protection from apoptosis, and regulation of gene expression, including translational control (reviewed in 40, 237, 238). The best-studied function of Akt/PKB is protection from apoptosis. Expression of constitutively active Akt/PKB (in several cell

types) prevents the induction of apoptosis induced by a wide array of pro-apoptotic stimuli (including growth factor withdrawal, DNA damage, matrix detachment, etc.; reviewed in 237, 238). Endogenous c-Akt/PKB is thought to mediate (at least in part) the anti-apoptotic function of growth factors. The identification of protein targets which could mediate the anti-apoptotic functions of Akt/PKB has been facilitated by the identification of an Akt/PKB consensus phosphorylation motif, (RXRXXS/TΦ, where Φ is a bulky hydrophobic residue). This motif is present in several members of the apoptotic machinery (e.g. Bcl2 family members, caspases and caspase inhibitors), as well as in transcription factors regulating the expression of genes involved in death (e.g. Forkhead family members; reviewed in 238).

1.8.6 FRAP/mTOR

1.8.6.1 Rapamycin

Rapamycin, a lipophilic macrolide, was isolated from a strain of *Streptomyces hygroscopicus* found in a soil sample from Easter Island (known by the inhabitants as Rapa Nui; 245). This compound potently inhibits yeast cell growth, as well as the proliferation of several types of mammalian cells, including B and T lymphocytes. Rapamycin treatment results in a G1 arrest in a variety of cell types and species (reviewed in 246, 247). Due to its inhibitory effects on lymphocyte proliferation, rapamycin is a potent immunosuppressant, and effectively prevents allograft rejection (247, 248, and references therein).

1.8.6.1.1 Effects of rapamycin on translation rates in yeast

In yeast, rapamycin treatment leads to a precipitous translational arrest, accompanied by polysome disaggregation (249). The translational arrest occurs prior to, and is presumed to be the cause of, cell cycle arrest in G1 (249), which could be due to inhibition of cyclin 3 (*CLN3*) mRNA translation. Cln3p activates the transcription of late G1-specific genes,

including *CLN1* and *CLN2*, and is subject to translational control (250). *CLN3* mRNA translation is rapidly repressed following rapamycin treatment or nitrogen deprivation (251). The cycle arrest (but not the general decrease in translation initiation) can be overcome if the *CLN3* coding sequence is fused to the promoter and 5'UTR of the polyubiquitin-encoding gene *UBI4*, which renders *CLN3* translation less dependent on eIF4F. Consistent with this, in *S. cerevisiae* mutants defective in eIF4E (which also arrest in G1), expression of *CLN3* is sufficient to restore the G1 to S phase transition (252).

1.8.6.1.2 Effects of rapamycin on translation rates in mammalian cells

In mammalian cells, rapamycin treatment does not result in complete polysome dissociation; however, the translation of certain mRNAs is strongly affected by rapamycin. Many of the same mRNAs that respond to hormone and growth factor stimulation are extremely sensitive to rapamycin treatment (e.g. 253). The best studied example of rapamycin-sensitive mRNAs is the class possessing a 5'TOP (254, 255). Most 5'TOP-containing mRNAs code for components of the translational machinery, such as ribosomal proteins, elongation factors, and the poly(A) binding protein (reviewed in 21, 256, 257). Translation of these mRNAs is modulated through S6K1, as overexpression of a rapamycin-resistant form of S6K1 protects eEF1α (eukaryotic elongation factor 1α, a 5'TOP-containing mRNA) from the effects of rapamycin (258). Conversely, overexpression of a kinase dead (dominant-negative) S6K1 prevents translational up-regulation after mitogen treatment (258). How S6K1 modulates 5'TOP translation is not understood. For many years the ribosomal S6 protein was the only known S6K1 target. It was therefore postulated that 5'TOP translation was regulated through S6 phosphorylation. However, a direct proof for the involvement of S6 in 5'TOP mRNA translation control is lacking. It is noteworthy that 5'TOP translation during early *Xenopus* embryogenesis is regulated by S6K, but not by the ribosomal S6 protein. In this

system, 5'TOP mRNAs are rapamycin-sensitive, but S6 phosphorylation is unaffected by rapamycin (259). Whether this is an isolated case remains to be established.

A few examples of rapamycin-sensitive non-5'TOP mRNAs have also been identified. Rapamycin-sensitivity of the translation of some mRNAs could be mediated by the binding of specific mRNA binding proteins to *cis*-acting elements. For example, the mRNA coding for IGF-II leader 3 is found in inactive mRNPs (messenger ribonucleoproteins) in resting cells, but translation is induced in growing cells in a rapamycin-sensitive manner (260). This regulation is conferred by the 5'UTR, since translation of IGF-II leader 4, which differs only in the 5'UTR, is constitutive (260). One possible mechanism for this regulation is via the binding of a family of inhibitory proteins in the 5'UTR of the IGF-II leader 3 (and not to the leader 4; 261).

A third category of rapamycin-sensitive mRNAs are those presumed to require high amounts of eIF4F helicase activity, due to the presence of long, structured 5'UTRs (as described above). Indeed, the translation of two such mRNAs, those coding for ODC and c-myc, is strongly inhibited by rapamycin treatment (e.g. 140, 262, 263). Several other mRNAs whose translation is specifically affected by rapamycin (including Bcl3, GLUT1, and Cyclin D1) have also been identified (e.g. 2, 264, 265), but the mechanism mediating the rapamycin-sensitivity of these transcripts remains to be established. As previously discussed, a genomic-based approach will be necessary to obtain a clearer picture of the prevalence and identity of rapamycin-sensitive mRNAs.

1.8.6.2 FK506 binding proteins

FKBP12 (FK506-binding protein, MW of 12 kDa), an abundant, ubiquitously expressed protein of 108 amino acids (in humans), is the primary intracellular rapamycin receptor (266-268). FKBPs are peptidyl-prolyl cis/trans isomerases, catalyzing the

interconversion of peptidyl-prolyl bonds, and are thus presumed to play a role in protein folding (reviewed in 269). While rapamycin binding inhibits the isomerase activity of FKBP12 *in vitro* (270-272), inhibition does not appear to be the cause of rapamycin sensitivity. In fact, deletion of the four yeast FKBP genes (termed FPR1 - 4, FPR1 being the FKBP12 homolog; reviewed in 273) is not lethal, and does not significantly affect growth. Rather, deletion of FPR1 confers recessive rapamycin-resistance, indicating that the presence of Fpr1p (but not its catalytic activity) is required for rapamycin action - (270-272).

1.8.6.3 Identification of the yeast Target Of Rapamycin (TOR) proteins

A subsequent genetic screen in *S. cerevisiae* for rapamycin-resistant mutants identified two genes, *TOR1* and *2* (Target Of Rapamycin 1 and 2; a.k.a. *DRR1* and *2*, for Dominant Rapamycin Resistant), coding for two large proteins (>280kDa) sharing 67% identity at the amino acid level. Unlike the FKBPs, TOR mutants (which are point mutants, see below) act in a dominant manner (274-278). The curious results of the *FPR1* deletion experiments were explained when Tor1p and Tor2p were demonstrated to interact with an Fpr1p-rapamycin gain-of-function complex (but not with Fpr1p or rapamycin alone). Disruption of the *TOR1* gene is not lethal, but leads to a slight decrease in cell growth rate (276), whereas disruption of *TOR2* is lethal and is accompanied by random arrest throughout the cell cycle (277). Strains disrupted for both *TOR1* and *TOR2* recapitulate the phenotype observed with rapamycin treatment, in which cells arrest in G1 within one generation (276, 277). Taken together, these data indicate that Tor function (provided by either Tor1p or Tor2p) is necessary for G1 progression. The lethality of the *TOR2* deletion is not restored by overexpression of wild type or rapamycin-resistant *TOR1* proteins (279), indicating that Tor2p, in addition to a rapamycin-inhibitable activity, also possesses a rapamycin-resistant activity which is essential and not shared with Tor1p

(277, 279). The Tor2p-specific function appears to be linked to cell cycle-dependent organization of the actin cytoskeleton, which is disrupted in a *tor2* mutant (280, 281).

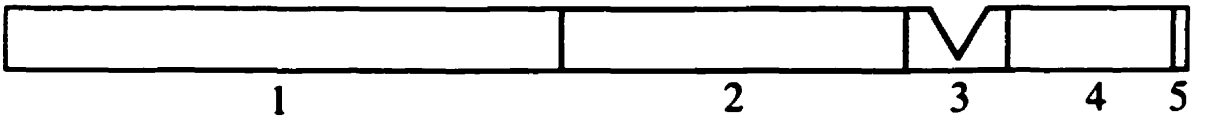
1.8.6.4 Cloning of a mammalian homolog of the TOR proteins

A mammalian homolog of the Tor proteins was purified and cloned from various species, and alternatively termed FRAP, mTOR, RAFT (rapamycin and FKBP12 target), SEP (sirolimus effector protein) and RAPT (rapamycin target; 282, 283-286). Here we refer to the mammalian protein as FRAP/mTOR. FRAP/mTOR is 289 kDa and shares 45% identity with the yeast Tor proteins (282, 285, 286). Only one homolog has been found in mammals, and the human, rat and mouse FRAP/mTOR proteins share > 95% identity at the amino acid level (reviewed in 247).

1.8.6.5 Modular structure of the Tors and FRAP/mTOR

The Tor and FRAP/mTOR proteins possess a C-terminal region (Fig. 1.8) sharing significant homology with lipid kinases (especially with PI3 kinases, and with weaker homology to PI4 kinases). Based on this homology, the Tors and FRAP/mTOR have been assigned to a larger protein family termed the PIKKs (PhosphoInositide Kinase-related Kinases). In mammalian cells, the PIKK family is comprised of FRAP/mTOR, ATM (Ataxia Telangiectasia Mutated), ATR/FRP (Ataxia Telangiectasia and Rad3 related; also known as FRP, FRAP Related Protein), and DNA-PKc (DNA-activated Protein Kinase, catalytic subunit). TRRAP (Transformation/Transcription domain-Associated Protein) also exhibits significant homology to the PIKKs, although it lacks conserved residues in its catalytic domain, and is therefore not expected to possess kinase activity. Most PIKKs are conserved from yeast to mammals, with the exception of the DNA-PKc protein, which appears to be restricted to vertebrates. PIKK family members are very large proteins, all of which have been posited to be involved in cell cycle checkpoint control.

FKBP12 rapamycin



1- HEAT domain
2- FAT domain (or toxic effector) domain
3- FRB domain
4- PI3-like kinase domain
5- PIKK-specific C-terminal fragment

In addition to the lipid kinase homology domain, all PIKKs also possess a short domain at their extreme C-terminus not present in PI3 and PI4 kinases, termed FATC (for FRAP, ATM and TRRAP, C-terminal homology domain), and a region of weaker homology termed the FAT domain (between amino acids 1382-1982 in the human FRAP/mTOR; 287, 288). Since the FAT domain is always found in combination with the FATC region, intramolecular interactions between FAT and FATC have been postulated to modulate kinase activity. The Tor FAT domain was also described independently as a "toxic effector domain", which inhibits yeast growth when overexpressed. How this portion of Tor confers a toxic effect in yeast is unknown, although it is presumed to interfere with the activity of the endogenous Tor by titrating out an effector or activator (287).

Immediately N-terminal to the kinase domain (and downstream of the FAT domain), lies the FKBP12-rapamycin binding (FRB) domain (274, 276, 277, 289). The structure of the FRB domain complexed with FKBP12 and rapamycin has been determined by X-ray crystallography, and reveals that rapamycin simultaneously occupies two hydrophobic pockets, one in FKBP12 and the other in FRAP/mTOR (290). While limited interactions occur between the two proteins, rapamycin establishes several contacts with both polypeptides, and is thus able to induce an FKBP12-FRAP/mTOR interaction (290). The yeast Tor mutants selected in the original genetic screens for rapamycin resistance possess mutations at Ser1972 or Ser1975 (in Tor1p and Tor2p, respectively, corresponding to Ser2035 in FRAP/mTOR). Mutation of this serine to bulkier residues (isoleucine, threonine, or glutamic acid) engenders rapamycin-resistance, while mutation of this serine to alanine, a smaller amino acid, does not affect rapamycin binding. The structure of the FRB domain complexed with FKBP12 and rapamycin explains the requirement for a small residue at this position, as a bulkier residue would be expected to prevent rapamycin binding in the hydrophobic pocket (290).

The first 1200 amino acids of the Tor and FRAP/mTOR proteins comprise a "HEAT" domain (named for Huntingtin, Elongation factor 3, the regulatory A subunit of PP2A and Tor1p, the first proteins found to possess such a motif; 291, 292, 293). This motif consists of stretches of ~40 amino acids in at least three repeats, and displays a consensus pattern of hydrophobic, proline, aspartic acid and arginine residues. HEAT repeats have also been described in several other proteins (including eIF4G; 294), many of which function as adapters or scaffolds. HEAT domains form curved rods consisting of α-loop-α repeats, providing a large hydrophobic surface area for protein-protein interactions (293-297).

A protein termed gephyrin interacts with a region of the FRAP/mTOR HEAT domain (aa 1017 – 1046; 298). Gephyrin is a tubulin-binding protein involved in postsynaptic clustering of neuronal glycine receptors, and is presumed to modulate the intracellular localization of FRAP/mTOR. Mutations that abrogate gephyrin binding also prevent FRAP/mTOR signaling to two downstream translational targets, the ribosomal S6 kinase 1 (S6K1) and 4E-BP1 (298). In yeast, the HEAT repeats are responsible for localization of Tor2p to the plasma membrane, most likely through protein-protein interactions with membrane-associated proteins (299).

1.9 Objectives of the studies

At the time I joined the laboratory, in 1994, the eIF4E-binding proteins 1 and 2 (4E-BP1 and 2) had just been cloned by Arnim Pause, through interaction with eIF4E (195). Computer searches had revealed that 4E-BP1 was the human homolog of the rat protein called PHAS-I, which had been cloned in the laboratory of Dr. John C. Lawrence a few months earlier. Interestingly, PHAS-I was cloned by Lawrence's group because it was one of the major phosphorylation substrates detected after insulin stimulation in rat adipocytes. Dr. Graham Belsham, then a visiting scientist in our laboratory, had been one

of the first to describe the phosphorylation of 4E-BP1 in the early 1980's, and he was able to demonstrate that stimulation of freshly isolated rat adipocytes with insulin resulted in a drastic decrease in the ability of 4E-BP1 to bind to eIF4E. Drs Pause and Belsham also demonstrated that expression of 4E-BP1 and 4E-BP2 decreased cap-dependent, but not cap-independent, translation, both *in vitro* and *in vivo* (195). We were thus immediately interested in assessing whether the 4E-BPs could take part in the shutoff of cellular protein synthesis induced following infection with viruses that translate in a cap-independent manner, or with a reduced requirement for eIF4E. In particular, we were interested in monitoring the phosphorylation state and the abundance of these translational inhibitors following infection. These experiments are presented in chapter 2. As discussed in section 1.8, the initial data from Dr. Lawrence's group had suggested that the MAP kinases (ERKs) were responsible for phosphorylation of 4E-BP1, and that phosphorylation occurred on a single residue, Ser65 (204). However, in 1996, the involvement of MAP kinases in 4E-BP1 phosphorylation was ruled out. Instead, we and others described that phosphorylation of 4E-BP1 occurred through a rapamycin- and wortmannin-sensitive pathway (128, 138, 166). We were thus interested in delineating the components of the signaling pathway to 4E-BP1 phosphorylation (chapter 3). In addition, it had also became clear that phosphorylation of 4E-BP1 occurred on multiple residues *in vivo*; we wished to study these residues, and the functional consequences of phosphorylation on the different sites. These experiments are presented in chapters 4 and 5.

CHAPTER 2

EFFECT OF VIRAL INFECTIONS ON THE TRANSLATIONAL INHIBITORS 4E-BP1 AND 4E-BP2

2.1 Introduction and perspectives

Several classes of viruses have evolved strategies to usurp their host translational machinery for the translation of viral proteins. This phenomenon, referred to as the "shutoff" of host protein synthesis, results in the selective production of viral proteins. The basis for this selective up-regulation lies in differences in the structure and/or sequence of the viral RNA versus cellular mRNAs, and in the factors required for their translation. Because 4E-BP1 can repress cap-dependent, but not cap-independent, translation, it is a likely target for those viruses that translate by a cap-independent mechanism or via a shunt mechanism, requiring less eIF4E.

This chapter describes (section 2.2) the dephosphorylation of 4E-BP1 following picornavirus (poliovirus and encephalomyocarditis virus, or EMCV) infection, and the possible implication of 4E-BP1 in EMCV-induced host protein synthesis shutoff. As described in section 1.5.2, picornavirus RNAs are naturally uncapped, and translate via internal initiation. Infection with most picornaviruses results in the shutoff of host cell protein synthesis (reviewed in 22, 91). The shutoff of host protein synthesis by picornaviruses was known to be exerted at the level of translation, since cellular mRNAs can be recovered from virus-infected cells in an intact and functionally active form (300-304). The mechanism of the shutoff was partly understood for some picornaviruses (enteroviruses, rhinoviruses and aphthoviruses). At the time when these studies were undertaken, it had been described that eIF4GI was cleaved into two fragments following poliovirus infection (22, 91, 305). Infection with several other members, but not all, of the picornavirus family, including other enteroviruses (coxsackie viruses), aphthoviruses (foot-and-mouth disease virus), and rhinoviruses, also results in the cleavage of eIF4GI (22). However, cleavage of eIF4GI alone cannot account for the virus-induced shutoff of host protein synthesis, because it precedes the shutoff of host protein synthesis by at least 30 minutes (e.g. 305). Furthermore, in cells treated with inhibitors of poliovirus

replication, such as guanidine hydrochloride, monensin, or nigericin, the shutoff of host protein synthesis is incomplete, yet the kinetics of eIF4GI cleavage are unaffected (306-308). EMCV, a cardiovirus, also causes a shutoff of host protein synthesis, but this inhibition occurs late in infection as compared to poliovirus (309). Also, in contrast to poliovirus, no cleavage of eIF4G occurs in EMCV infected cells (310). *In vitro* translation studies showed a general decrease in the ability of EMCV-infected cell extracts to translate both viral and cellular exogenous mRNAs, rather than a specific inhibition of cellular mRNA translation, such as is observed in extracts from poliovirus-infected cells (309, 311-313). This is in contrast to the *in vivo* studies showing a selective shutoff of host protein synthesis.

In the second part of this chapter (section 2.3), I investigated the phosphorylation of 4E-BP1 following adenovirus infection. Transcription of the adenovirus genome is divided into two phases: early and late expression. Early adenovirus gene expression can be detected within one hour after infection. During this phase, transcripts encoding proteins necessary for viral DNA replication, activation of late viral genes, and suppression of the host immune response are produced. Early phase translation is cap-dependent (for a thorough review, refer to 210). Late adenovirus gene expression (detected between 10 and 18 hours after infection of HeLa cells) is responsible for the production of large quantitites of polypeptides required for the assembly of the viral capsid. Most late adenovirus transcription is initiated from the major late promoter, which directs the synthesis of a single RNA precursor. This RNA is then alternatively spliced, polyadenylated and translated to generate the various late protein products. All of the transcripts from the major late promoter possess a common 5'UTR of 212 nucleotides called the tripartite leader (formed by the splicing of three small exons). Adenovirus mRNAs, which are transcribed by host RNA polymerases, are naturally capped. However, translation of the late viral mRNAs, which possess the tripartite leader, occurs

via ribosome shunting, a mechanim that requires less eIF4F activity. The effects of adenovirus infection on cellular protein synthesis is consistent with these differences between translation of early and late gene products. Early in infection, translation of cellular mRNAs is mildly increased in infected cells, consistent with a requirement for the virus to translate its early proteins by a cap-dependent fashion. However, in the late phase of adenovirus infection, a sharp decrease in host protein synthesis is observed. This has been attributed partly to inhibition of cellular mRNA export by the virus (the tripartite leader-containing mRNAs can be efficiently exported under these conditions). In addition, viral mRNAs are selectively translated; even those cellular mRNAs which are transported into the cytoplasm are not efficiently translated. Adenovirus late mRNAs can translate in poliovirus-infected cells or in cells arrested in mitosis (115, 314, 315), two conditions that interdict translation of cellular mRNAs. In the late phase of adenovirus infection, eIF4E becomes dephosphorylated by an unknown mechanism, coincident with the shutoff of host protein synthesis (115). When eIF4E is dephosphorylated, ribosome jumping is more prominent (86), although the role of eIF4E dephosphorylation per se in this effect is unknown. Several drugs that inhibit the shutoff of protein synthesis by adenovirus also inhibit eIF4E dephosphorylation (316, 317).

2.2 Activation of the translational suppressor 4E-BP1 following infection with encephalomyocarditis virus and poliovirus

2.2.1 Abstract

Infection of cells with picornaviruses, such as poliovirus and encephalomyocarditis virus (EMCV), causes a shutoff of host protein synthesis. The molecular mechanism of the shutoff has been partly elucidated for poliovirus, but not for EMCV. Translation initiation in eukaryotes is facilitated by the mRNA 5' cap structure to which the multisubunit translation initiation factor eIF4F binds to promote ribosome binding. Picornaviruses use a mechanism for the translation of their RNA which is independent of the cap structure. Poliovirus infection engenders the cleavage of the eIF4G component of eIF4F and renders this complex inactive for cap-dependent translation. In contrast, EMCV infection does not result in eIF4G cleavage. Here, we report that both EMCV and poliovirus activate a translational repressor, 4E-BP1, which inhibits cap-dependent translation by binding to the cap-binding subunit eIF4E. Binding of eIF4E occurs only to the underphosphorylated form of 4E-BP1 and this interaction is highly regulated in cells. We show that 4E-BP1 becomes dephosphorylated upon infection with both EMCV and poliovirus. Dephosphorylation of 4E-BP1 temporally coincides with the shutoff of protein synthesis by EMCV, but lags behind the shutoff and eIF4G cleavage in poliovirus infected cells. Dephosphorylation of 4E-BP1 may participate in the shutoff phenomenon in EMCV infected cells, by specifically inhibiting cap-dependent translation.

2.2.3 Results

2.2.3.1 4E-BP1 phosphorylation is reduced in EMCV infected cells.

Infection of Krebs II ascites cells with EMCV caused a reduction in host cell protein synthesis (Fig. 2.1A). Shutoff of host protein synthesis was detectable 4 h after infection and was almost complete at 5h post-infection (Fig. 2.1A, lanes 4 to 9). Two different strains of EMCV varied in the kinetics of the shutoff. The shutoff was more pronounced for the EMCV K-2 than for the EMCV R strain (Fig. 2.1A, compare lanes 5 and 6). Therefore, the strain EMCV K-2 was chosen for subsequent studies. To determine whether the 4E-BP1 phosphorylation state changes upon virus infection, a Western analysis was performed with a polyclonal antibody against 4E-BP1. Two forms of 4E-BP1 were present in mock-infected cells (Fig. 2.1B, indicated by the two upper arrowheads; lane 1). Between 3 and 4h after infection, the intensity of the upper band began to diminish and a faster migrating band appeared (Fig. 2.1B, indicated by an arrow; lanes 10 and 11). According to previous studies in mouse cells, the new band is probably the unphosphorylated form of 4E-BP1, which migrates faster than the phosphorylated forms (138, 157, 166). In a recent report, Lin et al. resolved by two-dimensional gel three forms of 4E-BP1. The faster migrating form did not contain any phosphate (138). A Far-western analysis, using a heart muscle kinase-eIF4E probe, was performed on the same samples to determine whether the modification of 4E-BP1 affected its binding to eIF4E. In mock-infected cells, eIF4E interacted only weakly with the two forms of 4E-BP1 (Fig. 2.1C, lanes 1-6), again suggesting that these are the phosphorylated forms, which have a low affinity for eIF4E. The signal on the Far-Western blot with mock-infected cells slightly increased with time (Fig. 2.1C, lanes 1-6), probably as a consequence of serum deprivation (A.-C. G., unpublished data). In sharp contrast, when cells were infected with EMCV, a strong interaction of eIF4E with the faster migrating form of 4E-BP1 was detected (Fig. 2.1C, lanes 11 and 12), further supporting the notion that this infection-induced 4E-BP1 species is the

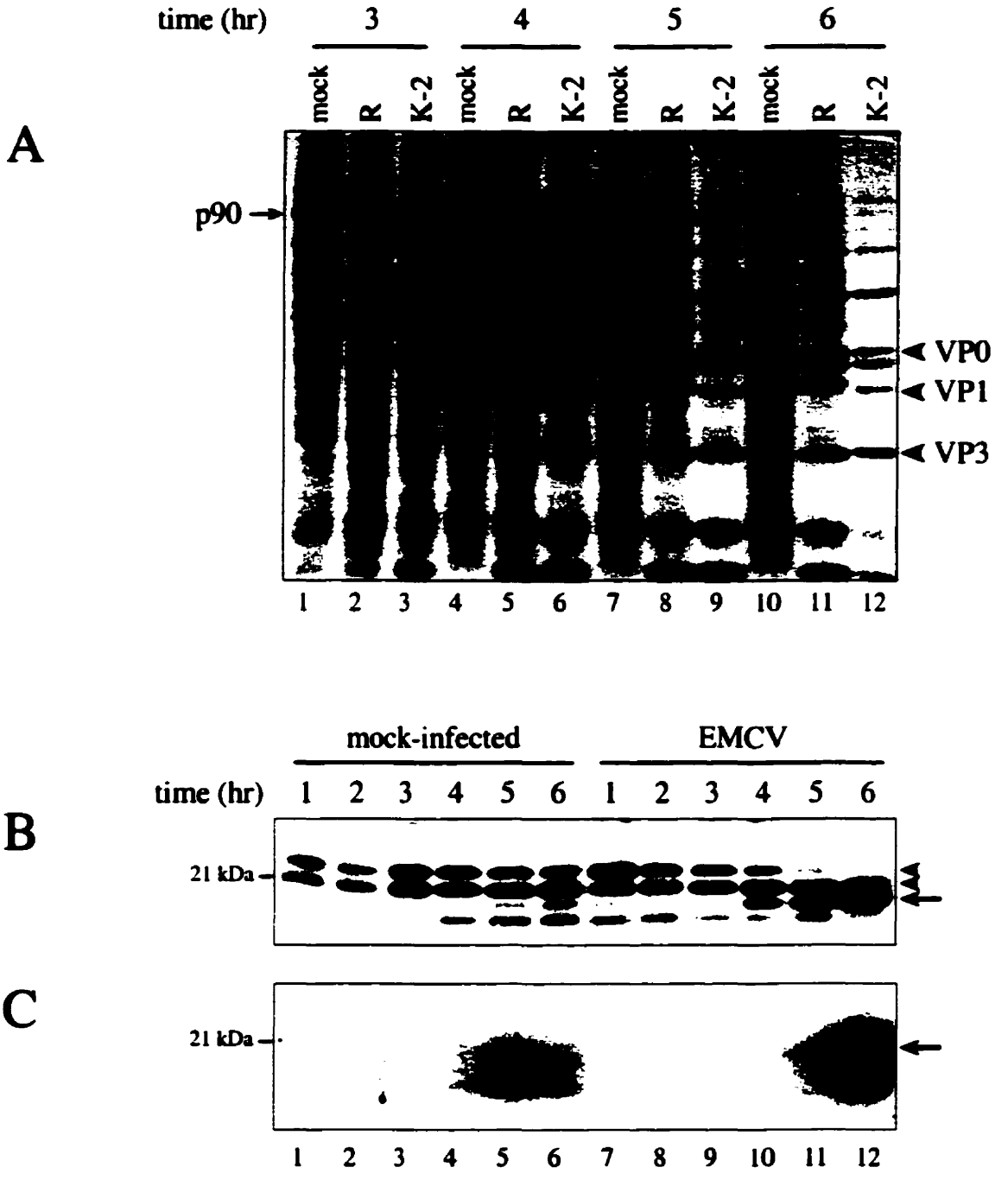


Figure 2.1. EMCV infection leads to 4E-BP1 dephosphorylation. (A) Time course of the shutoff. Krebs II mouse ascites were labeled with [^{35}S] methionine at the indicated times after infection (R and K-2 strains of EMCV) and cytoplasmic protein extracts were analyzed by SDS-PAGE. The arrow indicates an arbitrarily chosen cellular protein (p90), which was used to measure the inhibition of host protein synthesis, and the arrowheads indicate virus capsid proteins. (B) Heat-treated total extracts were subjected to Western blotting and probed with a polyclonal 4E-BP1 antibody. (C) Extracts prepared and blotted as in (B) were probed with ^{32}P-labeled eIF4E. The analyses were performed as described in Materials and Methods.

hypophosphorylated form. Taken together, these results show that the kinetics of dephosphorylation of 4E-BP1 temporally coincides with that of the shutoff of the host protein synthesis, and with the increased binding of 4E-BP1 to eIF4E. Similar results were also obtained with the EMCV R strain, but with slower kinetics of dephosphorylation of 4E-BP1 (data not shown), in accordance with the finding that this virus causes a slower shutoff.

2.2.3.2 Translation extracts prepared from EMCV-infected Krebs II cells do not reproduce the in vivo inhibition of cap-dependent translation and 4E-BP1 dephosphorylation.

Because eIF4E is necessary for cap-dependent, but not for cap-independent translation, its sequestration by 4E-BP1 would be expected to specifically inhibit cap-dependent translation in an *in vitro* translation extract prepared from EMCV-infected cells. However, numerous earlier studies reported that extracts prepared from EMCV-infected cells did not exhibit any selectivity in cap-independent versus cap-dependent translation (302, 309, 312, 313). These findings are in apparent contradiction with the *in vivo* data that point to a role for 4E-BP1 in the mediation of the shutoff of host protein synthesis exerted by EMCV. To address this apparent discrepancy, we analyzed the status of 4E-BP1 phosphorylation in translation extracts prepared from EMCV-infected cells. Krebs II ascites translation extracts were prepared either from mock- or 5h post-infected cells. Globin RNA and CAT RNA (which are capped transcripts) and the EMC-CAT (which is uncapped and translates in a cap-independent fashion, driven by the EMCV IRES) have been used in this study. In accordance with previous studies (302, 309, 312, 313), all mRNAs tested, regardless of their dependency on the cap for translation, were translated less efficiently in extracts from EMCV-infected as compared to their translation in mock-infected cells. The cap-dependent globin mRNA translation decreased 2.5 and 8 fold in EMCV R and K-2-infected Krebs II ascites translation extracts, respectively, as

compared to translation in extracts from mock-infected cells (Fig. 2.2A, lanes 1 to 3). Translation of CAT mRNA was also reduced 2.5 and 6 fold (lanes 4 to 6; the position of the CAT protein is indicated by an arrowhead; the lower band is probably due to initiation at a downstream AUG). Significantly, cap-independent translation of EMC-CAT mRNA was also reduced to the same extent as globin and CAT translation (lanes 7 to 9).

The phosphorylation state of 4E-BP1 in the translation extracts was monitored by Western analysis. No difference in the migration pattern of the different forms of 4E-BP1 between mock-infected and 5h post-infected cells was evident with the two different strains of EMCV (Fig. 2.2A, inset). Cap-dependent translation thus occurred in translation extracts prepared from EMCV-infected cells, probably because of the absence of the dephosphorylated form of the repressor 4E-BP1. The difference between the phosphorylation state of 4E-BP1 in cell lysates and in *in vitro* translation extracts can therefore explain the discrepancy between the *in vivo* and *in vitro* patterns of protein synthesis after EMCV infection. A kinase activity which rephosphorylates 4E-BP1 could therefore be active in translation extracts.

An alternative, but less likely, explanation is that cap-dependent translation in extracts prepared from ascites cells are resistant to inhibition by 4E-BP1. To rule out this possibility, the effects on translation of the addition of GST-4E-BP1 to ascites extracts were examined. Addition of GST-4E-BP1 reduced by 2.5 fold the translation of globin mRNA and by twofold that of CAT mRNA (Fig. 2.2B, compare lane 2 and 4 to lanes 1 and 3). GST-4E-BP1 had only a marginal effect on the translation of EMC-CAT mRNA (Fig. 2.2B, lanes 5 and 6). Similar results were also obtained using a bicistronic RNA, in which the expression of the CAT is cap-dependent, and the expression of the luciferase (LUC) is cap-independent (data not shown). Furthermore, addition of GST-

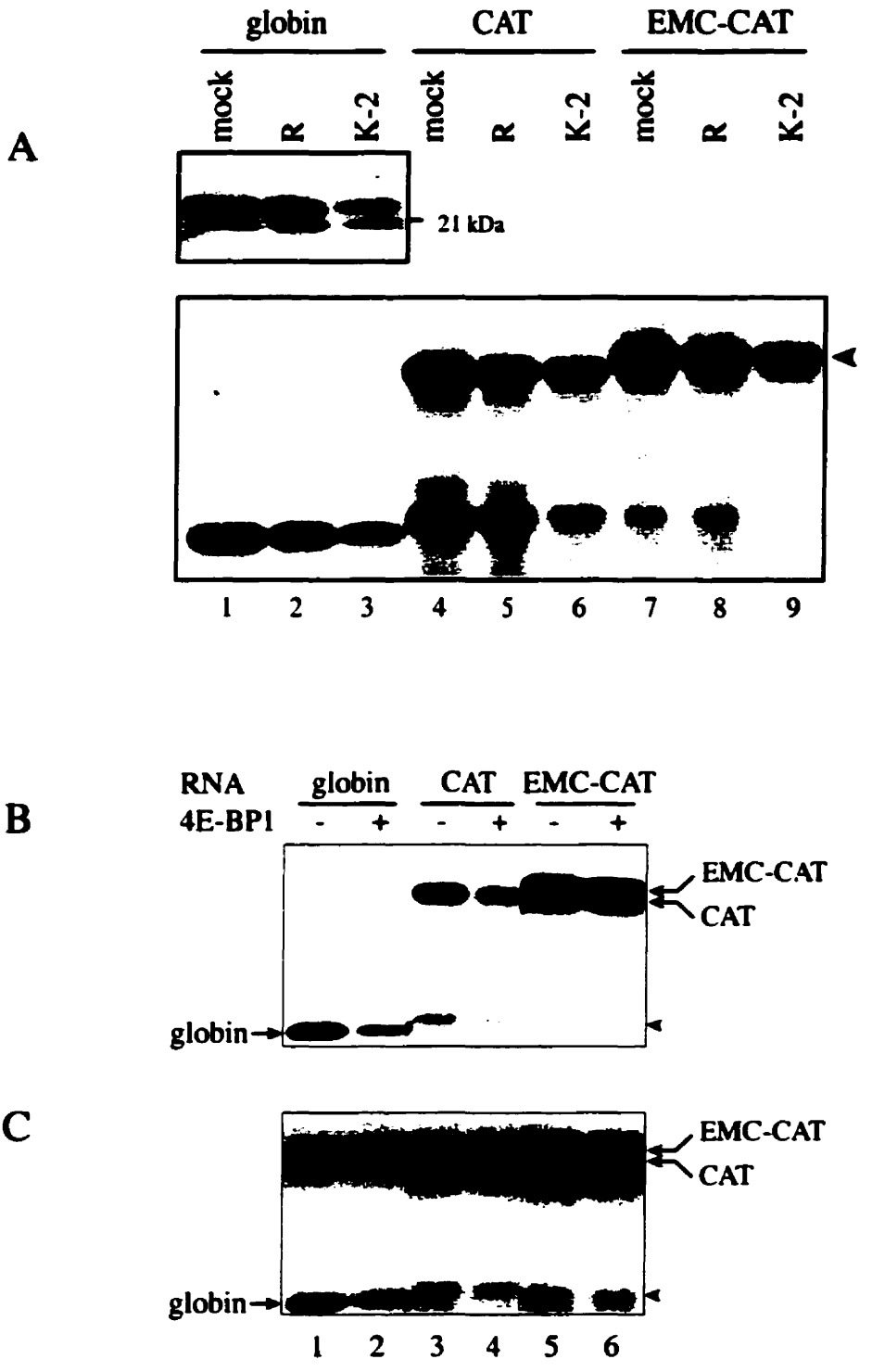


Figure 2.2. EMCV-infected cell extracts do not show selective cap-independent translation. (A) Extracts for translation were prepared from mock or EMCV-infected (R and K-2 strain) Krebs II ascites cells and used to translate globin, CAT (cap-dependent) or EMC-CAT (cap-independent) mRNAs. Extracts were heat-treated and analyzed by Western blotting with a polyclonal 4E-BP1 antibody (inset). (B) Extracts prepared from mock-infected Krebs II ascites cells were used to translate globin, CAT and EMC-CAT mRNAs in the presence or absence of 0.1μg GST-4E-BP1. (C) Translation was performed as described for (B), except that extracts were prepared from EMCV-K-2 infected cells.

4E-BP1 to a translation extract prepared from EMCV-infected ascites cells also selectively inhibited translation of cap-dependent mRNAs, although the extent of inhibition was smaller. The translation of globin mRNA and that of the CAT mRNA was decreased by approximately 1.5 fold (Fig. 2.2C, compare lanes 2 and 4 to lanes 1 and 3). In contrast, the translation of the EMC-CAT mRNA is not affected by the addition of 4E-BP1 (Fig. 2.2C, compare lane 6 to lane 5). Taken together, these results indicate that 4E-BP1 is inhibitory in *in vitro* translation extracts prepared from ascites cells, provided it is dephosphorylated. Thus, the lack of inhibition of cap-dependent translation *in vitro* could be explained by the absence of dephosphorylated 4E-BP1.

2.2.3.3 4E-BP1 is underphosphorylated upon infection of HeLa cells with poliovirus.
In poliovirus-infected cells, eIF4G is rapidly cleaved (22, 305). This event precedes the shutoff of host protein synthesis. However, this by itself is not sufficient for complete inhibition of protein synthesis (306, 308). We were therefore interested to determine the fate of 4E-BP1 phosphorylation in these cells. Cellular protein synthesis in poliovirus-infected HeLa cells was dramatically reduced as early as 2.5 hours after infection (Fig. 2.3A). The cleavage of eIF4G as assessed by Western blotting was first detected at 1.5h post-infection and was complete at 3h (Fig. 2.3B, compare lanes 2 and 4 to lanes 1 and 3), confirming that the cleavage of eIF4G precedes the shutoff of host protein synthesis. To analyze the status of 4E-BP1 phosphorylation following poliovirus infection, a Western blot analysis of 4E-BP1 was performed. In mock-infected cells (Fig. 2.3C, lane 1) three forms of 4E-BP1 were detected by the antibody (indicated by arrowheads; note that in human cells, three forms, as opposed to two species in mouse cells, are observed). At 4.5h after infection (4 h in other experiments), the slower migrating band completely disappeared, and the faster migrating bands became enhanced and shifted downwards on the gel (Fig. 2.3C, lanes 4 and 6). To determine whether the binding of 4E-BP1 to eIF4E was affected upon poliovirus infection, a Far Western analysis was performed. In mock-

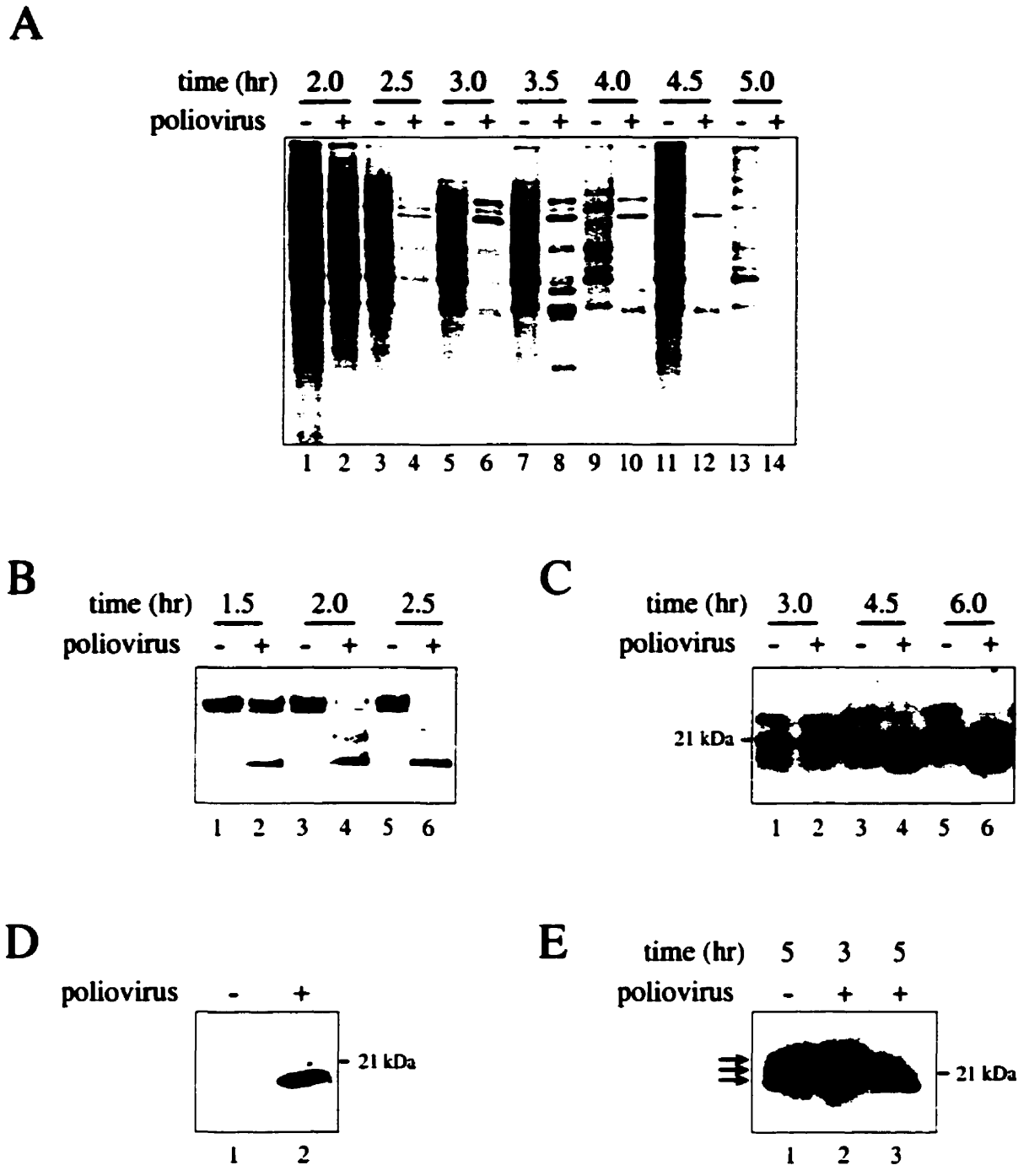


Figure 2.3. Modification of 4E-BP1 following infection by poliovirus. (A) Time course of the shutoff. HeLa cells infected with poliovirus and mock-infected cells were labeled for 30 min with [^{35}S]methionine at the indicated times after infection. Aliquots were analyzed by SDS-PAGE and autoradiography. (B) SDS-8% PAGE followed by Western blotting with an antibody against eIF4G was conducted using 25μg total protein. (C) Western analysis for 4E-BP1 was performed as described in Materials and Methods. (D) Far-Western analysis on 25μg of protein, using a ^{32}P-labeled eIF4E probe, was conducted as described in Materials and Methods. (E) ^{32}P-labeling of poliovirus-infected cells followed by immunoprecipitation with a polyclonal 4E-BP1 antibody.

infected HeLa cells, the eIF4E probe did not interact significantly with 4E-BP1 (Fig. 2.3D, lane 1). Binding of the eIF4E probe to 4E-BP1 was strongly enhanced 6 hours post-infection (Fig. 2.3D, lane 2), and the binding occurred with the bands that were shifted downwards on the Western blot shown in Fig. 2.3C. To further substantiate the conclusion that the two novel bands, which are shifted downwards, are due to 4E-BP1 dephosphorylation, 4E-BP1 was immunoprecipitated from [^{32}P] labeled cells. In mock-infected cells, three bands (Fig. 2.3E, lane 1, indicated by arrows) were detected. At 3h post-infection, these three bands were still present. However, the slower migrating band disappeared 5h post-infection and there was a global decrease of ~70% in [^{32}P] incorporation (Fig. 2.3E, compare lane 3 to 1). Thus, poliovirus infection causes the dephosphorylation of 4E-BP1. However, the dephosphorylation occurs almost two hours after the shutoff of host protein synthesis.

2.2.3.4 Guanidine prevents 4E-BP1 dephosphorylation induced by poliovirus infection. Guanidine hydrochloride inhibits poliovirus replication (311, 318). In its presence, infection results in a partial shutoff of host mRNA translation (306). Strikingly, guanidine hydrochloride does not prevent cleavage of eIF4G, suggesting that a second additional event is required for the complete shutoff of host protein synthesis (306). At 5.5h after infection, in the presence of 1.5mM guanidine hydrochloride, approximately 60% of host cell protein synthesis was inhibited (Fig. 2.4A, compare lanes 2 and 4). Under these conditions, all the eIF4G was cleaved (Fig. 2.4B). The kinetics of eIF4G cleavage was similar in guanidine-treated cells as compared to untreated cells (data not shown). However, the dephosphorylation of 4E-BP1 did not occur in the presence of guanidine (Fig. 2.4C, compare lanes 2 and 4), suggesting that replication of poliovirus and subsequent translation are required for dephosphorylation of 4E-BP1.

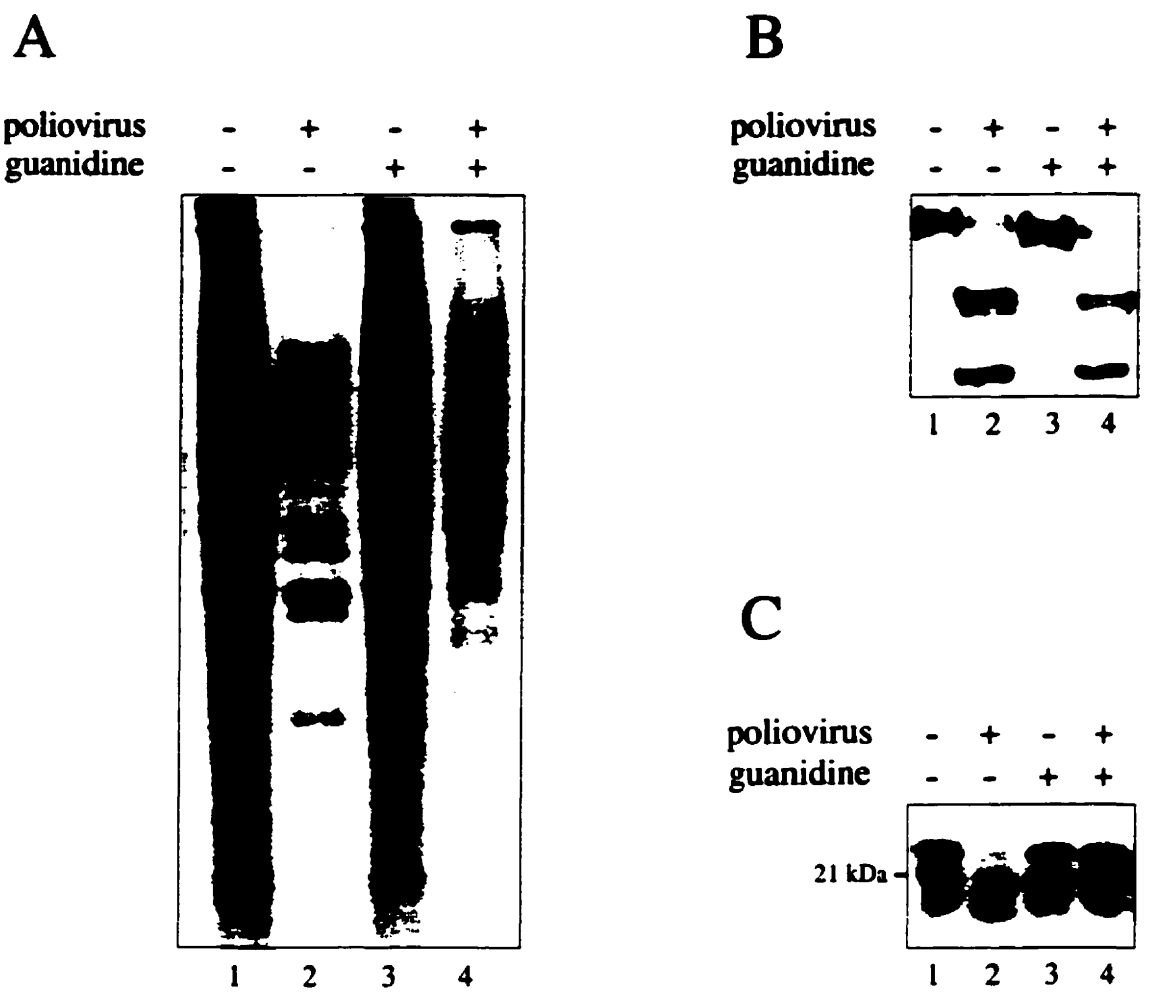


Figure 2.4. Effect of guanidine hydrochloride on eIF4G cleavage and 4E-BP1 dephosphorylation following poliovirus infection. HeLa cells were infected with poliovirus for 5 hr in the presence or absence of guanidine hydrochloride (1.5mM). Guanidine hydrochloride was added immediately following virus adsorption. (A) Shutoff of protein synthesis was monitored by [^{35}S] methionine labeling as described in Materials and Methods. (B) Extracts from cells treated as in (A) were analyzed by SDS-6% PAGE followed by Western blotting with an antibody directed against eIF4G. (C) Cell extracts as in (A) and (B) were heat-treated and analyzed by SDS-15% PAGE, followed by Western blotting for 4E-BP1 as described in Materials and Methods.

2.2.4 Discussion

The best characterized mechanism for the shutoff of host protein synthesis by viruses is that used by poliovirus. Numerous studies showed that the poliovirus 2A protease is responsible the inactivation of the cap-binding complex eIF4F by cleavage of its eIF4G component (for review, see 22, 91). However, cleavage of eIF4G does not occur following EMCV infection. Here, we demonstrated that 4E-BP1, a suppressor of cap-dependent initiation of translation, is activated by dephosphorylation following infection by EMCV, thus providing an attractive explanation for the shutoff of host protein synthesis by EMCV.

At the time of the publication of this report, the only initiation factor whose activity had been reported to be modified by EMCV was eIF2α. This protein is phosphorylated by the double-stranded (ds)RNA-dependent kinase (PKR) as a result of infection with several viruses (319). Phosphorylation of eIF2α would be expected to cause a general inhibition of translation rather than a specific shutoff of host protein synthesis, since this factor is required for the formation of the Met-tRNA ternary complex and ribosome binding to all mRNAs, including viral RNAs. However, it has been postulated that phosphorylation of eIF2α results in a stronger competition between cellular and viral RNA for the reduced amount of eIF2-GTP-Met-tRNA complex, and this competition was suggested to explain in part the shutoff (303, 320, 321). Another hypothesis posited that the changes in the intracellular ion concentrations upon EMCV infection selectively inhibit cellular mRNA translation (322). Our results provide a simple, alternative and attractive explanation for the preferential translation of EMCV RNA in infected cells. After the publication of this report, it was also reported that EMCV infection induced a dephosphorylation of eIF4E itself (121). Whether the dephosphorylation of 4E-BP1 and that of eIF4E both contribute to the shutoff is unclear. It is also possible that the two events are linked, especially because eIF4E is phosphorylated by an eIF4G-associated

kinase, MnkI (section 1.7.1). Thus if eIF4E is sequestered by 4E-BP1 and has no access to eIF4G and MnkI, its phosphorylation is expected to decrease, with kinetics depending on the turnover rate of phosphate.

A difficulty that has seriously hampered the studies on the mechanism of the shutoff of host protein synthesis by EMCV is that extracts prepared for *in vitro* translation do not recapitulate the *in vivo* discrimination against capped cellular mRNAs (302, 313, 322). As in previous studies, we did not observe a selective translation of viral RNA *in vitro*. However, the *in vitro* translation extracts from EMCV-infected cells did not contain underphosphorylated form of 4E-BP1. Addition of bacterially expressed 4E-BP1 (unphosphorylated) to Krebs extracts, infected or uninfected with EMCV, was sufficient to selectively inhibit cap-dependent translation, as was first observed in reticulocyte lysate. Therefore, the lack of effect seen in the *in vitro* extracts is likely due to the absence of the underphosphorylated 4E-BP1. Similarly, extracts prepared from poliovirus-infected cells did not contain underphosphorylated 4E-BP1 (A.-C. G., unpublished results). Thus, it appears that the dephosphorylation of 4E-BP1 in infected cells is a reversible phenomenon, and that 4E-BP1 is readily rephosphorylated upon preparation of *in vitro* translation extracts. Indeed, translation extracts are traditionally prepared in buffers lacking any kinase or phosphatase inhibitors (several of these compounds are inhibitory to translation), as opposed to the extracts used for the Western and Far-Westerns assays.

4E-BP1 dephosphorylation following poliovirus infection lags behind the shutoff of host protein synthesis, and therefore cannot explain it. It is possible, however, that in poliovirus-infected cells, the dephosphorylation of 4E-BP1 accentuates the inhibition of host mRNA translation late in infection. Alternatively, it is also conceivable that the dephosphorylation of 4E-BP1 does not play a role in the shutoff of host protein synthesis

by poliovirus, but is a relic of evolution. Dephosphorylation of 4E-BP1 might have been used by all picornaviruses early in their evolution, but cleavage of eIF4G has evolved for some picornaviridae family members as a more efficient means to inhibit host protein synthesis. The two events, cleavage of eIF4G and the dephosphorylation of 4E-BP1, share a common target - the cap recognition process.

The dephosphorylation of 4E-BP1 is a late event in poliovirus infection that requires RNA replication and extensive viral protein expression, because it is inhibited by guanidine. This differs from eIF4G cleavage, which occurs in the absence of virus replication. It is not established that the lack of 4E-BP1 dephosphorylation in the presence of guanidine can account for the residual cellular protein synthesis, since 4E-BP1 dephosphorylation occurs after the shutoff of host protein synthesis in the absence of guanidine. The dephosphorylation of 4E-BP1 is not likely to be the result of the inhibition of protein synthesis, because inhibition of host protein synthesis by adenovirus or vaccinia virus did not lead to dephosphorylation of 4E-BP1 (section 2.3; A.-C. G., unpublished observations).

Importantly, since the publication of this report, it was reported that eIF4GII is also a target for cleavage following poliovirus infection. Shutoff probably requires cleavage of both eIF4GI and eIF4GII. Consistent with this idea, eIF4GII is cleaved after poliovirus and human rhinovirus-14 infection, but more slowly than eIF4GI (323, 324). In contrast to eIF4GI, cleavage of eIF4GII coincides with the shutoff of host cell protein synthesis. Furthermore, although the addition of guanidine hydrochloride and monensin had only a marginal effect on eIF4GI cleavage, these drugs significantly delay the cleavage of eIF4GII. Even late after infection, a large fraction of eIF4GII remains intact (324). Thus, eIF4GII cleavage, in addition to eIF4GI cleavage, is probably necessary to shut off host protein synthesis.

The involvement of 4E-BP1 in EMCV-mediated shutoff of host protein synthesis reinforces the idea that even related viruses such as EMCV and poliovirus have evolved different mechanisms to maximize their translation, and that the shutoff is a complex event involving many factors. However, our data do not exclude the contribution of other mechanisms, such as competition between RNAs, or cellular permeabilization and differences in ionic conditions that favor the translation of viral mRNAs.

Since the publication of this manuscript, and in light of the inhibition of 4E-BP1 phosphorylation by rapamycin, it was demonstrated that treatment of cells with rapamycin mildly augments the rate of poliovirus and EMCV replication and the shutoff of host protein synthesis (325). The effects of rapamycin (and wortmannin) on viral protein synthesis are more pronounced with an EMCV replication mutant deficient in inducing host cell protein synthesis shutoff (326). It is thus possible that 4E-BP1 dephosphorylation after rapamycin or wortmannin treatment confers a translational advantage to the viral RNA due to the decrease in cap-dependent translation.

2.2.6 Materials and Methods

2.2.6.1 Virus strains. EMCV strains K-2 (313) and Rueckert (R) (327; a kind gift from Dr. Ann Palmemberg, Univ. Wisconsin, Madison) were grown in Krebs II cells and HeLa R19 cells, respectively. Mahoney strain of poliovirus type 1 was propagated in HeLa R19 cells.

2.2.6.2 Antibodies. Antibody 11208 was raised in rabbit (Pocono Farm, Canadensis, PA) against a glutathione S-transferase (GST)-4E-BP1 fusion protein. It does not cross-react with 4E-BP2, a protein that is 56% identical to 4E-BP1 (A.-C.G., unpublished). The antibody recognizes both human and mouse 4E-BP1. α-eIF4G rabbit polyclonal antibody was kindly provided by Luis Carrasco and Isabel Novoa (Universitad Autonoma de Madrid).

2.2.6.3 Cell culture, infections and treatments. Mouse Krebs II ascites carcinoma cells were grown in Balb/c mice for 7 days. Cells were washed twice with Earle's solution (Gibco) and mock-infected or infected with EMCV at a multiplicity of infection of 30 plaque-forming units (pfu)/cell. After adsorption of the virus for 30 min at 25°C, mock-infected or EMCV infected cells (10^7cells/ml) were incubated in S-MEM (Gibco) at 4°C for 14h. This incubation was performed to increase the efficiency of infection (328). Cells were then transferred to 37°C and incubated with gentle agitation in suspension for the times indicated in the figure legends. HeLa R19 cells were grown in DMEM containing 10% FBS and infected in serum-free medium at a multiplicity of infection of 100 pfu/cell with poliovirus.

2.2.6.4 Metabolic labeling. Cells were washed and incubated in 1ml of methionine-free DMEM (Gibco) for 30 min with methionine (10μCi/ml; 1Ci = 37 GBq). Cells were lysed in buffer containing 0.5% Nonidet P-40, 140mM NaCl and 30mM Tris-HCl, pH

7.5, and nuclei were removed by centrifugation. The supernatants from equal numbers of cells were analyzed on SDS/12.5% polyacrylamide gels. Radiolabeled proteins were quantified using a Phosphorimager (model no. Bas 2000; Fuji).

2.2.6.5 Western analysis. Cells grown in suspension were collected by centrifugation and cells grown in monolayers were scraped in cold buffer A (20mM Tris pH 7.5, 100mM KCl, 20mM ß-glycerol phosphate, 1mM DTT, 0.25mM Na_3VO_4, 10mM NaF, 1mM EDTA, 1mM EGTA, 10nM okadaic acid and 1mM phenylmethylsulfonyl fluoride). Lysis was performed by 3 freeze-thaw cycles, cell debris was pelleted by centrifugation, and the protein concentration in the supernatant was measured by the Biorad assay. To analyze 4E-BP1, 1mg of protein in 500μl was boiled for 7 min and then incubated on ice for 10 min. The precipitated material was removed by centrifugation and protein in the supernatant was precipitated with 15% TCA for 1 h, followed by two washes with diethyl ether. The pellet was dissolved in Laemmli sample buffer and aliquots (20%) were loaded on a SDS/15% polyacrylamide gel. Proteins were transferred onto a 0.22-μm nitrocellulose membrane, which was then blocked in 2% milk for 1 h followed by incubation for 2 h with rabbit polyclonal antiserum 11208 against 4E-BP1 (1:1000) in Tris buffered saline including 0.5% Tween-20 (TBST). For the analysis of eIF4G, 50μg of total protein extract lysed in cold buffer A (without heat-treatment) was loaded on a SDS/8% (or 6%) polyacrylamide gel. Proteins were transferred (5 h at 40V, without methanol in the transfer buffer) onto nitrocellulose and analyzed with rabbit anti-eIF4G polyclonal antibody (1:2500 in TBST). Incubation with secondary antibody was performed with peroxidase coupled donkey anti-rabbit Ig (1:5000 in TBST). Detection of peroxidase coupled antibody was performed using chemiluminescence (ECL, Amersham). In some experiments, ^{125}I-protein A (Amersham) was used (1:1000) and the signal was quantified using Phosphorimager analysis.

2.2.6.6 Far Western analysis of 4E-BP1. Heat-treated extracts were processed on a SDS/15% polyacrylamide gel as described for the Western analysis above, and proteins were transferred onto nitrocellulose membranes. Membranes were blocked in 5% milk in HBB (25mM Hepes-KOH, pH 7.7, 25mM NaCl, 5mM $MgCl_2$, 1mM DTT, 0.1% Nonidet P-40) for 2 h. Membranes were then incubated for 12 h in hybridization buffer (20mM Hepes-KOH, pH 7.7, 75mM KCl, 2.5mM $MgCl_2$, 0.1mM EDTA, 1mM DTT, 0.1% Nonidet P-40, 1% skim milk) containing ^{32}P-labeled HMK-eIF4E probe at 250,000 cpm/ml, as described (195, 329). After three washes with hybridization buffer, the membranes were dried and exposed against an X-ray film.

2.2.6.7 In vitro translation. Extracts prepared from mock-infected and EMCV-infected cells were treated with micrococcal nuclease (330) and translation was performed with [^{35}S]methionine in a volume of 12.5μl at 30°C for 90 min, as described (331). Following translation, reaction mixtures were supplemented with 2.5μl of a solution containing 6 mg RNAse A per ml and 60 mM EDTA and incubated at 30°C for an additional 5 min. Products of *in vitro* translation reactions were analyzed by electrophoresis on SDS/15% polyacrylamide gels. Gels were processed for fluorography with En^{3}Hance (Dupont).

2.2.6.8 In vivo labeling and immunoprecipitation. Monolayers of HeLa cells were infected with poliovirus as described. At 1h and 3h after infection, the medium was removed and cells were rinsed twice in phosphate-free DMEM (Gibco). [^{32}P]orthophosphate (0.5 mCi/ml; Dupont/NEN) was added and cells were incubated for 2h with occasional agitation. The medium was removed and cells were rinsed twice in cold TBS and lysed in RIPA buffer (50mM Tris-HCl pH 7.5, 150mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 0.25mM Na_3VO_4, 10nM okadaic acid and 10mM NaF. Debris was spun down and extracts were precleared with preimmune serum preadsorbed on protein-A agarose (Repligen) for 1h at 4°C. The

supernatant was immunoprecipitated for 4h at 4°C using 11208 antibody preadsorbed on protein-A agarose beads. Beads were washed three times in RIPA, once in 200mM LiCl, 1mM 2-mercaptoethanol, and resuspended in Laemmli sample buffer. Immunoprecipitated proteins were subjected to SDS/15% PAGE and autoradiography.

2.2.5 Acknowledgements

This paper was co-authored by Anne-Claude Gingras, Yuri Svitkin, Graham J. Belsham, Arnim Pause and Nahum Sonenberg. We thank members of the laboratory for helpful comments on the manuscript. We thank Dr. A. Palmenberg for the EMCV R strain and Dr. L. Carrasco and Isabel Novoa for the α-eIF4G antibodies. This work was supported by grants from the National Cancer Institute of Canada and the Medical Research Council of Canada to N.S. and a NATO grant CRG 950222 to G.J.B and N.S.; A.-C.G. was supported by an NSERC 67 studentship, and Arnim Pause by a Cancer Research Society of Montreal studentship.

2.3 Adenovirus infection inactivates the translational inhibitors 4E-BP1 and 4E-BP2

2.3.1 Abstract

Infection with many viruses results in the selective shutoff of host protein synthesis. A common target for virus interference with host protein synthesis is the cap-binding protein complex, eIF4F. The large subunit of the complex, eIF4G, is cleaved upon picornavirus (except cardiovirus) infection. Recently, it has been shown that infection with poliovirus or encephalomyocarditis virus (EMCV) activates 4E-BP1, which is a specific inhibitor of eIF4E. Adenovirus mRNAs expressed from the major late promoter (MLP) initiate translation by a ribosome shunt mechanism, and can be translated in cells superinfected by poliovirus. Consistent with the cap-independent translation of the late mRNAs, eIF4E is dephosphorylated upon adenovirus infection, coincident with the inhibition of host protein synthesis. Here we show that early in adenovirus infection 4E-BP1, and its related protein 4E-BP2, are phosphorylated and hence inactivated. This is not consistent with a role of 4E-BPs in adenovirus-induced shutoff, but could explain the increase in protein synthesis reported early in infection. Phosphorylation of 4E-BP1 and 4E-BP2 is consistent with earlier findings in adenovirus-infected cells on the activation of the protein kinase S6K1, whose phosphorylation lies on the same pathway as 4E-BPs, by E1A. Similar findings to those described here were reported for 4E-BP1 by Feigenblum and Schneider (123).

2.3.2 Results

We wished to determine whether 4E-binding proteins are dephosphorylated in adenovirus-infected cells similar to what we had shown for picornaviruses. First, HeLa cells were infected with wild type human adenovirus type 5 to assess 4E-BP1 phosphorylation at early times in infection. 4E-BP1 was detected by Western blotting, using an antiserum specific for 4E-BP1 (211; section 2.2.6.2). The hyperphosphorylated (γ and δ) forms of 4E-BP1 exhibit slow mobility on SDS-polyacrylamide gels relative to the unphosphorylated (α) and hypophosphorylated (β) species (195, 205, 211, 325). In mock-infected cells, three major 4E-BP1 forms were detected (Fig. 2.5A; isoforms α, β and γ). The bands corresponding to 4E-BP1 were shifted upward as early as 30 min post-infection and the pattern of 4E-BP1 isoforms did not change any further up to 3.5 hours after infection. A "Far-Western" analysis was performed to ascertain that the faster migrating forms (α and β), which correspond to the hypophosphorylated or unphosphorylated (166, 211) forms can interact with eIF4E. The binding of 4E-BP1 to eIF4E was strong in the mock-infected cells and at the onset of infection, but was barely detected as early as 30 min post-infection (Fig. 2.5B). Thus, the result of the Far-Western analysis is consistent with early phosphorylation of 4E-BP1 (Fig. 2.5B). The lower band whose intensity decreased in the Far-Western analysis early in infection in parallel with 4E-BP1, albeit less drastically, co-migrated with 4E-BP2 (Fig. 2.5B), suggesting that this protein is also a target for modification by adenovirus.

Next, we performed a time course of infection in HeLa cells to examine the phosphorylation status of 4E-BP1 or 4E-BP2 at late times after infection when the shutoff of protein synthesis occurs. The pattern of 4E-BP1 and 4E-BP2 isoforms was examined by Western blotting, using a rabbit polyclonal antiserum against 4E-BP1 (211) or an affinity purified rabbit polyclonal antiserum to 4E-BP2 (see Materials and Methods). The 4E-BP1 isoforms were shifted upward already at the first time point (4 hours post-

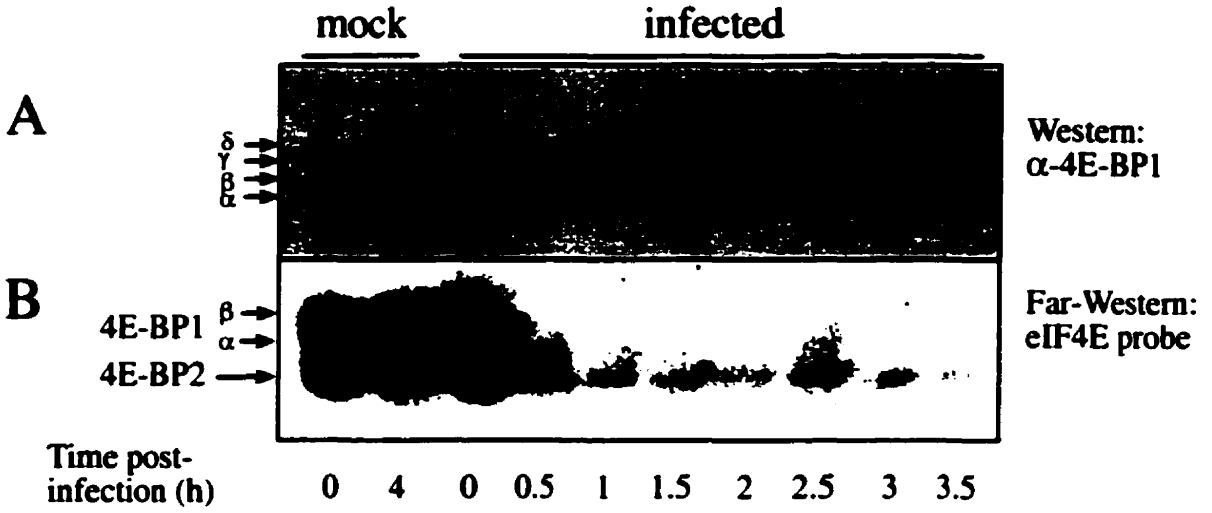


Figure 2.5. Adenovirus effect on 4E-BP1 phosphorylation early in infection. (A) Heat-treated extracts were analyzed by Western blotting using a rabbit polyclonal antibody directed against 4E-BP1. (B) Far-Western blotting was performed on the samples described in (A) using [^{32}P]-labeled eIF4E as a probe.

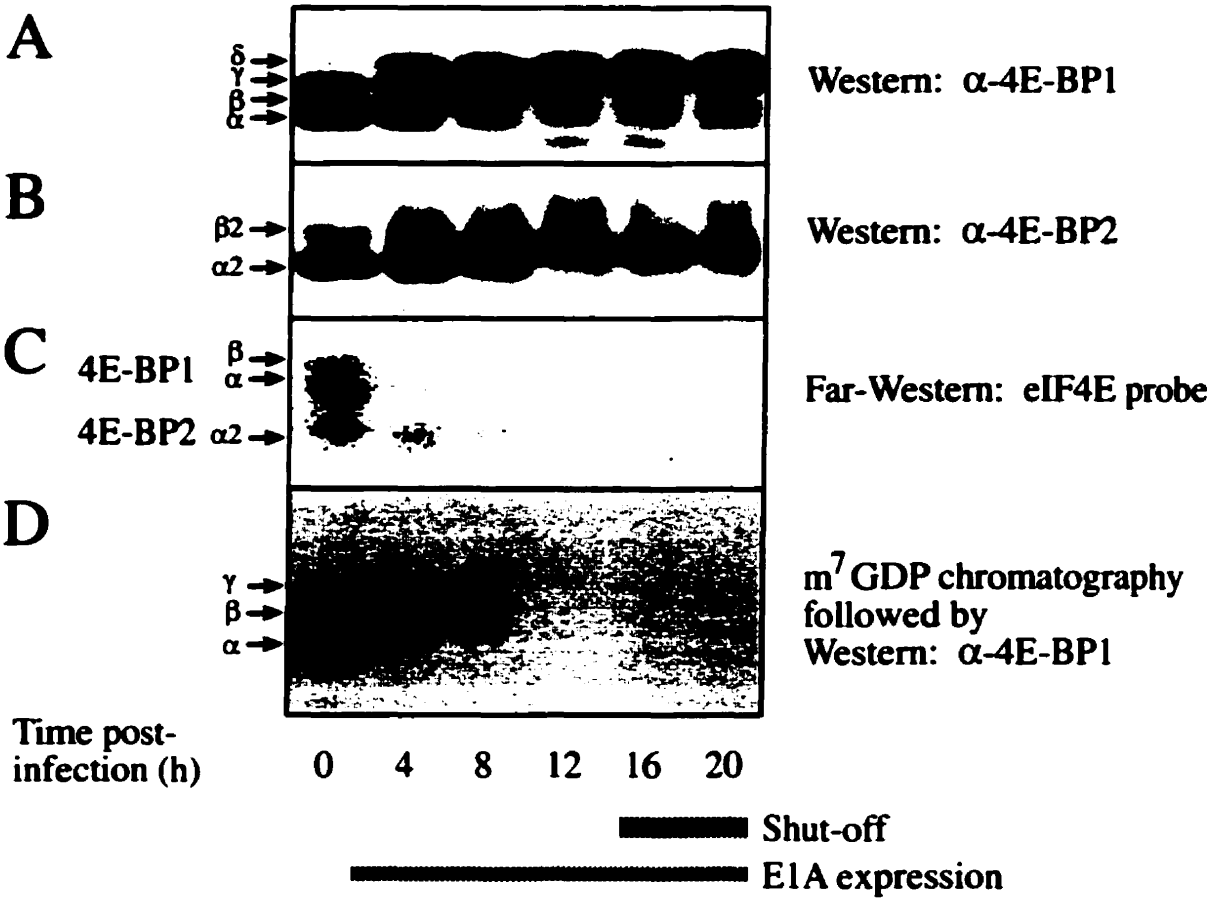


Figure 2.6. Adenovirus effect on 4E-BP1 and 4E-BP2 phosphorylation late in infection. (A) Heat-treated extracts were subjected to Western blotting and probed with a polyclonal 4E-BP1 antibody. (B) Extracts were subjected to Western blotting with an affinity-purified polyclonal antibody directed against 4E-BP2. (C) Extracts prepared and blotted as in (A) were probed with [^{32}P]-labeled eIF4E. (D) Extracts (250 µg, not heat-treated) were incubated with m^{7}GDP resin (25µl). Precipitated material was subjected to SDS-PAGE and Western blotting with an anti-4E-BP1 antibody.

infection), consistent with the results in Fig. 2.5 (Fig. 2.6A). The two faster migrating species of 4E-BP1 (α and β) gradually disappeared between 8 and 12 hours of infection and only the two slower migrating forms (γ and δ) were detected at 12 hours, without any further change at the time when the shutoff of host protein synthesis occurs (16-20 hours). Changes in the 4E-BP2 forms were studied in parallel. In HeLa cells, two bands were detected for 4E-BP2 (termed α2 and β2). As for 4E-BP1, the hypophosphorylated species (α2) migrated faster than the hyperphosphorylated (β2) form (Fig. 2.6B; A.-C.G. and N.S, unpublished data). At the onset of infection, most of the 4E-BP2 existed as an hypophosphorylated (α2) form. As noted for 4E-BP1, 4E-BP2 was also shifted upwards on the gel following adenovirus infection, starting at 4 hours. The shift was complete at 12 hours, as seen for 4E-BP1 (Fig. 2.6B). In other experiments, we observed a change in migration as early as 30 min after infection (data not shown). This effect was concomitant with the expression of the E1A protein, as assessed by Western blotting (data not shown). Phosphorylation of 4E-BP1 and 4E-BP2 following adenovirus infection caused a decreased affinity of both 4E-BPs towards eIF4E, as determined by a Far-Western assay using ^{32}P- labeled eIF4E as a probe. Binding of 4E-BP1 to eIF4E was abrogated, but binding of 4E-BP2 was only partially reduced (approximately 40% of the control; Fig. 2.6C). The significance of the residual binding of 4E-BP2 is not immediately clear. The decrease in 4E-BP1 binding to eIF4E was also confirmed by m^{7}GDP chromatography. Cell extracts were incubated on ice for 2 hours with m^{7}GDP-agarose resin as described before (195) and then washed extensively. Precipitated material was resolved by SDS-PAGE and analyzed by Western blotting with an anti-4E-BP1 antibody. Infection with adenovirus abrogated the recovery of 4E-BP1 on a cap-analog resin, in parallel with the mobility shift and the reduction of binding to the eIF4E probe as measured by Far-Western (Fig. 2.6D). This is similar to the situation observed after stimulation of cells with insulin or growth factors. Although the results show that 4E-BP1 and 4E-BP2 are most probably not involved in the shutoff of host protein

synthesis, the 4E-BPs could play a role in the increase of translation observed early in adenovirus infection. This hypothesis is strengthened by the data of Feigenblum and Schneider (123) who blocked the increase in translation following adenovirus infection in 293 cells by incubation with rapamycin, which prevents the phosphorylation of 4E-BP1 (128, 138, 166).

To confirm that the changes in electrophoretic mobility and in eIF4E-binding are due to the modulation of phosphorylation, we performed ^{32}P-labeling of the HeLa cells 5 hours post-infection. 4E-BP1 was immunoprecipitated and subjected to SDS-PAGE (211). In mock-infected cells, three bands corresponding to the β, γ and δ isoforms were resolved (Fig. 2.7, lane 1). Phosphorylation of 4E-BP1 is increased following infection with wild-type adenovirus, as evidenced by the increase in isoform δ (total increase 2.5 fold). This increase in phosphorylation is not observed when a virus containing a mutant form of E1A (dl 1101-1108/520) (332) is employed (Fig. 2.7, lane 3). This mutant contains deletions in the N-terminal domain of E1A as well as in the CR2 (conserved region 2) and is deficient in binding to Rb family members, as well as to other proteins such as p60cyclin A (332). The shift in 4E-BP1 electrophoretic mobility was not detected either with an E1A-deficient virus or with several E1A mutant viruses (data not shown), consistent with the data of Feigenblum and Schneider (123). These results indicate that E1A mediates 4E-BP's phosphorylation.

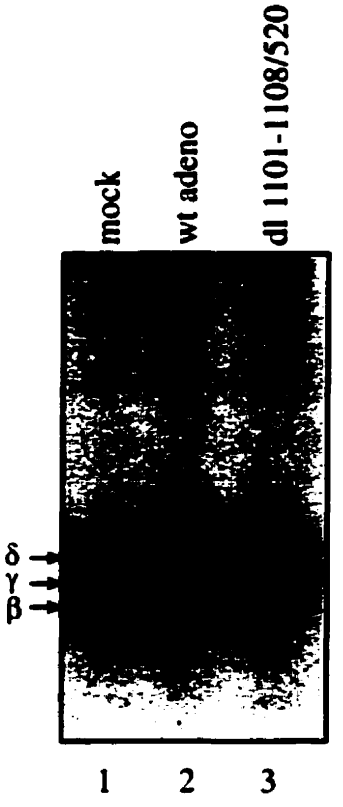


Figure 2.7. Incorporation of [^{32}P] into 4E-BP1 following adenovirus infection. 4E-BP1 was immunoprecipitated from [^{32}P] labeled cells that were were mock-infected, infected for 5 hours with wild-type adenovirus or infected with a virus carrying a mutant E1A (dl 1101-1108/520). Proteins were separated by SDS-PAGE, transferred onto a PVDF membrane and subjected to autoradiography.

2.3.4 Discussion

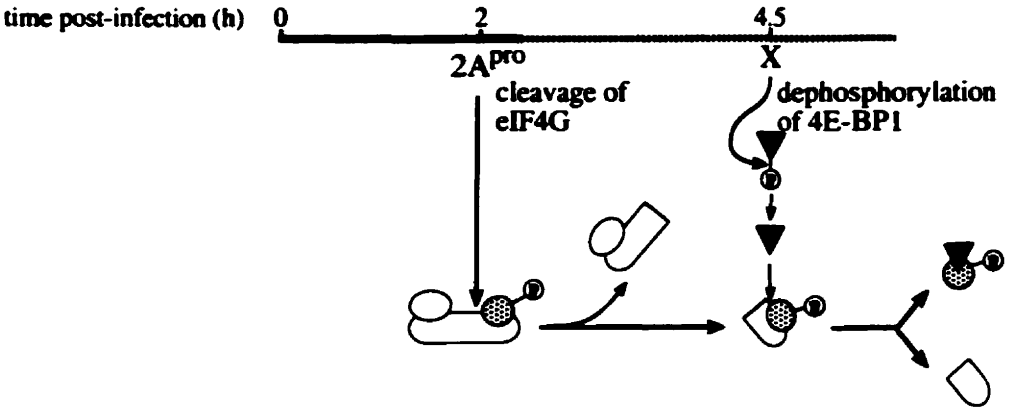
In summary, the results show that upon adenovirus infection, 4E-BP1 and 4E-BP2 become hyperphosphorylated. This is the first report to document a change in the phosphorylation status of 4E-BP2 following virus infection. As opposed to the effect of the picornaviruses EMCV and poliovirus (211), 4E-BP1 is not activated by infection, but inactivated early in infection. One of the reasons for this disparity could be explained by the difference in life cycles between picornaviruses and adenovirus. The poliovirus and EMCV life cycles are very short (around 5-6 hours in HeLa cells), and host protein synthesis shutoff occurs in the first few hours after infection. In addition, translation of the genome of these viruses completely proceeds in a cap- and eIF4E-independent fashion (22, 91). The adenovirus life cycle is much longer (18-24 hours). Adenovirus gene expression proceeds in two phases: early and late expression. Late expression is principally driven by the major late promoter (MLP), which directs transcription of mRNAs that translate via the shunt mechanism and exhibit reduced cap dependence (210). However, adenovirus early genes, such as E1A are translated in a cap-dependent fashion (210). Therefore, an early increase in eIF4F activity by inactivation of the translational repressors 4E-BP1 and 2 is likely to be of benefit to the virus.

Phosphorylation of 4E-BP1, following serum or insulin stimulation, occurs through a PI3-kinase dependent, rapamycin-sensitive pathway (128, 333). 4E-BP1 phosphorylation coincides with the activation of S6K1 (128). S6K1 does not phosphorylate 4E-BP1 directly (221), and does not appear to be upstream of 4E-BP1 (223). Indeed, transfection of a rapamycin-resistant form of S6K1 does not confer rapamycin resistance to 4E-BP1, as the case would be if 4E-BP1 was a downstream target of S6K1. Furthermore, co-transfecting a wild-type form of S6K1 together with 4E-BP1 reduced the phosphorylation of 4E-BP1 on rapamycin-sensitive sites (223), consistent with the idea that S6K1 and 4E-BP1 compete for a common upstream kinase. S6K1 is activated by the E1A protein upon

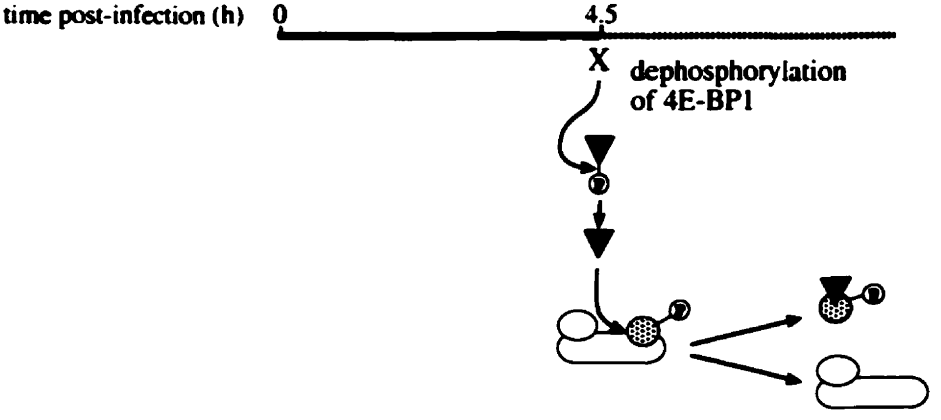
transfection (334), indicating that an element in the PI3-kinase signal transduction pathway upstream of S6K1 and the 4E-BPs is a target for adenovirus. Thus, 4E-BP1 and 2 are not likely to be direct targets for modification by the virus. It remains to be determined whether the inactivation of 4E-BPs contributes to transformation by Ad5, and to identify the direct cellular target in the signal transduction cascade leading to 4E-BP phosphorylation. Similar results to those documented here were reported earlier for 4E-BP1 by Feigenblum and Schneider (123). The data presented here confirm their report and, in addition, demonstrate that 4E-BP2, a related translation inhibitor, is also phosphorylated and inactivated by adenovirus infection.

Figure 2.8 summarizes the modifications involving eIF4F in several viral infections. 4E-BP1 is the target for picornaviruses, which activate it, but also of adenovirus, which phosphorylates and inactivates it (as well as 4E-BP2).

Poliovirus effects on eIF4G and 4E-BP1



Encephalomyocarditis effect on 4E-BP1



Adenovirus effects on eIF4E and 4E-BPs

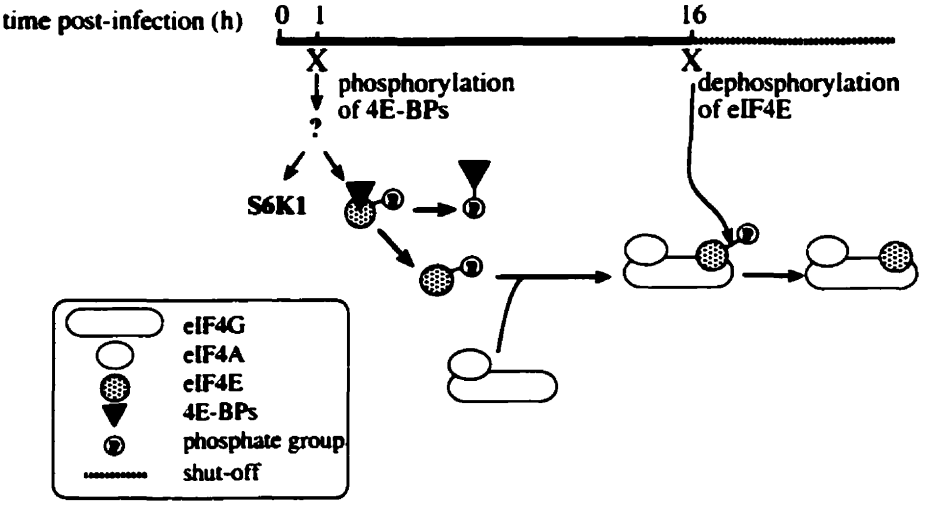


Figure 2.8. Modifications of eIF4F following infections with different viruses. Not shown is the effect of influenza virus which elicits dephosphorylation of eIF4E.

2.3.5 Materials and Methods

2.3.5.1 Cell culture and infection. HeLa cells were grown in DMEM supplemented with 10% FCS and infected with wild type human adenovirus type 5 (a kind gift from E. Querido, R. Marcellus and P.E. Branton) at a multiplicity of infection of 35 plaque forming units per cell.

2.3.5.2 Antibodies. Detection of 4E-BP1 was performed using a rabbit polyclonal antiserum (11208) specific for 4E-BP1 (211), which does not cross-react with 4E-BP2 or 4E-BP3 (data not shown). A fusion protein GST-HMK-4E-BP2 (195) was used to raise antisera to 4E-BP2. Because the crude antisera cross-reacted with 4E-BP1 and 4E-BP3, the antisera (11211) was affinity-purified to ensure specific recognition of 4E-BP2. Briefly, recombinant GST alone, GST-4E-BP1 and GST-HMK-4E-BP2 were expressed and purified from BL21 bacteria through glutathione-S-sepharose chromatography according to the manufacturer's instructions. The proteins were not eluted from the sepharose beads, but cross-linked directly to it, using a chemical crosslinker (dimethyl pipelimidate •2HCl, Pierce). The cross-linking efficiency was monitored and the affinity columns were extensively washed with CAPS buffer (3{cyclohexamino}-1-propanesulfonic acid; 100mM, pH 11), and equilibrated in phosphate-buffered saline (PBS). The crude antisera 11211 directed against GST-HMK-4E-BP2 was diluted 1:4 in PBS, and was passed several times over the GST and GST-4E-BP1 resins, in order to remove all antibodies which would recognize these two proteins. Removal of the antibodies directed against GST or 4E-BP1 was confirmed by Western blotting. Subsequently, the depleted antiserum was passed several times over the GST-HMK-4E-BP2 column to retain only those antibodies with a high affinity to 4E-BP2. After extensive washing of the column with PBS, the antibodies were eluted with 100mM glycine•HCl (decreasing pH of 3.0, 2.5, and 1.8). After neutralization (1M Tris•HCl, pH 9.0), fractions containing antibody were tested by Western blotting, and pooled. The

purified antibody against human 4E-BP2 does not cross-react with 4E-BP1 nor with 4E-BP3; A.-C.G and N.S., unpublished data).

2.3.5.3 Analysis of protein phosphorylation. Western and Far-Western blotting were as described (195, 211; section 2.2.6.6). For [^{32}P] labeling, Hela cells (mock infected, or infected) were incubated at 37°C for 5 h in serum-free DMEM containing 0.5 mCi/ml [^{32}P]orthophosphate (DuPont NEN; 3000 mCi/mmole). The medium was removed and the cells were rinsed twice in cold PBS. Cells were lysed in lysis buffer [10% glycerol, 50 mM Tris (pH 7.5), 60 mM KCl, 2 mM CDTA (trans,-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid), 1% Triton X-100, 2 mM DTT, 20 mM β-glycerolphosphate, 10 nM okadaic acid] for 30 min at 4°C. Lysate was harvested by scraping and cell debris was removed by centrifugation. The extract was precleared by incubation with protein A beads (50 μl per ml of extract) with end-over-end rotation at 4°C for 1 hr. The supernatant was transferred to a fresh tube, together with 30 μl of 11208 crude antisera per milliliter of extract, and incubated for 3 hr at 4°C. Protein A beads (30 μl packed beads) were added and incubation end-over-end was carried out for 2 hr at 4°C. Beads were spun down (microfuge, 6000 rpm, 2 min) and washed two times in lysis buffer, 2 times in RIPA buffer, and 2 times in LiCl solution (200 mM LiCl, 1 mM DTT). The immunoprecipitates were resuspended in Laemmli sample buffer and subjected to SDS-PAGE. The wet gel was subjected to autoradiography.

2.3.5 Acknowledgments

This paper was co-authored by Anne-Claude Gingras and Nahum Sonenberg. The authors are grateful to Emmanuelle Querido, Richard Marcellus and P.E. Branton for providing virus strains and for helpful discussions. We thank Dr. Robert Schneider for communicating his results before publication and Francis Poulin for critical reading of the manuscript. This work was supported by a grant from the Medical Research Council of Canada to N.S.; A.-C. G. was supported by a National Science and Engineering Research Council 67 (NSERC 67) studentship. N. S. is a Distinguished Scientist of the Medical Research Council of Canada, and a Howard Hughes Institute International Scholar.

CHAPTER 3

4E-BP1 IS PHOSPHORYLATED AND INACTIVATED BY THE AKT/PKB SIGNALING PATHWAY

3.1 Introduction and perspectives

As evidenced in the previous chapter, the phosphorylation state of 4E-BP1 is subject to extensive regulation. The state of phosphorylation of 4E-BP1 has been demonstrated over the past few years to be sensitive to a wide variety of stimuli (Table I), and to correlate, in most cases, with the growth status of cells. In this chapter, the signaling pathway leading to 4E-BP1 phosphorylation is studied. At the time when we undertook these studies (in collaboration with the laboratory of N. Hay), we and others had already demonstrated that 4E-BP1 phosphorylation is sensitive to inhibitors of PI3K (wortmannin) and FRAP/mTOR (rapamycin; 128, 138, 166). The role of PI3K in mediating 4E-BP1 phosphorylation was further suggested, through a collaboration with S. von Manteuffel and G. Thomas (Basel, Switzerland), by the fact that PDGF receptor mutants which are unable to activte PI3K, also failed to induce 4E-BP1 phosphorylation, while mutants deficient in the activation of other pathways had no effect on 4E-BP1 phosphorylation (128).

We were interested in more directly analyzing the effects of PI3K on 4E-BP1 phosphorylation. We also wanted to determine whether Akt/PKB (section 1.8.5) was involved in 4E-BP1 phosphorylation, and whether signaling through these molecules could reproduce the phosphorylation observed following insulin or serum stimulation. As mentioned above, 4E-BP1 phosphorylation is sensitive to rapamycin. However, it was unclear how the target of rapamycin protein (FRAP/mTOR) was linked to the PI3K pathway. We thus wished to determine the relative order (from the cell membrane to the substrate) of these signaling molecules.

3.2 Abstract

Growth factors and hormones activate protein translation by phosphorylation and inactivation of the translational repressors, the eIF4E-binding proteins (4E-BPs), through a wortmannin- and rapamycin-sensitive signaling pathway. The mechanism by which signals emanating from extracellular stimuli lead to phosphorylation of 4E-BPs is not well understood. Here we demonstrate that the activity of the serine/threonine kinase Akt/PKB is required in a signaling cascade that leads to phosphorylation and inactivation of 4E-BP1. PI3K elicits the phosphorylation of 4E-BP1 in a wortmannin- and rapamycin-sensitive manner, whereas activated Akt-mediated phosphorylation of 4E-BP1 is wortmannin resistant but rapamycin sensitive. A dominant negative mutant of Akt/PKB blocks insulin-mediated phosphorylation of 4E-BP1, indicating that Akt/PKB is required for the *in vivo* phosphorylation of 4E-BP1. Importantly, an activated Akt/PKB induces phosphorylation of 4E-BP1 on the same sites that are phosphorylated upon serum stimulation. Similar to what has been observed with serum and growth factors, phosphorylation of 4E-BP1 by Akt/PKB inhibits the interaction between 4E-BP1 and eIF4E. Furthermore, phosphorylation of 4E-BP1 by Akt/PKB requires the activity of FRAP/mTOR. FRAP/mTOR may lie downstream of Akt/PKB in this signaling cascade. These results demonstrate that the PI3K-Akt/PKB signaling pathway, in concert with FRAP/mTOR, induces the phosphorylation of 4E-BP1.

3.3 Results

3.3.1 p110α, the catalytic subunit of PI3K, and its downstream effector Akt/PKB mediate the phosphorylation of 4E-BP1

To study the role of Akt/PKB in the phosphorylation of 4E-BP1, a hemagglutinin-tagged 4E-BP1 (HA-4E-BP1) was generated. We first examined whether the transiently expressed HA-4E-BP1 exhibits a change in electrophoretic mobility after phosphorylation, as was observed for the endogenous 4E-BP1. Human embryonic kidney (HEK) 293 cells were transfected transiently with a HA-4E-BP1 expression vector. After transfection, the cells were deprived of serum for 36 hr and then stimulated with insulin for 30 min. Immunoblot analysis demonstrated a clear shift in mobility of HA-4E-BP1 with insulin stimulation (Fig. 3.1A, lanes 1,2). The mobility shift was not observed when cells were preincubated with either wortmannin or rapamycin (lanes 3,4), consistent with what has been observed previously for endogenous 4E-BP1 (128).

Previous studies have indicated a role for PI3K in the phosphorylation of 4E-BP1 by serum and growth factors (128, 153). However, it was also suggested that the effects of extracellular stimuli on 4E-BP1 phosphorylation could be explained by direct activation of FRAP/mTOR, as the *in vitro* autokinase activity of FRAP/mTOR is also inhibited by wortmannin (230). To examine whether PI3K can affect the phosphorylation state of 4E-BP1, we transiently cotransfected HA-4E-BP1 and PI3K expression vectors into serum-deprived 293 cells. Cotransfection of HA-4E-BP1 with the catalytic subunit of PI3K (p110α) induced phosphorylation of 4E-BP1, as manifested by a shift in its mobility (Fig. 3.1B, lane 2). This shift in mobility is similar to that observed with insulin stimulation (cf. Fig. 3.1A, lane 2, with Fig. 3.1B, lane 2). An activated form of p110α, p110α caax (p110α*), which is targeted to the plasma membrane by farnesylation (335; Fig. 3.1B, lane 3), also caused this mobility shift (interestingly, overexpression of the wild-type

p110α in 293 cells is sufficient to induce 4E-BP1 mobility shift). Thus, PI3K by itself affects the phosphorylation of 4E-BP1.

Next, we examined whether Akt/PKB mediates 4E-BP1 phosphorylation. We used two forms of Akt/PKB: the wild type c-Akt, and an activated form of Akt, MyrAkt. MyrAkt is comprised of the entire coding sequence of c-Akt fused in-frame to the Src myristoylation signal. This fusion protein is constitutively active, is independent of growth factors, and is wortmannin resistant (336, 337; see below). Cotransfection of either wild type c-Akt or MyrAkt and HA-4E-BP1 expression vectors caused a mobility shift of 4E-BP1 (Fig. 3.2A). These results suggest that both PI3K and its downstream effector Akt are intermediates in the signaling pathway leading to 4E-BP1 phosphorylation. Transient transfections of 293 cells produces a high level of c-Akt expression that was sufficient to elicit the 4E-BP1 mobility shift to the same extent as MyrAkt (Fig. 3.2A, cf. lanes 3 and 4).

To determine whether the endogenous PI3K/Akt signaling pathway is involved in mediating the phosphorylation of 4E-BP1 by growth factors, we used a kinase-deficient mutant of Akt/PKB containing a point mutation in the ATP-binding domain K179M, Akt(kin-) (239). When expressed at high levels, the kinase dead mutant acted in a dominant negative fashion to abolish the mobility shift of 4E-BP1 normally elicited by insulin treatment (Fig. 3.2B). Insulin treatment induces the mobility shift of 4E-BP1 (lanes 1,2). Coexpression of Akt(kin-) significantly reduced the insulin-mediated mobility shift (lanes 3,4). These results demonstrate that endogenous Akt is required to transmit the signal leading to 4E-BP1 phosphorylation.

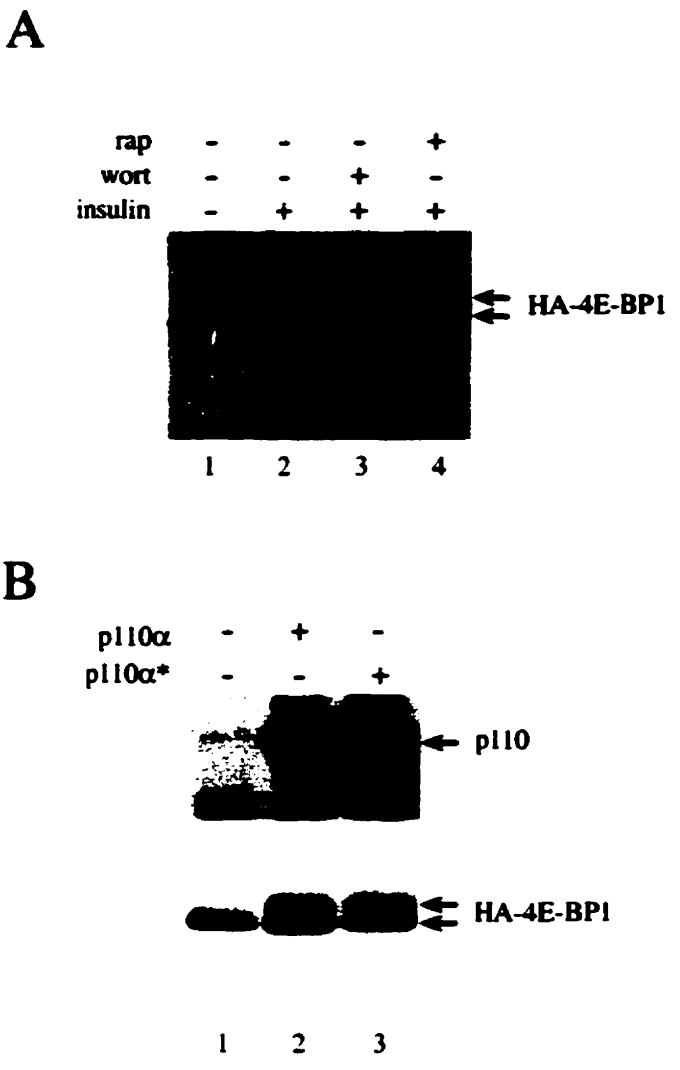


Figure 3.1. PI3K elicits phosphorylation of 4E-BP1. (A) Insulin-mediated phosphorylation of 4E-BP1 is both rapamycin and wortmannin sensitive. Human embryonic kidney (HEK) 293 cells were transiently transfected with a hemaglutinin (HA) epitope-tagged 4E-BP1 expression vector. After transfection, cells were deprived of serum for 36 hr, and either mock treated or stimulated with insulin (1 μg/ml) for 30 min in the presence of either wortmannin (wort, 200 nM) or rapamycin (rap, 20 ng/ml). Cell extracts were prepared as described in Materials and Methods, and HA-4E-BP1 was detected by immunoblot analysis with an anti-HA antibody. Arrows indicate the different phosphorylated isoforms of HA-4E-BP1. (B) The catalytic subunit of PI3K p110α elicits phosphorylation of HA-4E-BP1. HEK 293 cells were cotransfected with HA-4E-BP1 expression vector along with one of the following: control vector (lane 1), p110α expression vector, or p110α caax (p110α*) expression vector. After transfection, cells were deprived of serum for 36 hr. HA-4E-BP1 was detected as described in A. p110α and p110α caax were detected as described in Materials and Methods. The results shown are representative of three independent experiments.

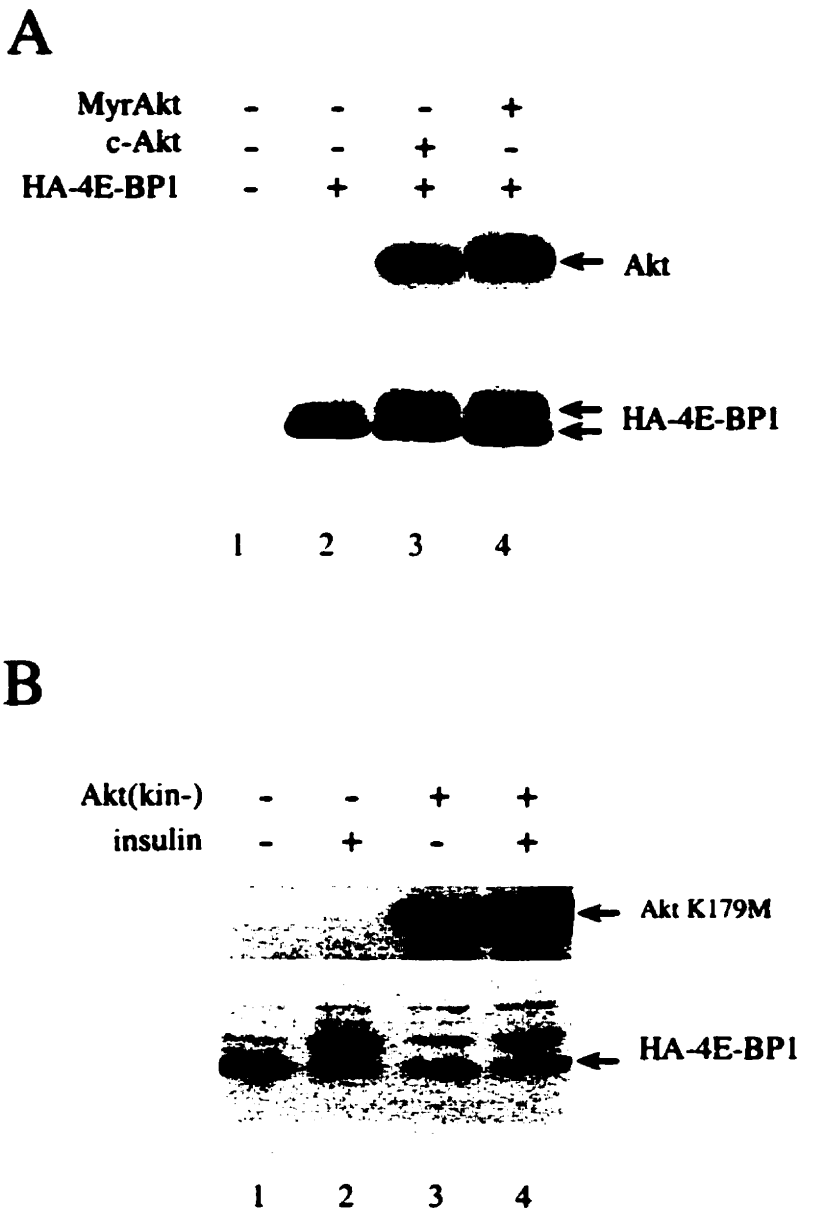


Figure 3.2. Akt/PKB elicits phosphorylation of HA-4E-BP1. (A) HEK 293 cells were mock transfected or cotransfected with HA-4E-BP1 expression vector and one of the following: control vector (lane 2), HA-c-Akt expression vector, or HA-MyrAkt expression vector. Cells were deprived of serum for 36 hr. HA-4E-BP1 was detected as described in Fig. 3.1, and Akt expression was detected with an anti-HA antibody. (B) A kinase-deficient mutant of Akt/PKB inhibits phosphorylation of 4E-BP1 by insulin. HEK 293 cells were cotransfected with a HA-4E-BP1 expression vector (100 ng) and the following: control vector (lanes 1,2) or HA-AktK179M expression vector, Akt(kin-) (lanes 3,4). After transfection, cells were serum-deprived for 36 hr, then stimulated with 100 ng/ml of insulin for 45 min. HA-4E-BP1 and HA-AktK179M expression were revealed using an anti-HA antibody. The results shown are representative of three independent experiments.

Recent results suggested the possibility that the wortmannin sensitivity of 4E-BP1 phosphorylation is attributable to direct inhibition of FRAP/mTOR activity by wortmannin (230). Therefore, we examined the sensitivity of PI3K and Akt/PKB-mediated phosphorylation of 4B-BP1 to wortmannin and rapamycin. Both wortmannin and rapamycin inhibited the ability of an activated form of p110α to cause the mobility shift of 4E-BP1 (Fig. 3.3A). However, the mobility shift elicited by the activated Akt/PKB was wortmannin resistant but rapamycin sensitive (Fig. 3.3B). These results indicate that the wortmannin sensitivity of 4E-BP1 phosphorylation by growth factors is attributable to inhibition of PI3K and not inhibition of FRAP activity.

Taken together these results suggest a linear pathway from growth factor receptors to the activation of PI3K, which in turn activates Akt/PKB and leads to phosphorylation of 4E-BP1.

3.3.2 Akt/PKB mediates the phosphorylation of 4E-BP1 and 4E-BP2 in vivo

To confirm that the gel mobility shift observed by cotransfection of Akt/PKB with 4E-BP1 is attributable to an increase in 4E-BP1 phosphorylation, we first established a stable 293-cell line overexpressing MyrAkt. 293-MyrAkt or wild-type 293 cells were then labeled metabolically with [^{32}P]orthophosphate, and 4E-BP1 and 4E-BP2 were immunoprecipitated and subjected to SDS-PAGE and autoradiography. In control cells, two phosphorylated isoforms termed β and γ (the fastest migrating isoform, α, is unphosphorylated and hence not detected by ^{32}P labeling) were detected (Fig. 3.4A, lane 1). After serum or insulin stimulation (lanes 2,3) a 2.5-fold increase in ^{32}P incorporation was observed, and two isoforms of slower mobility (δ and ε) appeared. Rapamycin or wortmannin treatment abrogated the effect of serum and insulin on 4E-BP1 phosphorylation (lanes 4,5). In 293-MyrAkt cells, the four phosphorylated species (β, γ, δ, ε) were present in the absence of stimuli (lane 6, 2.5-fold more total ^{32}P

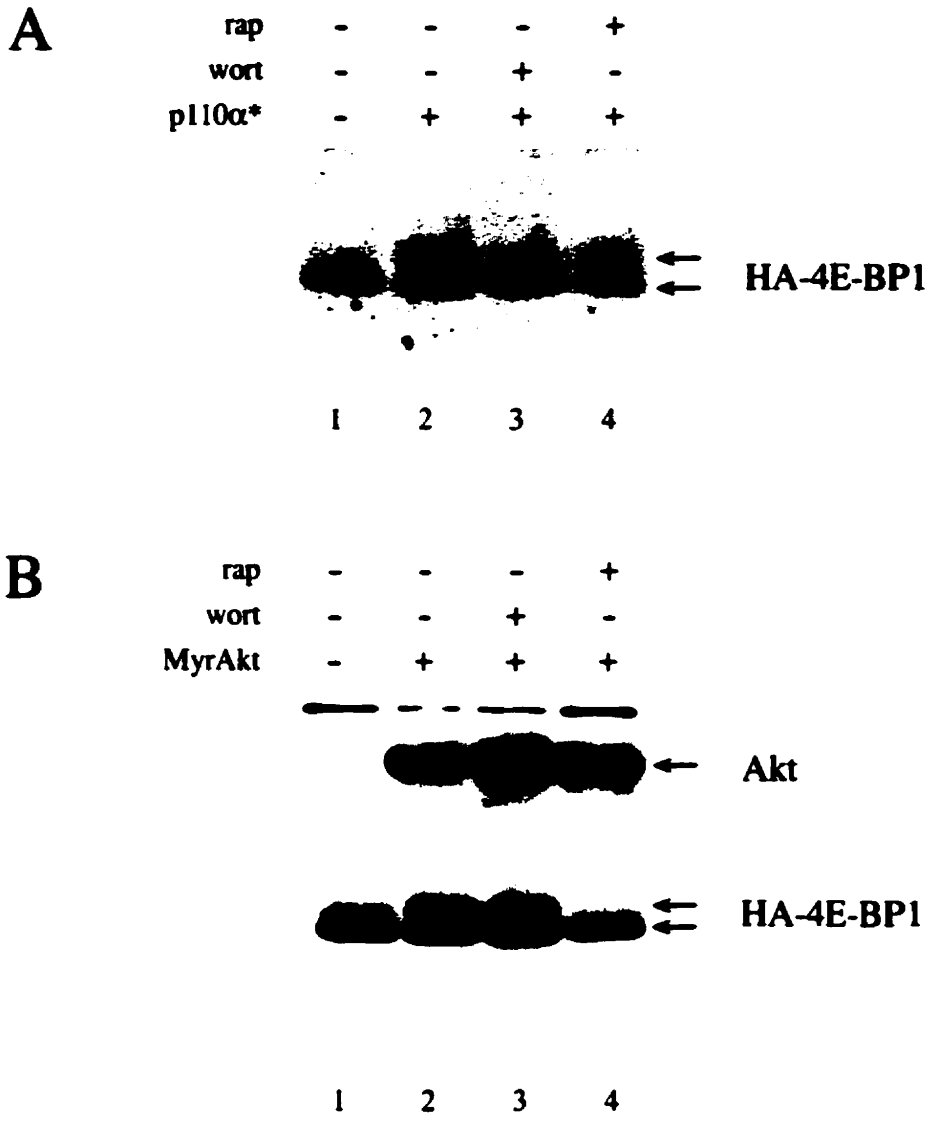


Figure 3.3. ***Effect of wortmannin and rapamycin on 4E-BP1 phosphorylation by Akt/PKB and PI3K.*** (A) Phosphorylation of 4E-BP1 by p110α caax is both wortmannin and rapamycin sensitive. HEK 293 cells were cotransfected with HA-4E-BP1 expression vector and one of the following: control vector (lane 1) or p110α caax (p110α*) expression vector. After transfection, cells were deprived of serum for 36 hr and treated with either wortmannin (wort, 200 nM) or rapamycin (rap, 20 ng/ml). Cell extracts were prepared and HA-4E-BP1 was detected as described in Fig. 3.1. (B) Phosphorylation of 4E-BP1 induced by an activated Akt/PKB is wortmannin resistant, but rapamycin sensitive. HEK 293 cells were cotransfected with HA-4E-BP1 expression vector and with control vector (lane 1), or with HA-MyrAkt expression vector. After transfection, cells were deprived of serum for 36 hr. Cells were treated with kinase inhibitors and 4E-BP1 was detected as described in A. HA-MyrAkt expression was detected on the same immunoblot. The figure is representative of two independent experiments.

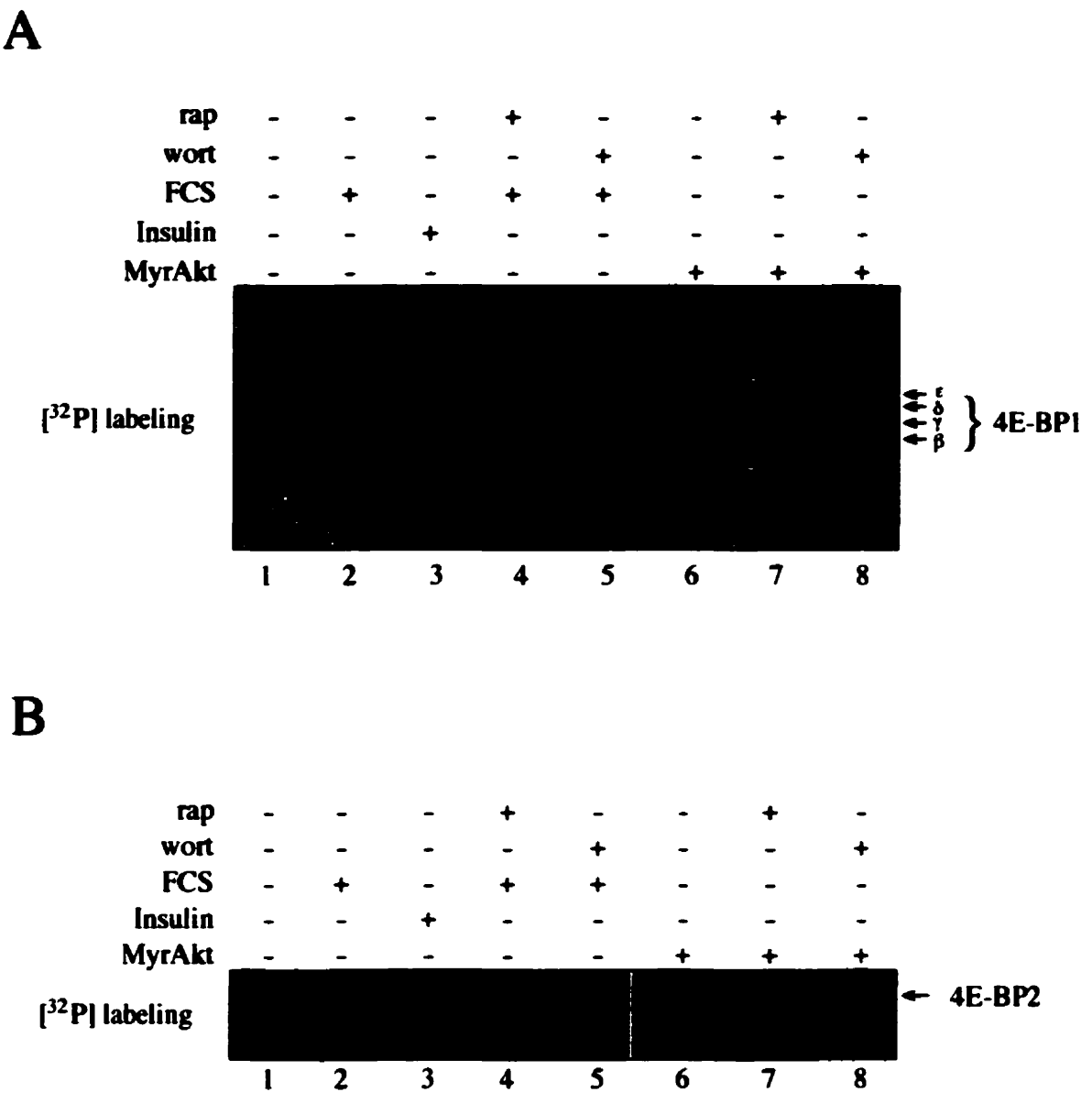


Figure 3.4. 4E-BP1 and 4E-BP2 ^{32}P incorporation is increased in 293 MyrAkt cells and is resistant to wortmannin treatment. Cells were labeled with [^{32}P]orthophosphate as described in Materials and Methods and 4E-BP1 (A) and 4E-BP2 (B) were immunoprecipitated successively with polyclonal antibodies, separated by SDS-PAGE, transferred to Immobilon-P^{SQ} and subjected to autoradiography. The various phosphorylated isoforms are indicated for 4E-BP1.

incorporation than in starved 293 cells). The pattern of phosphorylation is very similar to that of 293 cells stimulated with serum or insulin (cf. lane 6 with lanes 2 and 3). In 293-MyrAkt cells, the phosphorylation of 4E-BP1 was sensitive to rapamycin treatment, but completely resistant to wortmannin treatment (lanes 7,8). A similar effect was observed for 4E-BP2. 4E-BP2 was immunoprecipitated from the lysates as 4E-BP1. 4E-BP2 is phosphorylated on fewer residues than 4E-(218), and only one isoform incorporating ^{32}P is detected by SDS-PAGE (Fig. 3.4B). Total ^{32}P incorporation in 4E-BP2 was increased ~2- to 2.5-fold in serum- and insulin-stimulated cells (Fig. 3.4B, cf. lanes 2 and 3 with lane 1). This increase was diminished (1.5-fold) by rapamycin treatment and abolished by wortmannin treatment (lanes 4,5). In MyrAkt-expressing 293 cells, 4E-BP2 phosphorylation was increased approximately twofold, as compared to serum deprived 293 cells (cf. lane 6 with lane 1). As with 4E-BP1, the increase in 4E-BP2 phosphorylation in MyrAkt cells was rapamycin sensitive, but wortmannin insensitive (lanes 7,8). Although the effect of rapamycin on 4E-BP2 phosphorylation was modest (1.5-fold) in the experiment presented here, this inhibitory effect was reproduced several times in 293 cells, with an inhibition varying from 1.5- to 3-fold. This is similar to the inhibition of 4E-BP1 phosphorylation by rapamycin.

These results further confirm that the differences in the mobility shift observed for 4E-BP1 in Figures 3.1, 3.2 and 3.3 are attributable to changes in the phosphorylation state of 4E-BP1. To determine whether the effects observed on 4E-BP1 phosphorylation are specific to 293 cells, the same experiment was repeated in Rat1a cells stably expressing MyrAkt (336). Similar results to that observed with 293 cells (two- to threefold increase in phosphorylation) were obtained for both 4E-BP1 and 4E-BP2, and MyrAkt-induced phosphorylation was also sensitive to rapamycin, but resistant to wortmannin (A-C G, data not shown).

To determine whether Akt/PKB-mediated phosphorylation of 4E-BP1 occurs on the *in vivo* phosphorylation sites, phosphopeptide maps were performed on ^{32}P orthophosphate-labeled 4E-BP1. Ten phosphopeptides were detected in serum-starved 293 cells (Fig. 3.5A, labeled 1-10 in the order of decreasing intensity). When 293 cells were stimulated with serum, the intensity of some of the spots greatly increased (Fig. 3.5B, spots 8 and 9), whereas some new spots (11-14) appeared. Peptide 14 was not reproducibly detected in other experiments and will not be discussed further. Rapamycin treatment caused the decrease or disappearance of two serum-dependent spots (11 and 12) and of spots 8 and 9 (Fig. 3.5C). The effects of wortmannin were even more striking, resulting, for example, in the diminution of spot 7 (Fig. 3.5D). Serum-starved 293-MyrAkt cells exhibited a phosphopeptide pattern almost identical to that of 293 cells stimulated with serum. Phosphopeptides 11-13 were present in the absence of serum, although the increase in spots 9 and 13 was not as marked as in serum-treated cells (cf. E with A and B). As expected, rapamycin treatment caused a marked decrease in spots 11 and 12 (Fig. 3.5F). Wortmannin treatment had practically no effect on phosphopeptides 8, 11, 12 (Fig. 3.5G). Taken together, these data indicate that the increase in phosphorylation of 4E-BP1 in 293-MyrAkt cells occurs on physiologically relevant sites and confirm our data that MyrAkt expression confers resistance to wortmannin, but not to rapamycin on 4E-BP1.

To determine whether the changes in 4E-BP1 phosphorylation correlate with 4E-BP1 activity, the interaction of 4E-BP1 with eIF4E was examined, using cap-affinity chromatography. HEK 293 and other human cells contain multiple 4E-BP1 isoforms (128, 211). The isoform pattern observed in mouse and rat cells is much simpler, because of a smaller number and better separation of the isoforms. Thus, we performed the analysis in stably transfected Rat1a/MyrAkt and wild-type Rat1a cells. In rodent cells, a total of three 4E-BP1 forms can be detected: (1) a hyperphosphorylated slow migrating isoform (γ), which does not interact with eIF4E; (2) a middle form (β), which is

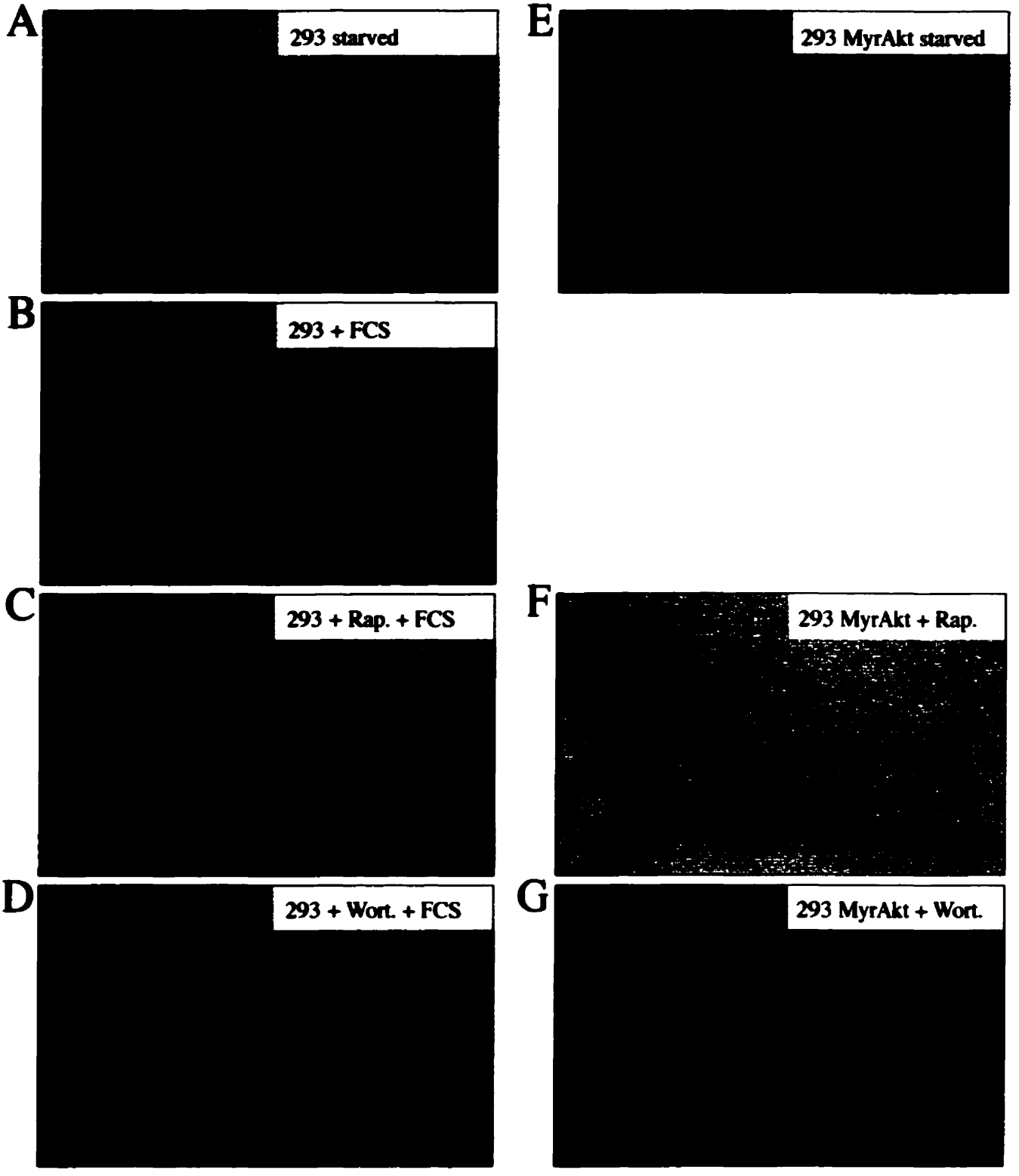


Figure 3.5. The phosphopeptide map of 4E-BP1 in 293 MyrAkt cells is identical to that of serum-stimulated 293 cells. ^{32}P-Labeled 4E-BP1 (Fig. 3.4) was excised from an Immobilon membrane, digested with trypsin-chymotrypsin, and analyzed by two-dimensional phosphopeptide mapping, as described in Materials and Methods. HEK 293 cells (A-D) and HEK 293/ MyrAkt cells (E-G) were deprived of serum for 36 hr. Cells were labeled with ^{32}P as described in Materials and Methods. (A,E) Untreated cells. (B-D,F,G) were treated as follows: 15% FCS for 30 min (B); pretreated with rapamycin (20 ng/ml) for 20 min before addition of FCS (C); pretreated with wortmannin (100 nM) for 20 min before addition of FCS (D); with rapamycin (20 ng/ml) for 20 min (F); with wortmannin (100 nM) for 20 min (G).

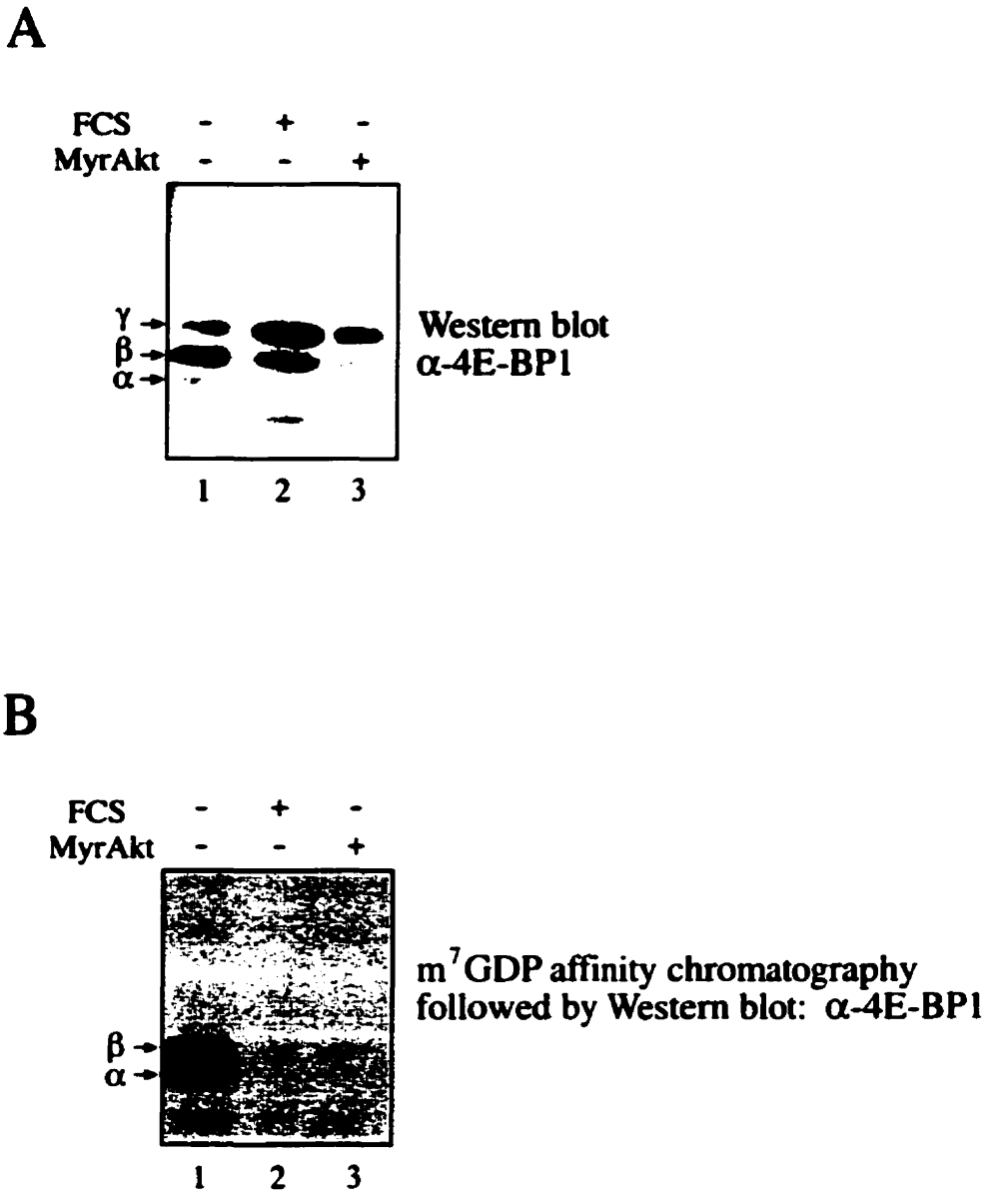


Figure 3.6. Phosphorylation of 4E-BP1 by Akt/PKB inhibits its interaction with eIF4E. Rat1a and Rat1a/MyrAkt cells were incubated in 0.5% FCS overnight. Rat1a cells were then treated with 20% FCS for 40 min. Cells were lysed by three freeze-thaw cycles and extracts were either heat treated (total extract; 100 μg) or incubated (750 μg) with m^7GDP-agarose resin, as described in Materials and Methods. Samples were separated by SDS-PAGE and 4E-BP1 protein was analyzed by Western blotting. (A) Total extract (100 μg). (B) Material bound to the m^7GDP-agarose resin. Positions of the 4E-BP1 isoforms are indicated.

phosphorylated and binds eIF4E with a low affinity; and (3) an unphosphorylated fast migrating species (α), which interacts very strongly with eIF4E (138, 166, 211). Rat1a and Rat1a MyrAkt cells were serum starved and then serum stimulated (Rat1a). A Western blot performed on total extracts indicated that the three isoforms (α, β, γ) were present in serum-starved cells (Fig. 3.6A, lane 1, the β form is predominant), only the two slower migrating forms (β and γ) were detected in serum-stimulated Rat1a cells (lane 2, the γ form is predominant). Only the hyperphosphorylated form (γ) was detected in Rat1a MyrAkt cells (lane 3; significantly less 4E-BP1 was present consistently in the MyrAkt cells, for reasons that are not immediately clear), indicating that 4E-BP1 is hyperphosphorylated. A cap-affinity isolation of eIF4E was conducted. In serum-starved Rat1a cells, there was a significant amount of 4E-BP1 (isoforms α and β) that bound eIF4E (Fig. 3.6B, lane 1). Binding was abolished in serum-stimulated cells (lane 2), and in Rat1a (MyrAkt) cells (lane 3). Taken together, these results suggest that phosphorylation of 4E-BP1 in MyrAkt cells prevents its association with eIF4E and that Akt plays a pivotal role in regulating 4E-BP1 activity in cells.

3.3.3 Akt/PKB cannot directly phosphorylate 4E-BP1

To address the question of whether Akt/PKB can phosphorylate 4E-BP1 directly, an *in vitro* kinase assay was used. The kinase reaction was performed using histone H2B as a control substrate (336). Histone H2B phosphorylation was increased gradually with time of incubation with immunoprecipitated HA-MyrAkt (Fig. 3.7, lanes 1-3). After a 45-min incubation, background phosphorylation of H2B was detected even with an immunoprecipitate from mock transfected cells (lane 7). In contrast, only background phosphorylation of GST-4E-BP1 was observed upon incubation with immunoprecipitated HA-MyrAkt (lanes 4-6, and lane 8). Phosphorylation of 4E-BP1 was not observed even after prolonged exposure (data not shown). Therefore, we conclude that Akt/PKB cannot

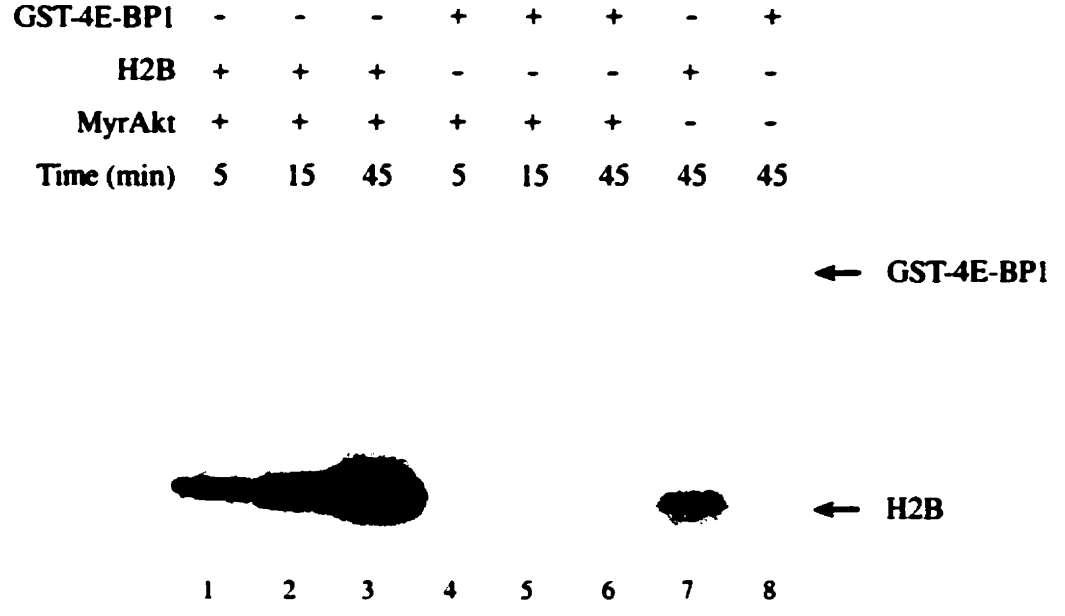


Figure 3.7. ***Akt/PKB does not phosphorylate 4E-BP1 in vitro.*** HEK 293 cells were transfected transiently with an HA-MyrAkt expression vector or with vector alone. Forty-eight hours after transfection, MyrAkt was immunoprecipitated with an anti-HA antibody (HA.11). Immunoprecipitates from mock transfected (lanes 7,8) or from HA-MyrAkt transfected cells (lanes 1-6) were used for kinase reactions, as described in Materials and Methods. GST-4E-BP1 (2 μg) and histone H2B (2 μg) were used as substrates and incubated with the immunoprecipitates for the indicated times. Samples were analyzed by SDS-PAGE. An equal amount of immunoprecipitate was used for each reaction. Equal protein loading was visualized by Coomassie Blue staining. The results shown are representative of two independent experiments.

phosphorylate 4E-BP1 *in vitro* and is unlikely to serve as the kinase that directly phosphorylates 4E-BP1 *in vivo* (see also below).

3.3.4 FRAP/mTOR activity is required for 4E-BP1 phosphorylation by Akt/PKB

Previous data and our results suggested that FRAP/mTOR is required for 4E-BP1 phosphorylation. The transfection of wild-type FRAP into serum-deprived wild-type 293 cells did not result in a mobility shift of 4E-BP1 (S.G. Kennedy and N. Hay, unpublished). To evaluate the role of FRAP/mTOR in our system, we transfected wild-type FRAP and a rapamycin-resistant mutant (S2035T) form of FRAP (338) together with HA-4E-BP1 into 293 cells stably expressing MyrAkt. The transiently transfected HA-4E-BP1 exhibited a mobility shift even after 36 hr of serum deprivation (Fig. 3.8, lane 1). These results are consistent with the increased phosphorylation of 4E-BP1 observed in serum-deprived 293-MyrAkt cells (see Fig. 3.4). Phosphorylation of 4E-BP1 in 293-MyrAkt cells was unchanged by transfection with wild-type FRAP and FRAP(S2035T) (Fig. 3.87, lanes 2,5) and was resistant to wortmannin treatment (lanes 3,6). However, phosphorylation of 4E-BP1 in 293-MyrAkt cells transfected with wild-type FRAP was still sensitive to rapamycin treatment (lane 4), whereas transfection of FRAP(S2035T), conferred rapamycin resistance to 4E-BP1 phosphorylation (lane 7). Therefore, we conclude that Akt/PKB-induced phosphorylation of 4E-BP1 requires FRAP/mTOR.

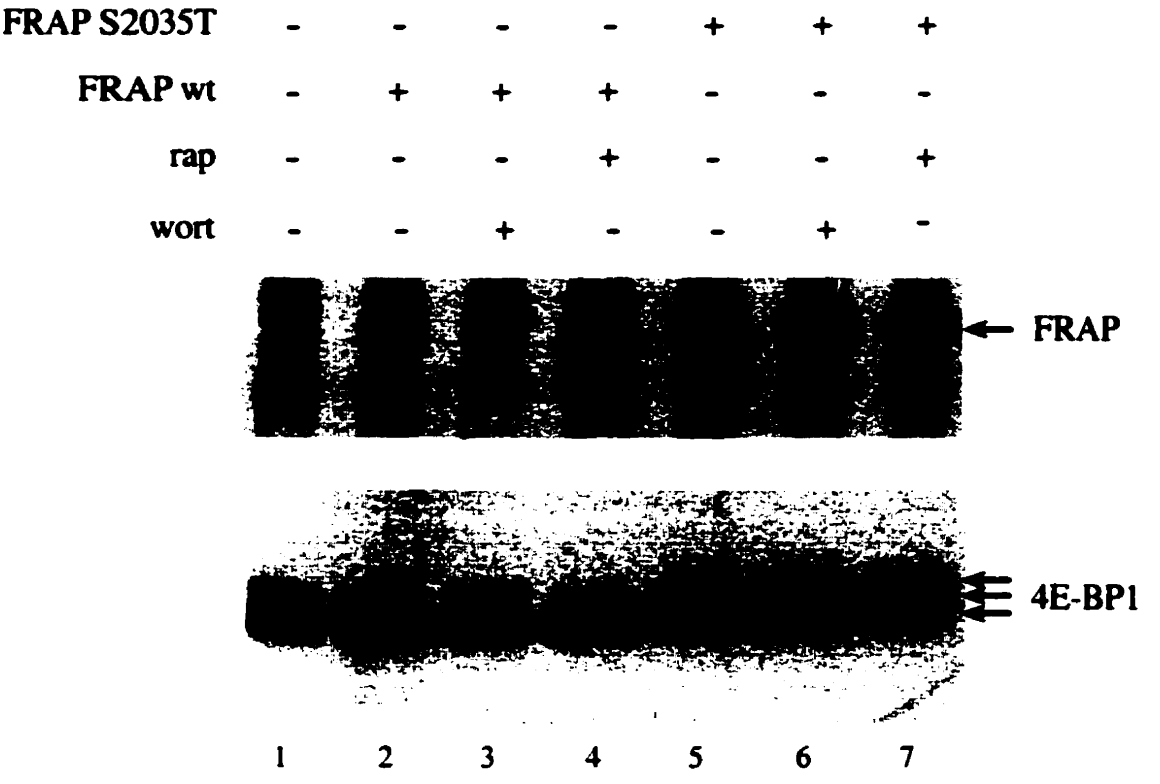


Figure 3.8. FRAP/mTOR activity is required for phosphorylation of 4E-BP1. HEK 293 MyrAkt cells were cotransfected with HA-4E-BP1 expression vector and with control vector (lane 1) or with wild-type epitope-tagged flag-FRAP expression vector, or a rapamycin-resistant mutant flag-FRAP S2035T expression vector. After transfection, cells were serum deprived for 36 hr and were either left untreated or treated with wortmannin (wort, 200 nM) or rapamycin (rap, 20 ng/ml). Cell extracts were prepared, and HA-4E-BP1 was detected as described in Fig. 3.1. Equal amounts of extract from the same experiment were used for detection of flag-FRAP with an anti-flag antibody. The positions of flag-FRAP and HA-4E-BP1 are indicated. The results shown in this figure are representative of three independent experiments.

3.4 Discussion

Studies using pharmacological agents such as wortmannin and LY294002, which inhibit PI3K activity, have indicated a role for PI3K in signaling to 4E-BP1 (e.g. 128). This hypothesis was reinforced by the use of mutants in the PDGF receptor and in the insulin-receptor substrate-1 (IRS-1) that cannot bind PI3K (128, 153). However, a recent report showing that wortmannin and LY294002 inhibit the kinase activity of FRAP/mTOR, which is required for 4E-BP1 phosphorylation, challenged the idea that PI3K is required for 4E-BP1 phosphorylation (230). In the present study we have provided direct evidence that growth factors mediate the phosphorylation of 4E-BP1 via PI3K, which is the wortmannin-sensitive component in this signaling pathway (Figs. 3.1B and 3.3A). Moreover, we have demonstrated that the downstream effector of PI3K, Akt/PKB, is a critical intermediate in the signal transduction pathway leading from growth factors to the phosphorylation of 4E-BP1. Both the wild-type catalytic subunit of PI3K and the wild-type c-Akt promote the phosphorylation of 4E-BP1 when overexpressed transiently in 293 cells. It is possible that the expression levels of these wild-type kinases are sufficiently high that even if only a fraction of the molecules are active, phosphorylation of 4E-BP1 is observed. We found that a constitutively active form of Akt/PKB promotes phosphorylation of 4E-BP1 in the absence of growth factors, in a wortmannin-resistant manner, whereas a dominant-interfering mutant of Akt/PKB blocks the ability of insulin to induce phosphorylation of 4E-BP1 (Fig. 3.2B). An activated Akt/PKB can also mediate the *in vivo* phosphorylation of 4E-BP2 (Fig. 3.4B). The phosphorylation of 4E-BP1 by an activated Akt/PKB and in response to growth factors occurred at apparently identical sites, as was demonstrated by phosphopeptide mapping (Fig. 3.5A,E). An activated Akt/PKB inhibits the binding of 4E-BP1 to eIF4E, even in the absence of growth factors, and therefore is presumed to increase eIF4E-dependent translation (Fig. 3.6).

The fact that identical phosphorylation sites on 4E-BP1 are diminished by rapamycin in both serum-induced and Akt/PKB-induced 4E-BP1 phosphorylation (Fig. 3.5) implies that the rapamycin-sensitive component lies downstream of Akt/PKB in this signaling cascade. Indeed, a rapamycin-resistant mutant of FRAP/mTOR confers rapamycin resistance to 4E-BP1 phosphorylation induced by an activated Akt/PKB (Fig. 3.8). Thus, these data allowed us to determine the order of these signaling events (Fig. 3.9). Although these results suggest a linear pathway from PI3K through Akt/PKB and to FRAP/mTOR, we cannot exclude a parallel signaling pathway that includes FRAP/mTOR and that subsequently converges upon a common downstream effector leading to phosphorylation of 4E-BP1. A recent study that demonstrated that FRAP/mTOR immunoprecipitated from cells is able to phosphorylate 4E-BP1 *in vitro* (339), is consistent with a linear signaling pathway leading from growth factor receptor to PI3K, Akt/PKB, and FRAP/mTOR. However, the observation that immunoprecipitated FRAP/mTOR can phosphorylate 4E-BP1 does not preclude the possibility that a downstream effector of both FRAP/mTOR and Akt/PKB can be coimmunoprecipitated with FRAP/mTOR and is actually responsible for 4E-BP1 phosphorylation *in vitro*. This will be addressed in more detail in chapter 6.

Since the publication of this report, several other groups have confirmed that Akt/PKB is involved in mediating 4E-BP1 phosphorylation (e.g. 340, 341, 342). These studies are complementary to ours. For example, Kohn et al. (342) constructed a conditionally active Akt/PKB (a membrane-targeted Akt/PKB fused to the hormone binding domain of a mutant murine estrogen receptor that selectively binds 4-hydroxytamoxifen). The estrogen receptor fusion keeps the Akt/PKB inactive in the absence of 4-hydroxytamoxifen. However, after addition of 4-hydroxytamoxifen, Akt/PKB is derepressed, and rapidly induces phosphorylation of 4E-BP1 (342). Takata et al. were able to prevent insulin-induced phosphorylation of 4E-BP1 in CHO cells expressing a

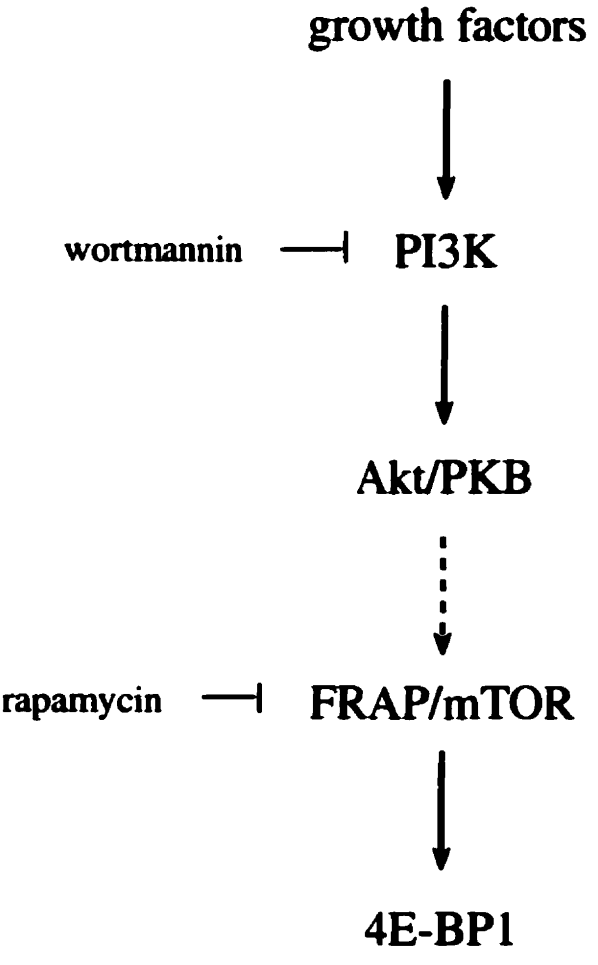


Figure 3.9. Order of the events leading to 4E-BP1 phosphorylation. Growth factors activate PI3K. This leads to the activation of Akt/PKB. The signal from Akt/PKB is relayed to 4E-BP1 only when FRAP/mTOR is active. Dashed lines indicate that a direct link between Akt/PKB and FRAP/mTOR has not been demonstrated. See text for details.

different dominant negative Akt/PKB than that we have utilized (a protein in which the two phosphorylation sites have been replaced by alanines; 340). Dufner et al. have also showed that full 4E-BP1 phosphorylation can be elicited by a non membrane-targeted Akt/PKB, providing Akt/PKB phosphorylation sites are replaced by acidic residues (341). Also, since the publication of this manuscript, it was reported that 4E-BP1 is found in a hyperphosphorylated state in the absence of stimuli in cells do not contain the phosphatase PTEN (section 1.8.4; 343).

Consequences of Akt/PKB signaling on translation were also studied: it was reported that activation of Akt/PKB results in translational activation (265, 344), consistent with the effects of Akt/PKB in inactivating 4E-BP1 and 4E-BP2. On the other hand, while an early report had indicated that S6K1 was activated downstream of a membrane-targeted Akt/PKB (240), this hypothesis was challenged by the recent observation that Akt/PKB activated by acidic substitutions of its phosphorylation sites (without artificial membrane targeting) did not induce S6K1 phosphorylation, but rather repressed it (341). This is consistent with the recent finding that Akt/PKB and S6K1 are in fact phosphorylated by the same kinase, PDK1 (reviewed in 106). Phosphorylation of S6K1 on Thr229, the PDK1 site, is absolutely critical for activation (345). Presumably, expression of the Akt/PKB constructs results in squelching of PDK1, leading to an inactivation of S6K1. It remains to be determined whether the endogenous Akt/PKB can signal to S6K1 under any condition. It is possible that conditions similar to those encountered in v-akt (fusion of Akt/PKB to membrane targeting signal) will be found in mammalian cells. This protein would be expected to increase the activity of S6K1 as well as of the physiological Akt/PKB targets, probably increasing its transforming potential.

Akt/PKB has been implicated in protection from apoptosis in various systems (section 1.8.5 and references therein). Many targets for Akt/PKB anti-apoptotic signaling were

proposed, several of which having established roles in induction of, or protection from, apoptosis. In addition, Akt/PKB targets include a number of transcription factors, such as the Forkhead family, which is involved in the synthesis of the component of the apoptotic machinery (reviewed in 237, 238). The role of the eIF4F complex as an anti-apoptotic effector of Akt/PKB needs to be addressed. Interestingly, overexpression of eIF4E induces resistance to apoptosis in serum-restricted primary fibroblasts with enforced expression of Myc (346). Consistent with a role for eIF4E as an anti-apoptotic agent, forced overexpression of 4E-BP1 wt in transformed rat embryo fibroblasts overexpressing oncogenic Ras sensitized cells to apoptosis induced by serum withdrawal or by cytostatic drugs (347).

In addition to its well-described anti-apoptotic roles, Akt/PKB also relays signals inducing cell proliferation (reviewed in 237). eIF4F could play a role in this process, as overexpression of eIF4E accelerates the proliferation (notably through shortening of the cell cycle), increases DNA synthesis, and induces malignant transformation of immortalized rodent fibroblasts (reviewed in 113). Conversely, the 4E-BPs negatively regulate cell proliferation, and partially reverse the transformed phenotype due to the expression of eIF4E, or oncogenic ras or src (348). Because of its pivotal role in cell survival and proliferation, modulation of Akt/PKB activity *in vivo* might have an impact on therapies of cancer and degenerative diseases. 4E-BPs, the new downstream targets of Akt/PKB identified in these studies, can facilitate research on the activity of this multipotent kinase because the mobility-shift assay of 4E-BP1 can provide a simple and attractive read-out for Akt/PKB activity *in vivo*.

3.5 Materials and methods

3.5.1 Plasmids and antibodies. The human 4E-BP1 coding sequence was amplified by PCR and introduced in-frame into the cytomegalovirus (CMV)-based vector pACTAG-2 (a kind gift from A. Charest and M. Tremblay, McGill University, Montreal, Canada) to express a fusion protein with three amino-terminal HA tags. Philip Tsichlis and Alfonso Bellacosa (Fox Chase Cancer Center, Philadelphia, PA) generously provided HA-c-Akt, HA-c-Akt K179M (239), and HA-MyrAkt (337) expression vectors. The BamHI/BglII fragment of HA-c-Akt K179M was introduced into pcDNA3 (Invitrogen) to generate the c-Akt (kin-) expression vector used in this study. FRAP and FRAP(S2035T) (338) expression vectors were generously provided by Stuart Schreiber and Eric Brown (Harvard University, Cambridge, MA). Myc-epitope-tagged p110α and p110α* (335) expression vectors were generously provided by Julian Downward (Imperial Cancer Research Fund, London, UK). Antibody 11208 against human 4E-BP1 was described previously (211). Antibody 11209 is a rabbit polyclonal antibody (Pocono Rabbit Farm, Canadensis, PA) raised against human 4E-BP1 (expressed as a GST fusion protein). Neither antibody cross-reacts with 4E-BP2 in immunoprecipitation studies. Anti-4E-BP2 rabbit polyclonal antibody was raised against a GST-HMK-4E-BP2 fusion protein (construct described in 195). The 4E-BP2 crude antisera cross-reacts (as indicated by Western blotting and by immunoprecipitation studies) with 4E-BP1 and, to a lesser extent, with a novel 4E-BP family member, 4E-BP3; the migration of these three proteins on SDS-PAGE is different, enabling us to still use this antibody for immunoprecipitation. The anti-HA antibody 12CA5 (mouse monoclonal) was concentrated from tissue culture supernatant using protein G-Sepharose beads (Pharmacia). The anti-HA mouse monoclonal antibody HA.11 was purchased from BabCO and was used at a dilution of 1:1000. The anti-flag mouse monoclonal antibody M5 was purchased from Kodak and was used at a dilution of 1:400. The anti-Myc-epitope mouse monoclonal antibody 9E10 was used at a dilution of 1:500 for immunoprecipitation and 1:1000 for Western blotting.

3.5.2 Cell culture and viral infection. Cell culture was performed as described previously (336, 349). Ecotropic MyrAkt retrovirus was made by transient transfection of Bosc23 cells as described previously (349). Virus was used to infect PA317 cells and a stable producer cell line was generated. The resultant amphotropic virus was used to infect HEK 293 cells, and stable clones were selected with G418 (500 μg/ml, 349). Clones were pooled and maintained as the stable cell line 293-MyrAkt.

3.5.3 Transient transfection. Transient transfections were conducted using either LipofectAMINE (GIBCO BRL) or calcium phosphate, as indicated in the figure legends. For LipofectAMINE transfection, HEK 293 cells were plated at 1 x 10^6/6-cm plate and grown overnight in DMEM/10% FCS. Cells were rinsed once with PBS and placed in 600 μl of DMEM. Four hundred nanograms of HA 4E-BP1 and 2.5 μg of the p110α and Akt/PKB expression constructs were added to 600 μl of DMEM containing 25 μl of LipofectAMINE and incubated at room temperature for 30 min. This reaction mixture was added to cells and incubated at 37°C for 4 hr. DMEM with 20% FCS (3 ml) was added for 2 hr, then cells were rinsed once with PBS and incubated in DMEM overnight. The medium was changed and cell extracts were prepared after 48 hr. Calcium phosphate transfection was conducted according to (350). The calcium phosphate/DNA precipitate was incubated with cells overnight in DMEM with 10% FCS. Cells were rinsed once with PBS and placed in DMEM. The following day cells were rinsed again with DMEM.

3.5.4 Extract preparation and Western blotting. Cells were rinsed twice with cold buffer A [20 mM Tris-HCl (pH 7.5), 100 mM KCl, 20 mM β-glycerolphosphate, 1 mM DTT, 0.25 mM Na3VO4, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 10 nM okadaic acid, 1 mM phenylmethylsulfonyl fluoride] and scraped into a minimal volume of the same

buffer. Lysis was performed by three freeze-thaw cycles. Cell debris was pelleted by centrifugation, and the protein concentration in the supernatant was measured using the Bio-Rad assay. For analysis of endogenous 4E-BP1, 75 μg of total cell extract was incubated at 100°C for 7 min to enrich for 4E-BP1, which is heat-stable. Samples were incubated on ice for 5 min, and precipitated material was removed by centrifugation (13,000 rpm, 5 min). When necessary, the extract was TCA precipitated (195). Laemmli sample buffer was added to the supernatant, which was then subjected to SDS-15% PAGE. For analysis of transfected HA-4E-BP1, cells were lysed by three freeze-thaw cycles, and 50 μg of protein was analyzed by SDS-10% PAGE. Western blotting, using chemiluminescence detection, was performed as described (211) using either the anti-4E-BP1 11208 antibody (1:1500) or the anti-HA 12CA5 monoclonal antibody (0.5 μg/ml). For analysis of p110α, p110α and p110α* expression vectors were transfected into HEK 293 cells. After 36 hr cells were lysed into 500 μl of RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS] containing 1 mM PMSF and subjected to immunoprecipitation for 3 hr with 1 μg of the mouse monoclonal anti-Myc antibody 9E10. Immunoprecipitates were rinsed three times with RIPA buffer and resuspended in Laemmli buffer. Samples were boiled and subjected to SDS-8% PAGE. p110α was detected by incubation with the 9E10 antibody.

3.5.5 Chromatography on m^7GDP-agarose. Cell extracts prepared by three freeze-thaw cycles, in buffer containing 20 mM HEPES-KOH (pH 7.5), 75 mM KCl and 1 mM EDTA, were incubated for 1 hr with m^7GDP coupled to agarose adipic resin [30 μl of packed beads per reaction; beads were prepared according to (177). Beads were spun down in a microfuge (3000 rpm, 30 sec), washed three times with 20 volumes of the same buffer, and resuspended in Laemmli sample buffer. Samples were then analyzed by SDS-PAGE and Western blotting as described above.

3.5.6 Metabolic labeling and immunoprecipitation. HEK 293 cells starved for 30-36 hr or Rat1a cells starved for 16-24 hr were incubated at 37°C for 3 hr in serum-free DMEM containing 0.5 mCi/ml [^{32}P]orthophosphate (DuPont NEN; 3000 mCi/mmole). Rapamycin (20 ng/ml) or wortmannin (100 nM) were added for 20 min, followed by the addition of dialyzed FBS (15%; GIBCO) for 30 min. The medium was removed and the cells were rinsed twice in cold PBS. Cells were lysed in lysis buffer [10% glycerol, 50 mM Tris (pH 7.5), 60 mM KCl, 2 mM CDTA (trans,-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid), 1% Triton X-100, 2 mM DTT, 20 mM β-glycerolphosphate, 10 nM okadaic acid] for 30 min at 4°C. Lysate was harvested by scraping and cell debris was removed by centrifugation. Total radioactivity in the lysate was monitored by spotting 1, 2, 5, and 10 μl of the extract onto a phosphocellulose (P81) paper, which was washed extensively with 75 mM phosphoric acid and dried. Bound radioactivity was measured by scintillation counting (111). The extract (equivalent quantities of radioactivity) was precleared by incubation with protein A beads (50 μl per ml of extract) with end-over-end rotation at 4°C for 1 hr. The supernatant was transferred to a fresh tube, together with 30 μl of 11209 crude antisera per milliliter of extract, and incubated for 3 hr at 4°C. Protein A beads (30 μl packed beads) were added and incubation end-over-end was carried out for 2 hr at 4°C. Beads were spun down (microfuge, 6000 rpm, 2 min) and washed two times in lysis buffer, 2 times in RIPA buffer, and 2 times in LiCl solution (200 mM LiCl, 1 mM DTT). In some experiments, the supernatant (unbound fraction) was further incubated with 11211 anti-4E-BP2 antibody as for 4E-BP1. Immunoprecipitated material was subjected to SDS-15% PAGE and transferred to PVDF membranes (Immobilon-P or Immobilon-PSQ, Millipore), which were dried and autoradiographed. Radioactive bands corresponding to 4E-BPs were excised and Cerenkov counted.

3.5.8 Phosphopeptide maps. Tryptic-chymotryptic digestion of 4E-BP1 immobilized on the PVDF membranes was performed essentially as described (351), with the following modifications. The digest was performed using a 200:1 mixture of TPCK-treated trypsin and chymotrypsin (5 μg, Worthington) for 10 hr, followed by the addition of 5 μg enzyme for 3 hr. The sample was then lyophilized (speed-vac, Savant), resuspended in 200 μl of water, lyophilized again, resuspended in 100 μl of water, lyophilized a third time, resuspended in 100 μl (pH 1.9) buffer (2.5% vol/vol formic acid 88% and 7.8% vol/vol glacial acetic acid) and lyophilized a fourth time. For chromatography, first dimension (electrophoresis) was performed in pH 1.9 buffer using the HTLE 7000 apparatus (CBS Scientific); second dimension was performed in phosphochromatography buffer (37.5% vol/vol n-butanol, 25% vol/vol pyridine, 7.5% vol/vol glacial acetic acid). Plastic-coated cellulose thin-layer chromatography plates (Kodak; 20 cm x 20 cm) were used.

3.5.9 Akt/PKB kinase assay. HEK 293 1 x 10^6 cells were transfected with 3 μg of HA-MyrAkt DNA using LipofectAMINE. Cells were grown in 10% DMEM for 48 hr, then lysed into Akt/PKB lysis buffer (336). Extracts were incubated with 3 μg of monoclonal mouse anti-HA (BabCO, HA.11) for 3 hr. Immunoprecipitations and kinase reactions with histone H2B (2 μg) and GST-4E-BP1 (2 μg) were conducted according to (239).

3.6 Acknowledgments

This paper was co-authored by Anne-Claude Gingras, Scott G. Kennedy, Maura A. O'Leary, Nahum Sonenberg, and Nissim Hay. A.-C.G and S.G.K contributed equally to this paper. The authors are grateful to S. Schreiber, A. Bellacosa, P. Tsichlis, and J. Downward for plasmids and antibodies. We thank C. Palfrey, B. Raught, and S. Conzen for critical comments and valuable discussions. This work was supported by American Cancer Society (ACS) grant CB-133 and by National Institutes of Health (NIH) grant CA71874 (N.H.), and by the National Cancer Institute of Canada (N.S.). N.S. is a Medical Research Council of Canada Distinguished Scientist and a Howard Hughes Medical Institute International Scholar. A.-C.G. was supported by a Natural Science and Engineering of Canada 1967 Studentship. S.G.K. was supported by training grant GM07151.

CHAPTER 4

REGULATION OF 4E-BP1 PHOSPHORYLATION: A NOVEL TWO-STEP MECHANISM

4.1 Introduction and perspectives

In the previous chapter, we established FRAP/mTOR as the most proximal known kinase in the 4E-BP1 phosphorylation pathway. Reports published during the course of these studies suggested that FRAP/mTOR could in fact phosphorylate 4E-BP1 directly. Indeed, immunoprecipitates of FRAP/mTOR from rat brain, or from cells transfected with a tagged form of FRAP/mTOR, contain a kinase activity capable of phosphorylating 4E-BP1, while immunoprecipitates of a catalytically inactive FRAP/mTOR mutant fail to phosphorylate 4E-BP1 (339, 352, 353). Similarly, addition of a complex of FKBP12-rapamycin to the *in vitro* kinase assay abrogates phosphorylation of 4E-BP1, indicating that the kinase activity present in the FRAP immunoprecipitates is sensitive to rapamycin (339, 352, 353). There was disagreement, however, as to the number and identity of the sites phosphorylated by FRAP/mTOR. Brunn et al. (339) reported the phosphorylation of five 4E-BP1 Ser/Thr-Pro sites by a FRAP/mTOR immunoprecipitate *in vitro*, and concluded that FRAP/mTOR was responsible for the phosphorylation of all *in vivo* 4E-BP1 phosphorylation sites (205). However, Burnett et al. (353) subsequently reported that only two 4E-BP1 sites, threonines 37 and 46 (the numbering throughout this paper is according to the human 4E-BP1 protein), were phosphorylated by FRAP/mTOR. When threonines 37 and 46 were mutated to alanines, binding to eIF4E was reported to be constitutive (353). These authors thus concluded that phosphorylation of Thr37 and Thr46 leads to the dissociation of 4E-BP1 from eIF4E. Another report demonstrated, however, that 4E-BP1 phosphorylated on Thr37 is associated with eIF4E (205). Thus, at the onset of these studies, it was known that 4E-BP1 is phosphorylated *in vivo* on multiple residues (205, 214). However, the order of phosphate addition and the functional significance of each phosphorylation event regarding the interaction of 4E-BP1 with eIF4E were unknown. The relative sensitivity of each site to different stimuli and pharmacological inhibitors also remained unknown. We thus attempted to study 4E-BP1 phosphorylation, at individual sites, using a combination of phosphopeptide

mapping and mass spectrometry. We first applied these techniques to elucidate the number and identity of the sites phosphorylated by FRAP/mTOR immunoprecipitates and to study the functional consequences of phosphorylation at these residues. While we could obtain phosphorylation of 4E-BP1 *in vitro* with an immunoprecipitated FRAP/mTOR or with a baculovirus-expressed FRAP/mTOR, we only observed phosphorylation of two phosphopeptides by phosphopeptide mapping. We then went on to identify these sites and determine the functional consequences of phosphorylation at these two residues by FRAP/mTOR. In the next chapter, we describe that these two residues, Thr37 and Thr46 are phosphorylated under basal conditions in 293 cells (i.e. in the absence of serum), but that their replacement to alanines prevent incorporation of phosphate in the remaining phosphorylation sites. Thus, phosphorylation at Thr37 and Thr46 acts as a priming event for the phosphorylation of the other residues, suggesting a two-step model for the phosphorylation of 4E-BP1.

4.2 Abstract

The multisubunit eukaryotic translation initiation factor (eIF) 4F recruits 40S ribosomal subunits to the 5' end of mRNA. The eIF4F subunit eIF4E interacts directly with the mRNA 5' cap structure. Assembly of the eIF4F complex is inhibited by a family of repressor polypeptides, the eIF4E-binding proteins (4E-BPs). Binding of the 4E-BPs to eIF4E is regulated by phosphorylation: Hypophosphorylated 4E-BP isoforms interact strongly with eIF4E, whereas hyperphosphorylated isoforms do not. 4E-BP1 is hypophosphorylated in quiescent cells, but is hyperphosphorylated on multiple sites following exposure to a variety of extracellular stimuli. The PI3K-Akt/PKB pathway and the kinase FRAP/mTOR signal to 4E-BP1. FRAP/mTOR has been reported to phosphorylate 4E-BP1 directly *in vitro*. However, it is not known if FRAP/mTOR is responsible for the phosphorylation of all 4E-BP1 sites, nor which sites must be phosphorylated to release 4E-BP1 from eIF4E. To address these questions, a recombinant FRAP/mTOR protein and a FRAP/mTOR immunoprecipitate were utilized in *in vitro* kinase assays to phosphorylate 4E-BP1. Phosphopeptide mapping of the *in vitro*-labeled protein yielded two 4E-BP1 phosphopeptides that comigrated with phosphopeptides produced *in vivo*. Mass spectrometry analysis indicated that these peptides contain phosphorylated Thr37 and Thr46. Thr37 and Thr46 are efficiently phosphorylated *in vitro* by FRAP/mTOR when 4E-BP1 is bound to eIF4E. However, phosphorylation at these sites was not associated with a loss of eIF4E binding. Phosphorylated Thr37 and Thr46 are detected in all phosphorylated *in vivo* 4E-BP1 isoforms, including those that interact with eIF4E. Finally, mutational analysis demonstrated that phosphorylation of Thr37/Thr46 is required for subsequent phosphorylation of several carboxy-terminal serum-sensitive sites. Taken together, our results suggest that 4E-BP1 phosphorylation by FRAP/mTOR on Thr37 and Thr46 is a priming event for subsequent phosphorylation of the carboxy-terminal serum-sensitive sites.

4.3 Results

4.3.1 Both recombinant FRAP/mTOR and a FRAP/mTOR immune complex phosphorylate 4E-BP1.

Phosphopeptide mapping has proven to be a powerful method in the study of the sensitivity of specific phosphorylation sites to various stimuli (see, e.g., 124, 127, 223, 353). This technique is also applied to the identification of putative kinases by comparing the pattern of the phosphopeptides generated *in vitro* with those phosphorylated *in vivo*. Here it was used to better define the role of FRAP/mTOR in 4E-BP1 phosphorylation. A rat brain FRAP/mTOR immunoprecipitate and a purified baculovirus-expressed Flag-tagged FRAP/mTOR fusion protein were used in an *in vitro* kinase assay with recombinant human 4E-BP1 as a substrate. ^{32}P-Labeled 4E-BP1 was separated via SDS-PAGE, then transferred to nitrocellulose membranes, which were subjected to autoradiography. 4E-BP1 was readily phosphorylated by both preparations of FRAP/mTOR (data not shown). The membrane piece bearing the labeled 4E-BP1 protein was excised and incubated in a trypsin/chymotrypsin mixture, and two-dimensional phosphopeptide mapping was performed on the liberated peptides. The map prepared from 4E-BP1 phosphorylated by the rat brain FRAP/mTOR immunoprecipitate yielded two major phosphopeptides (indicated as 1 and 2; Fig. 4.1A). In parallel, serum-stimulated 293 cells were labeled *in vivo* with [^{32}P]orthophosphate, and endogenous 4E-BP1 was subjected to tryptic/chymotryptic mapping (Fig. 4.1B). Mixing of the *in vitro*- and the *in vivo*-labeled products demonstrated that the two phosphopeptides generated by FRAP/mTOR labeling comigrate with the two most prominent *in vivo* phosphopeptides (Fig. 4.1C). The same major phosphopeptides were observed in 4E-BP1 phosphorylated by a recombinant baculovirus-expressed FRAP/mTOR (Fig. 4.1D), consistent with the notion that 4E-BP1 phosphorylation on these sites is catalyzed by FRAP/mTOR itself, and not by a contaminating kinase in the immunoprecipitate.

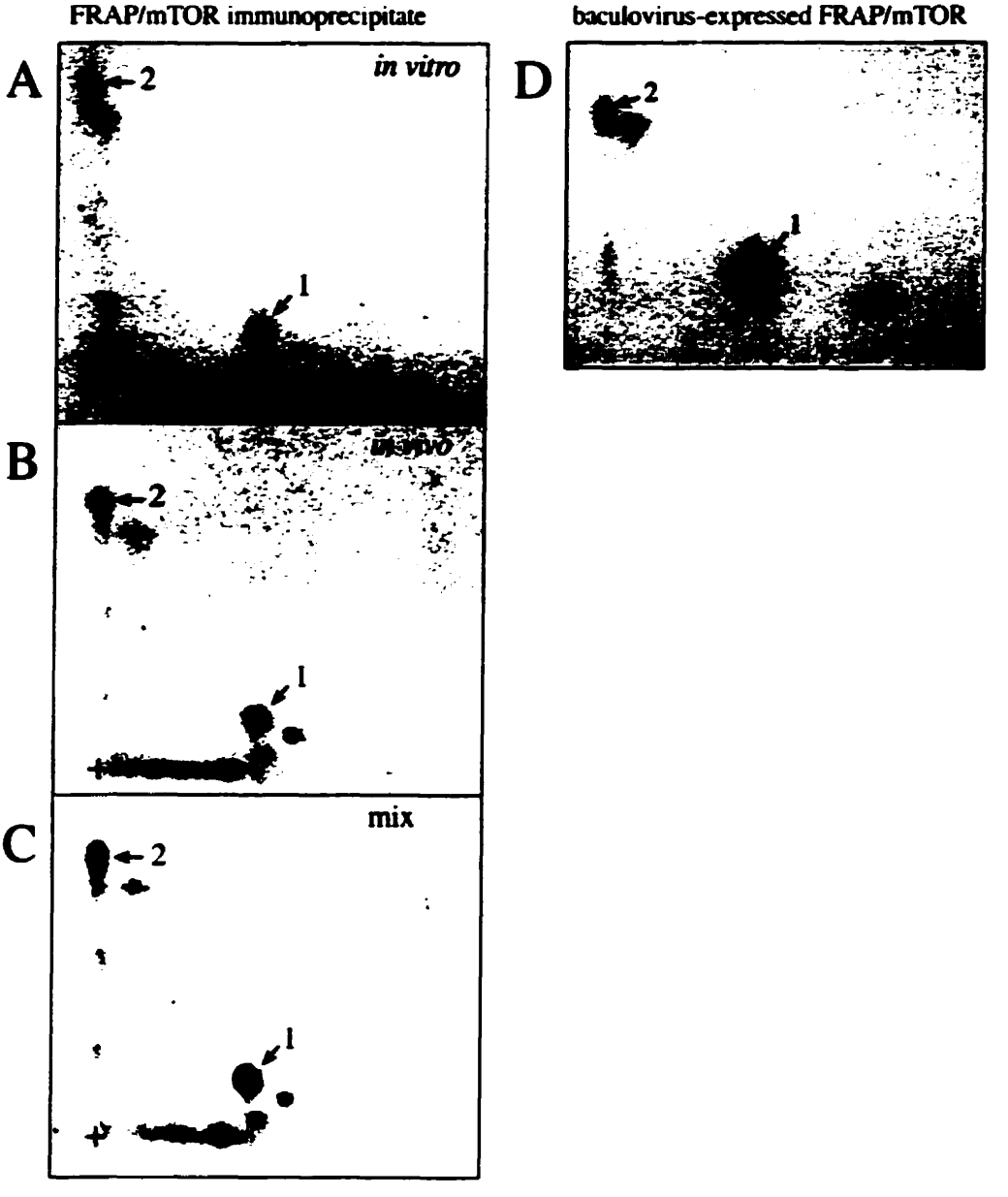


Figure 4.1. FRAP/mTOR phosphorylates 4E-BP1 in vitro on two sites that are also phosphorylated in vivo. (A) FRAP/mTOR immunoprecipitated from rat brain was utilized in an *in vitro* kinase assay with human 4E-BP1 (expressed as a GST fusion protein, which was then cleaved and HPLC purified) as a substrate. Phosphopeptide mapping was performed on phosphorylated 4E-BP1. The two most intense phosphopeptides are numbered 1 and 2. (+) Origin of migration. (B) Tryptic/chymotryptic map of 4E-BP1 phosphorylated in 293 cells. The positions of phosphopeptides that comigrate with the FRAP/mTOR phosphorylated peptides 1 and 2 are indicated. (C) An equal number of cpm from the *in vitro*- and the *in vivo*-phosphorylated 4E-BP1 were combined and phosphopeptide mapping was performed. (D) Tryptic/chymotryptic map of 4E-BP1 phosphorylated *in vitro* with purified FRAP/mTOR produced using the baculovirus system.

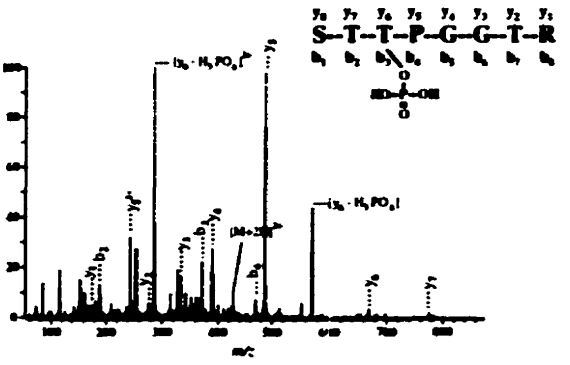
It should be noted that it remains possible that an endogenous kinase from the insect cells coprecipitates with FRAP/mTOR during the purification procedure; this possibility would be excluded by the use of bacterially-expressed FRAP/mTOR. However, attempts to express FRAP/mTOR in bacteria have been unsuccessful in our laboratory and others. The peptide of lower intensity observed in Figure 4.1A next to peptide 1 was obtained in several experiments and appears to comigrate with an *in vivo* peptide. However, unlike peptides 1 and 2, its intensity decreases drastically if the FRAP/mTOR immunoprecipitate is washed at higher stringency, or if recombinant 4E-BP1 is incubated for shorter times with the FRAP/mTOR immunoprecipitate. Also, phosphorylation of this peptide was extremely weak in the 4E-BP1 sample phosphorylated with baculovirus-expressed FRAP/mTOR. Thus, phosphorylation at this residue may arise as a consequence of a contaminating kinase activity in our preparations of FRAP/mTOR. Other peptides are visible in some experiments, but also appear to be due to a contaminating kinase activity. Because the immunoprecipitated material is more readily available, FRAP/mTOR immunoprecipitates were used in all subsequent experiments.

4.3.2 Identification of in vivo 4E-BP1 phosphorylation sites

To identify *in vivo* 4E-BP1 phosphorylation sites, 293 cells were grown to confluence, then starved of serum. A portion of the cells was then incubated with [^{32}P]orthophosphate, stimulated with serum, and lysed. The remainder of the cells was treated in the same manner, without the labeling step. 4E-BP1 was then immunoprecipitated and the labeled and unlabeled immunoprecipitates were mixed, separated by SDS-PAGE, and transferred onto a nitrocellulose membrane. After autoradiography, the protein was subjected to two-dimensional phosphopeptide mapping (a representative map is shown in Fig. 4.2A). Peptides 1 and 2, which comigrated with the *in vitro* FRAP/mTOR phosphorylated 4E-BP1 peptides, as well as peptides 3-5, which migrated in the same area as peptide 2, were scraped from the two-dimensional

A

B



C

phosphopeptide	peptide sequence	location
1	(F)ST**T**PGGTR	44-51
2	(R)VVLGDGVQLPPGDYST**T**PGGTLF	21-43
3	(R)RVVLGDGVQLPPGDYST**T**PGGTLFST**T**PGGTR	20-51
4	(R)VVLGDGVQLPPGDYST**T**PGGTLFST**T**PGGTR	21-51
5	(R)RVVLGDGVQLPPGDYST**T**PGGTLF	20-43

D

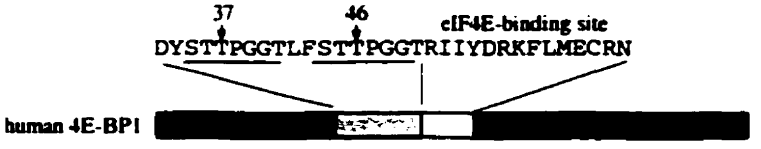


Figure 4.2. Thr-37 and Thr-46 are phosphorylated in vivo on phosphopeptides that comigrate with FRAP/mTOR phosphorylated peptides. 4E-BP1 immunoprecipitated from 2 x 10^8 cells (1/20 labeled with [^{32}P]orthophosphate) was separated by SDS-15% PAGE, electroblotted onto a nitrocellulose membrane, and proteolytically cleaved with trypsin/chymotrypsin as described in Materials and Methods. (A) Tryptic/chymotryptic two-dimensional phosphopeptide map of 4E-BP1. Phosphopeptides (labeled 1-5) were isolated from the plates and identified by mass spectrometry. (B) Tandem mass spectrum of m/z 428.9 revealing the sequence and phosphorylation site of the phosphopeptide contained in spot 1 of the map shown in A. The position of the phosphorylated threonine residue (Thr-46) is shown. (C) Identification of the phosphorylated peptides in spots 1-5 from the phosphopeptide map shown in A. Phosphorylated amino acids are in boldface type; the amino acid amino terminal to the cleavage site for trypsin or chymotrypsin is indicated in parentheses. (D) The positions of Thr-37 and Thr-46 in 4E-BP1, relative to the eIF4E-binding site.

phosphopeptide map and eluted. Phosphopeptides were identified by capillary liquid chromatography-electrospray ionization tandem mass spectrometry (LC-MS/MS). The tandem mass spectrum for phosphopeptide 1 corresponds to 44-STTPGGTR-51, with the phosphorylation site being Thr46 (Fig. 4.2B). Peptide 2 was identified as 21-VVLGDGVQLPPGDYSTTPGGTLF-43, with the phosphorylation site being Thr37 (data not shown). Peptides 3-5, whose intensities varied from experiment to experiment, contain both phospho-Thr37 and phospho-Thr46 and are derived from partially digested products (data not shown). The sequence of each identified phosphopeptide and the position of the phosphorylated amino acids are shown in Figure 4.2C. In agreement with these findings, all of these peptides contained only phosphothreonine, as detected by phosphoaminoacid analysis (data not shown). Threonines 37 and 46 are located in the middle of 4E-BP1, immediately amino-terminal to the eIF4E-binding site (Fig. 4.2D).

4.3.3 FRAP/mTOR phosphorylates Thr37 and Thr46

To confirm that the *in vivo* phosphorylation sites were the same as those phosphorylated *in vitro* by FRAP/mTOR, histidine-tagged mutants of 4E-BP1 containing either a Thr37Ala or a Thr46Ala mutation were phosphorylated by a FRAP/mTOR immunoprecipitate. Quantitatively, both mutant proteins were phosphorylated to approximately half the extent of wild-type 4E-BP1 (data not shown). Tryptic/chymotryptic mapping of the mutants was also performed. Whereas the map of the wild-type protein shows the presence of the two phosphopeptides containing Thr37 and Thr46, the map of the Thr37Ala mutant contains only phosphorylated Thr46 (cf. Fig. 4.3, B and A). Similarly, the map from the Thr46Ala mutant confirms Thr46 as a target for FRAP/mTOR (Fig. 4.3C). When both sites are mutated to alanines, or when the region containing both threonines (amino acids 34-52 in human 4E-BP1) is deleted, the phosphorylation of 4E-BP1 by FRAP/mTOR is almost abolished (these mutants are

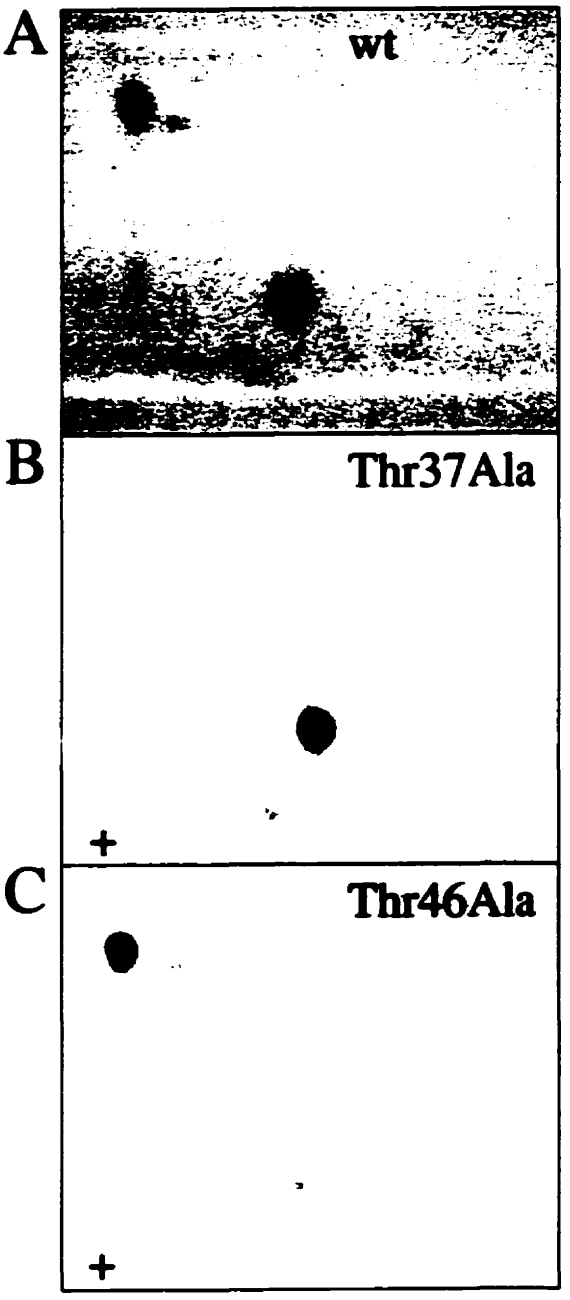


Figure 4.3. In vitro kinase assay of Thr37Ala and Thr46Ala mutant proteins confirm that they are the primary FRAP/mTOR phosphorylation sites. Histidine-tagged murine 4E-BP1 was phosphorylated *in vitro* in an immune-complex kinase assay with FRAP/mTOR, and tryptic/chymotryptic maps were performed. (A) Wild type; (B) Thr37Ala; (C) Thr46Ala.

labeled to <5% of the levels observed for the wild-type protein; data not shown). Thus, Thr37 and Thr46 are the two primary amino acids phosphorylated by FRAP/mTOR.

4.3.4 FRAP/mTOR phosphorylates a 4E-BP1/eIF4E complex

Hypophosphorylated 4E-BPs interact strongly with eIF4E, and one or more kinases are posited to induce the disruption of the eIF4E/4E-BP complex by phosphorylation of the 4E-BPs. Thus, the *in vivo* substrate for a 4E-BP kinase should be a 4E-BP/eIF4E complex, and a physiological 4E-BP kinase(s) should be able to phosphorylate 4E-BP when bound to eIF4E. To determine whether the FRAP/mTOR immunoprecipitate can phosphorylate a 4E-BP1/eIF4E complex *in vitro*, an equimolar quantity (Fig. 4.4A, lane 2) or a twofold molar excess of eIF4E (lane 3) was preincubated with 4E-BP1, then a kinase assay was performed. As a control for the specificity of the kinase reaction, 4E-BP1 was also phosphorylated under the same conditions with the MAP kinase ERK2. ERK2 was reported previously to phosphorylate 4E-BP1 preferentially on Ser65 (221), but is unable to phosphorylate 4E-BP1 complexed with eIF4E (138). The presence of eIF4E did not reduce FRAP/mTOR phosphorylation of 4E-BP1 (Fig. 4.4A, cf. lanes 2 and 3 to lane 1). In contrast, although ERK2 readily phosphorylated free 4E-BP1, it failed to phosphorylate 4E-BP1 in the presence of a twofold molar excess of eIF4E (cf. lanes 6 and 4). eIF4E prevented phosphorylation of the wild-type 4E-BP1 by ERK2, but not of a 4E-BP1 mutant lacking the eIF4E binding site (Fig. 4.4A, lanes 7 and 8), indicating that the inhibition by eIF4E is a direct consequence of its interaction with 4E-BP1. eIF4E was not a substrate for FRAP/mTOR, nor for ERK2 (Fig. 4.4A). 4E-BP1 phosphorylated by FRAP/mTOR in the presence (Fig. 4.4B) or absence (Fig. 4.4C) of eIF4E was analyzed by two-dimensional phosphopeptide mapping. In both cases, Thr37 and Thr46 were the primary phosphorylation sites. Phosphorylation of the 4E-BP1/eIF4E complex by a FRAP/mTOR immunoprecipitate is consistent with it (or an associated kinase) being a physiological 4E-BP1 kinase.

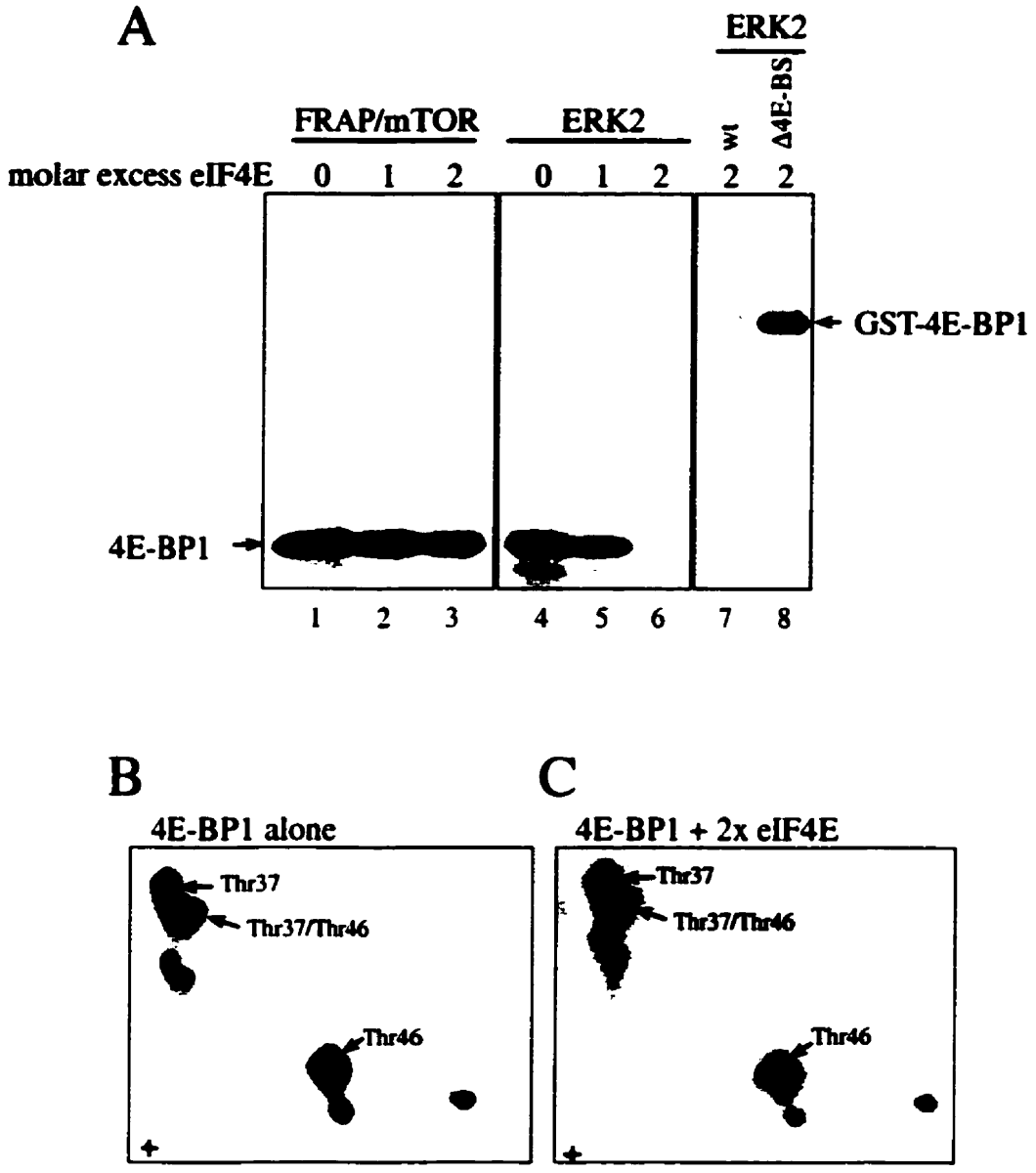


Figure 4.4. FRAP/mTOR phosphorylates 4E-BP1 complexed with eIF4E. (A) 4E-BP1 was pre-incubated without eIF4E, with an equimolar quantity or with a twofold molar excess of eIF4E, and subjected to a kinase assay using FRAP/mTOR immunoprecipitated from rat brain (lanes 1-3) or recombinant ERK2 (lanes 4-8). Phosphorylated proteins were separated via SDS-15%-PAGE, transferred to nitrocellulose membranes, and subjected to autoradiography. The substrates used were as follows. (Lanes 1-6) A thrombin-cleaved and HPLC-purified GST-4E-BP1; (lane 7) an uncleaved GST-4E-BP1 wild-type protein; (lane 8) an uncleaved GST-4E-BP1 mutant lacking the eIF4E-binding site. (B) Tryptic/chymotryptic map of 4E-BP1 alone (A, lane 1). (C) Tryptic/chymotryptic map of 4E-BP1 complexed with a twofold molar excess eIF4E (A, lane 3).

4.3.5 FRAP/mTOR phosphorylation of 4E-BP1 does not disrupt the 4E-BP1/eIF4E complex

After establishing that FRAP/mTOR can phosphorylate 4E-BP1 complexed with eIF4E, it was important to determine whether phosphorylation of Thr37/Thr46 disrupts the 4E-BP1/eIF4E complex. A 4E-BP1/eIF4E complex was phosphorylated by FRAP/mTOR, then incubated with a cap analog (m^7GDP) coupled to agarose beads, washed, and subjected to SDS-PAGE. As demonstrated in Figure 4.4, the addition of eIF4E did not affect the phosphorylation of 4E-BP1 by FRAP/mTOR (Fig. 4.5A, bottom panel, cf. lanes 1 and 2). Importantly, most of the ^{32}P-labeled 4E-BP1 (~65%) was retained on the m^7GDP beads in the presence of eIF4E (Fig. 4.5A, cf. lane 2, top and lane 2, bottom panel). A portion of 4E-BP1 (~35%) was found in the unbound fraction after the m^7GDP-agarose pull-down (cf. middle and bottom panels, lane 2). This procedure does not quantitatively sequester eIF4E either, however, as ~20% of the eIF4E is also present in the unbound fraction (data not shown). 4E-BP1 in the bound and unbound fractions was analyzed by two-dimensional phosphopeptide mapping. Similar levels of phospho-Thr37 and phospho-Thr46 were observed in each case (Fig. 4.5B), demonstrating that the phosphorylation of 4E-BP1 on Thr37 and Thr46 does not disrupt the 4E-BP1/eIF4E complex. It cannot be ruled out, however, that only monophosphorylated 4E-BP1 molecules interact with eIF4E and that the proteins phosphorylated on both residues do not bind. Nevertheless, more phosphorylated 4E-BP1 remains bound to eIF4E than dissociates after incubation with FRAP/mTOR. This indicates that the kinase activity present in the FRAP/mTOR immunoprecipitate is insufficient to disrupt the 4E-BP1:eIF4E complex. Consistent with this result, a deletion of the region encompassing Thr37 and Thr46 (amino acids 34-50) binds to eIF4E to the same extent as the wild-type protein, indicating that these amino acids are not involved in mediating eIF4E binding (data not shown). Furthermore, a bacterially expressed double mutant of 4E-BP1

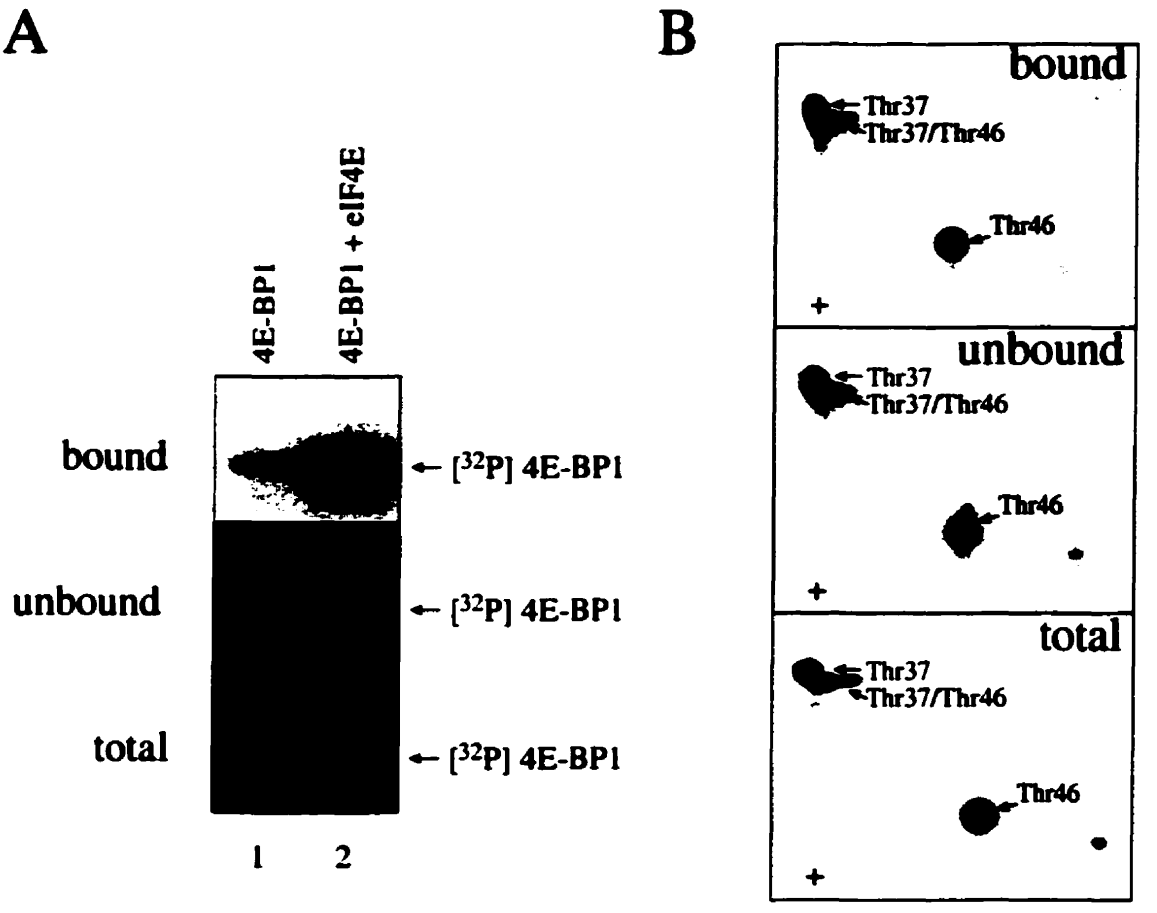


Figure 4.5. FRAP/mTOR phosphorylation of Thr-37 and Thr-46 does not disrupt the 4E-BP1/eIF4E complex. (A) 4E-BP1 alone, or a 4E-BP1/eIF4E complex, was incubated with FRAP/mTOR, as in Fig. 4.4. The kinase reaction was stopped and one-half of the material was immediately subjected to SDS-PAGE (bottom). The remaining material was incubated with m^7GDP-agarose beads to purify eIF4E and associated proteins. The bound (top) and unbound (middle) fractions were analyzed by SDS-PAGE. (B) Phosphorylated 4E-BP1 (A, lane 2; bound, unbound, and total) was analyzed by two-dimensional phosphopeptide mapping.

Thr37Glu/Thr46Glu (designed to mimic phosphorylation at these residues) was also without effect on eIF4E binding (data not shown).

4.3.6 Thr37 and Thr46 are phosphorylated to a high stoichiometry in serum-starved cells and are detected in all phosphorylated isoforms in vivo

Multiple sites on 4E-BP1 are phosphorylated following the addition of serum to serum-starved cells. However, 4E-BP1 is phosphorylated to a significant extent even in 293 cells deprived of serum. To map the phosphoaminoacids present in serum-starved cells, starved or stimulated 293 cells were labeled with [^{32}P]orthophosphate *in vivo*, and phosphopeptide maps were generated from immunoprecipitated 4E-BP1. Phosphopeptides containing phospho-Thr37 and -Thr46 are present at relatively high levels in serum-starved cells, and their total quantity increased only marginally following serum-stimulation relative to the quantity of protein present, in contrast to the serum-induced phosphopeptides a-d (Fig. 4.6, cf. panels A and B; phosphopeptides a-d are very sensitive to minor variations in serum treatment. Depending on the confluency and passage number of the cells, the time span of serum-starvation and stimulation and even the batch of serum, the quantity of these phosphopeptides vary; see, e.g., Figs. 4.1B, 4.2A, and 4.6B).

An increase in 4E-BP1 phosphorylation is accompanied by a decrease in its electrophoretic mobility. To determine the order of phosphate addition, each of the forms separated by SDS-PAGE was mapped. As previously observed (e.g. 124, 211, 219, 223), multiple isoforms of human 4E-BP1 incorporate ^{32}P *in vivo* (3-5 distinct bands, depending on the cell type and resolution of the gel; in Fig. 4.7A, inset, three bands were separated). Tryptic maps from the slowest (Fig. 4.7A) and the fastest (Fig. 4.7B) migrating isoform are shown. Phosphopeptides containing Thr37 and Thr46 are present in both 4E-BP1 isoforms (cf. panels B and A). This observation is not restricted to

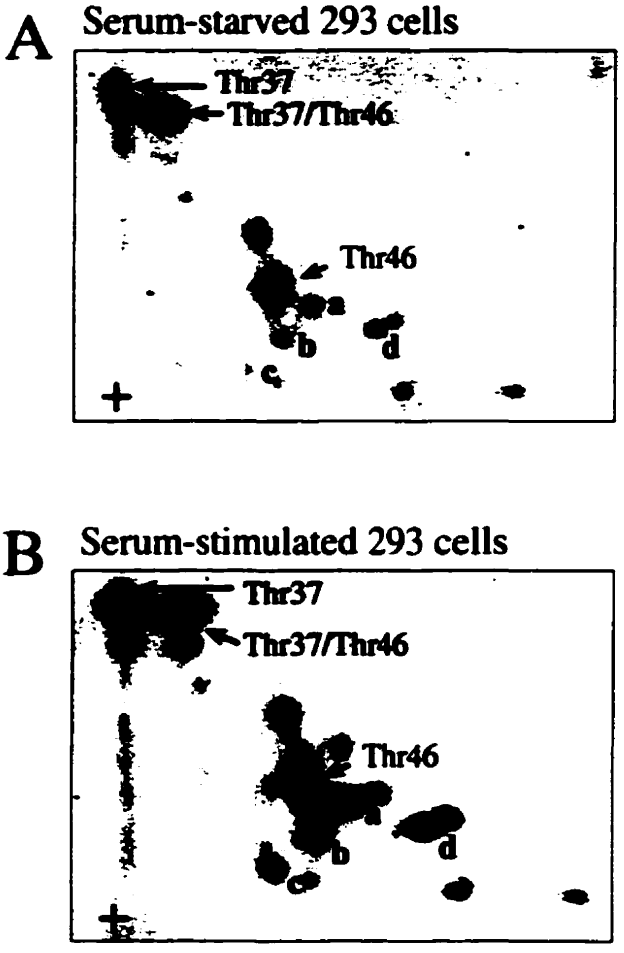


Figure 4.6. Phosphorylated Thr37 and Thr46 are present at high stoichiometries in serum-starved 293 cells. 293 cells were grown to confluence and deprived of serum for 30 hr. ^{32}P-Labeling was performed for 4 hr and phosphopeptide maps were generated from starved cells (A) or cells stimulated for 30 min with 10% dialyzed FCS (B). Phosphopeptides containing phospho-Thr37 or -Thr46 are identified. Serum-induced phosphopeptides are labeled a-d.

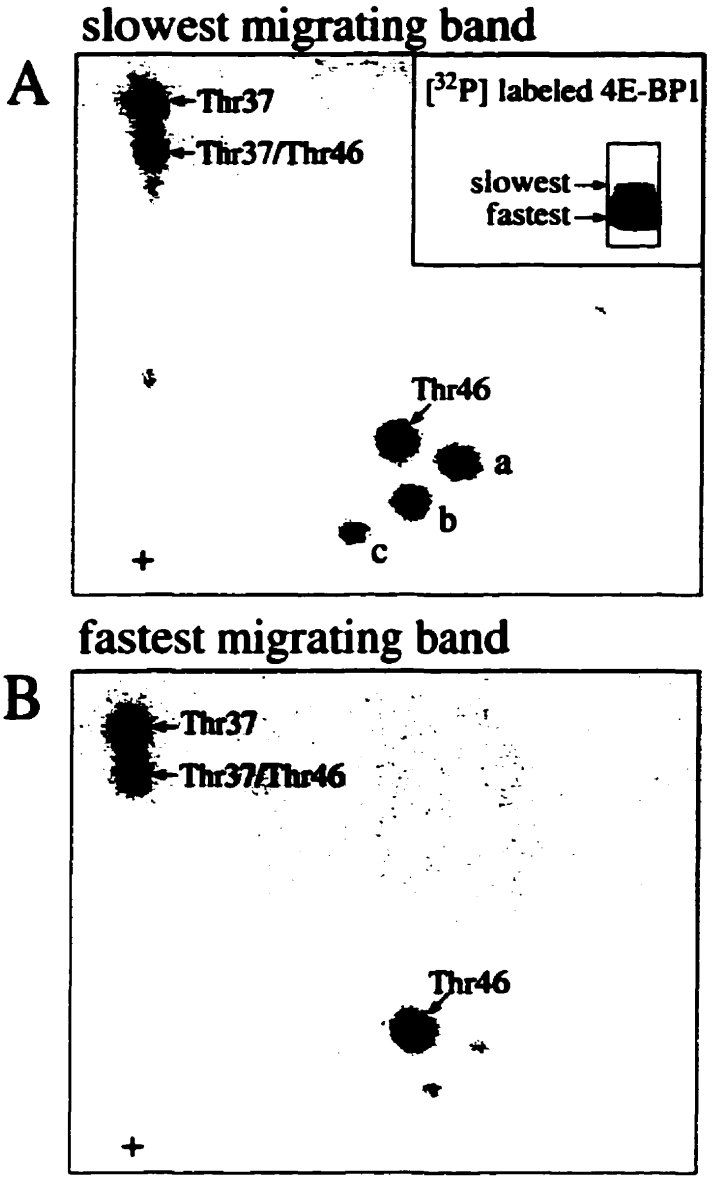


Figure 4.7. Phosphorylated Thr37 and Thr46 are present in the fastest migrating phosphorylated 4E-BP1 isoform. 293 cells were serum starved for 30 hr, incubated with [^{32}P]orthophosphate, stimulated with 10% dialyzed FCS, and extracts were subjected to SDS-PAGE and autoradiography (A, inset). The slowest (A) and fastest-migrating (B) 4E-BP1 isoforms were isolated and phosphopeptide maps were performed.

human cells, because phospho-Thr37 and phospho-Thr46 are also observed in the fastest migrating phosphorylated 4E-BP1 isoforms from Rat1a cells (data not shown). Thus, phosphorylation of Thr37 and Thr46 appears to be an early event in a sequential process of 4E-BP1 phosphorylation. Phosphorylated Thr37 and Thr46 are detected in isoforms that bind eIF4E as well as in isoforms that do not bind eIF4E (see, e.g., 128). These data also show that phosphorylation of Thr37 and Thr46 does not cause a shift in electrophoretic mobility upon 4E-BP1 hyperphosphorylation.

To confirm that phospho-Thr37 and -Thr46 are present in all phosphorylated isoforms and that their phosphorylation status is only marginally affected by serum addition, phosphospecific antibodies to Thr37 and Thr46 were developed. The sequences surrounding Thr37 and Thr46 are almost identical (Fig. 4.2D), thus the phosphospecific antibody that was generated against a peptide containing phosphorylated Thr37 (anti-phospho-Thr37) does not discriminate between phosphorylated Thr37 and phosphorylated Thr46 (data not shown). To confirm the specificity of the phosphospecific antibody, hemagglutinin-tagged wild-type 4E-BP1 and a 4E-BP1 mutant in which Thr37 and Thr46 were mutated to alanines were transfected into 293T cells. The phosphospecific antibody detected the wild-type 4E-BP1 protein, but failed to detect the Thr37Ala/Thr46Ala mutant, although both proteins were expressed to a similar level (Fig. 4.8A, cf. lanes 1 and 2 with lanes 3 and 4). Also, the phosphospecific antibody recognizes the phosphorylated peptide containing Thr37, but not the unphosphorylated peptide (data not shown), indicating that the phosphate group is an essential part of the antibody recognition motif.

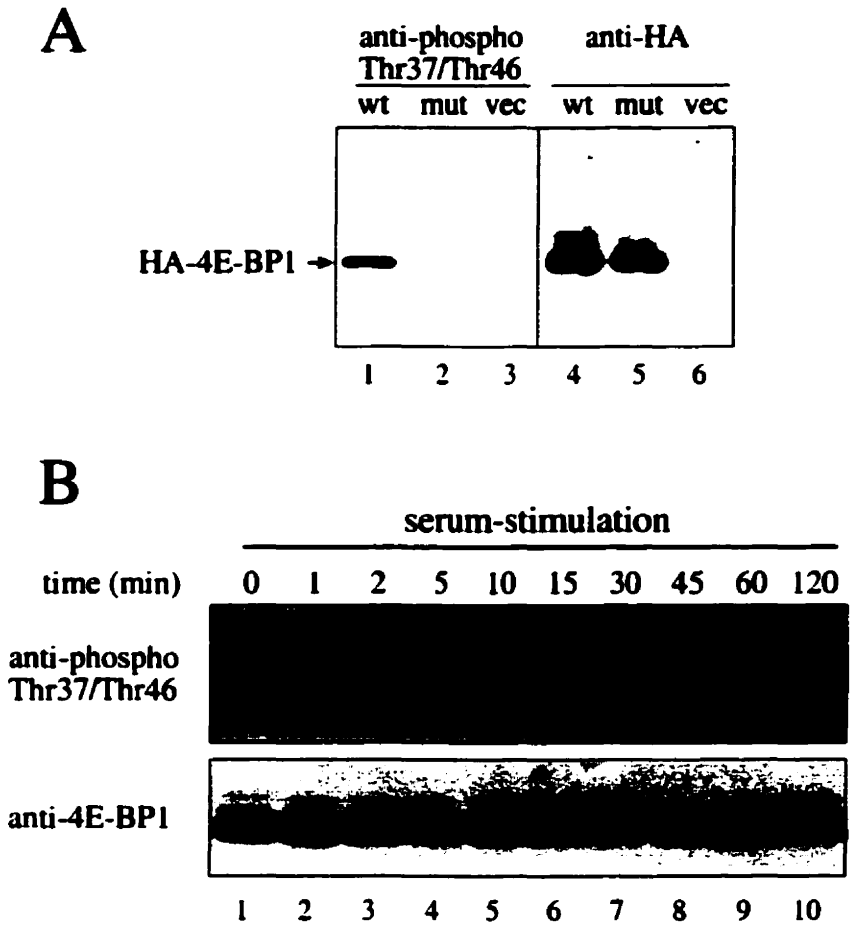


Figure 4.8. Phosphospecific antibodies confirm the relative lack of serum sensitivity of Thr37 and Thr46. (A) 293 cells were transfected with wild-type HA-4E-BP1 (wt), HA-4E-BP1 Thr37Ala/Thr46Ala (mut), or vector alone (vec). Cell extracts were prepared and analyzed by Western blotting with either the phosphospecific antibody directed against Thr37 and Thr46 or with an anti-HA antibody. (B) A time course of serum stimulation (after 30 hr of starvation) of 293 cells was performed for the indicated times. Extracts were prepared for Western blotting (see Materials and Methods) and duplicate blots were performed. Phosphospecific antibody to Thr37 and Thr46 (see Materials and Methods; top) and an anti-4E-BP1 antibody raised against the entire protein (bottom) were used to probe the membranes.

Following serum stimulation of 293 cells, duplicate blots were generated, and probed either with a polyclonal anti-4E-BP1 antiserum or with the phosphospecific antibody. The electrophoretic mobility of a significant portion of 4E-BP1 is retarded following serum stimulation (Fig. 4.8B, bottom panel). The phosphospecific antibody to Thr37/Thr46 recognizes all 4E-BP1 isoforms separated by SDS-PAGE (Fig. 4.8B, top). Using a secondary antibody coupled to ^{125}I, the amount of Thr37/Thr46 in all isoforms was quantified. The intensity of the signal obtained for phosphorylated Thr37 and Thr46 at the time of peak phosphorylation (time = 30 min; normalized for total expression) is ~1.7-fold greater than the signal obtained from lysates of serum-starved cells. In other experiments, this increase was between 1.3- and 1.8-fold (data not shown). Thus, as observed for the *in vivo* ^{32}P-labeling, phosphorylation of Thr37 and Thr46 is only marginally responsive to serum stimulation. These data are also consistent with reports demonstrating that FRAP activity is only marginally enhanced in response to insulin stimulation (354).

4.3.7 Thr37 and Thr46 phosphorylation is sensitive to rapamycin and LY294002 under starvation conditions

The response of Thr37 and Thr46 phosphorylation to inhibitors of PI3K or FRAP/mTOR was also examined. Rapamycin, LY294002, or wortmannin treatment of 293 cells prior to serum stimulation prevents the serum-induced hyperphosphorylation of 4E-BP1 (128). On two-dimensional phosphopeptide maps, treatment with rapamycin and wortmannin causes the disappearance of a subset of phosphopeptides, whereas others are relatively rapamycin and wortmannin resistant (124, 223). Interestingly, the most rapamycin-, LY294002-, and wortmannin-resistant phosphopeptides are those containing phosphorylated Thr37 and Thr46 (see chapter 3). This result is puzzling, as FRAP/mTOR itself is rapamycin sensitive and is inhibited, at least *in vitro*, by wortmannin and LY294002 (230). To address this apparent discrepancy, starved 293

cells were incubated with rapamycin, and half of the cells were serum stimulated in the presence of rapamycin, while the other half was not serum-stimulated. Thr37 and Thr46 phosphorylation decreased drastically and rapidly following rapamycin treatment in the absence of subsequent serum stimulation (Fig. 4.9, top, cf. lane 1 with lanes 2-5). However, when the cells were stimulated with serum subsequent to rapamycin treatment, this effect was much less pronounced (top panel, lanes 6-10). The expression level of 4E-BP1 protein was not affected by rapamycin treatment (Fig. 4.9, bottom). Pretreatment with the PI3K inhibitor LY294002 produced the same effect as rapamycin (data not shown). The extent of dephosphorylation was calculated to be ~sixfold in the absence of subsequent stimulation and 1.5-2-fold in the presence of serum. Thus, consistent with Thr37 and Thr46 being phosphorylated by FRAP/mTOR, their phosphorylation is sensitive to rapamycin and LY294002. This sensitivity is reduced, however, by ~threefold in the presence of serum. This difference will be addressed in section 4.4.

4.3.8 Thr37 and Thr46 phosphorylation is a priming step required for subsequent phosphorylation of the serum-sensitive sites

In view of the results described above, the biological significance of Thr37 and Thr46 phosphorylation remained elusive. To address this issue, constructs encoding hemagglutinin (HA)-tagged wild-type or point mutants of 4E-BP1 (Thr37Ala, Thr46Ala, Thr37Ala/Thr46Ala, and Thr37Glu/Thr46Glu) were transfected into 293T cells, and the pattern of phosphorylation was examined (Peptide maps prepared from 293T cells are identical to those prepared from 293 cells; data not shown). One set of transfected cells was used for ^{32}P-labeling and another served for quantitation of protein by Western blotting. HA-4E-BP1 from ^{32}P-labeled samples was immunoprecipitated using a monoclonal anti-HA antibody, and proteins were separated by SDS-PAGE. Incorporation of ^{32}P was monitored (a representative autoradiograph is shown in Fig.

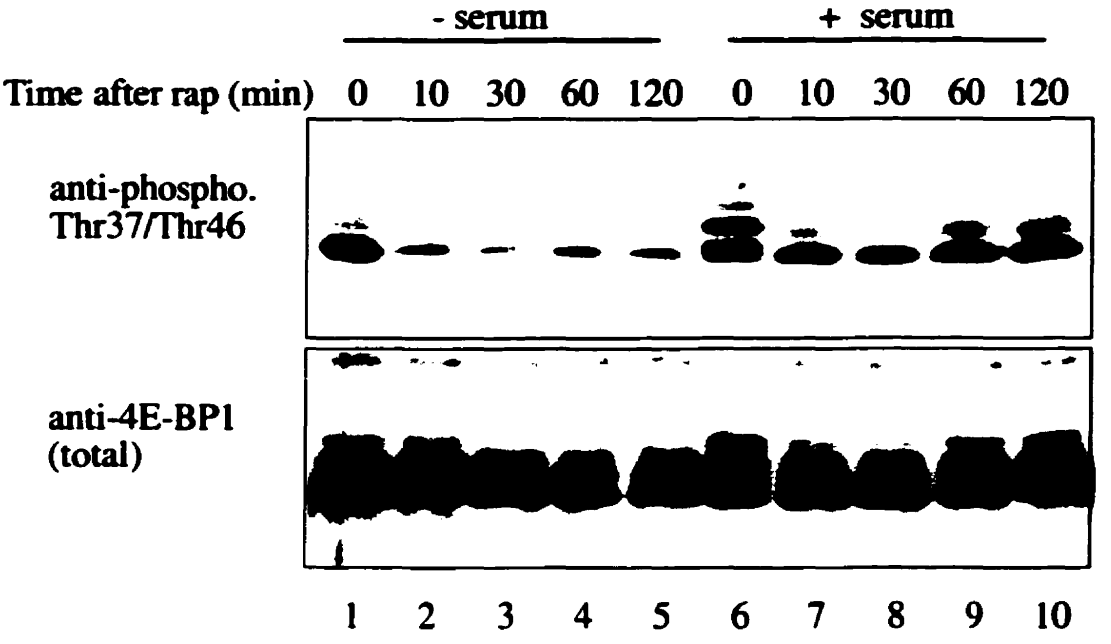


Figure 4.9. Sensitivity of Thr37 and Thr46 phosphorylation to rapamycin. Serum-starved 293 cells were incubated with rapamycin (20 ng/ml). One set of plates was lysed immediately (lanes 1-5); the other was serum stimulated for 30 min prior to lysis (lanes 6-10). Extracts were heat treated and analyzed by Western blotting, as described in Materials and Methods. The membranes were incubated with either an anti-phosphospecific antibody (top) or with an anti-4E-BP1 antibody (bottom).

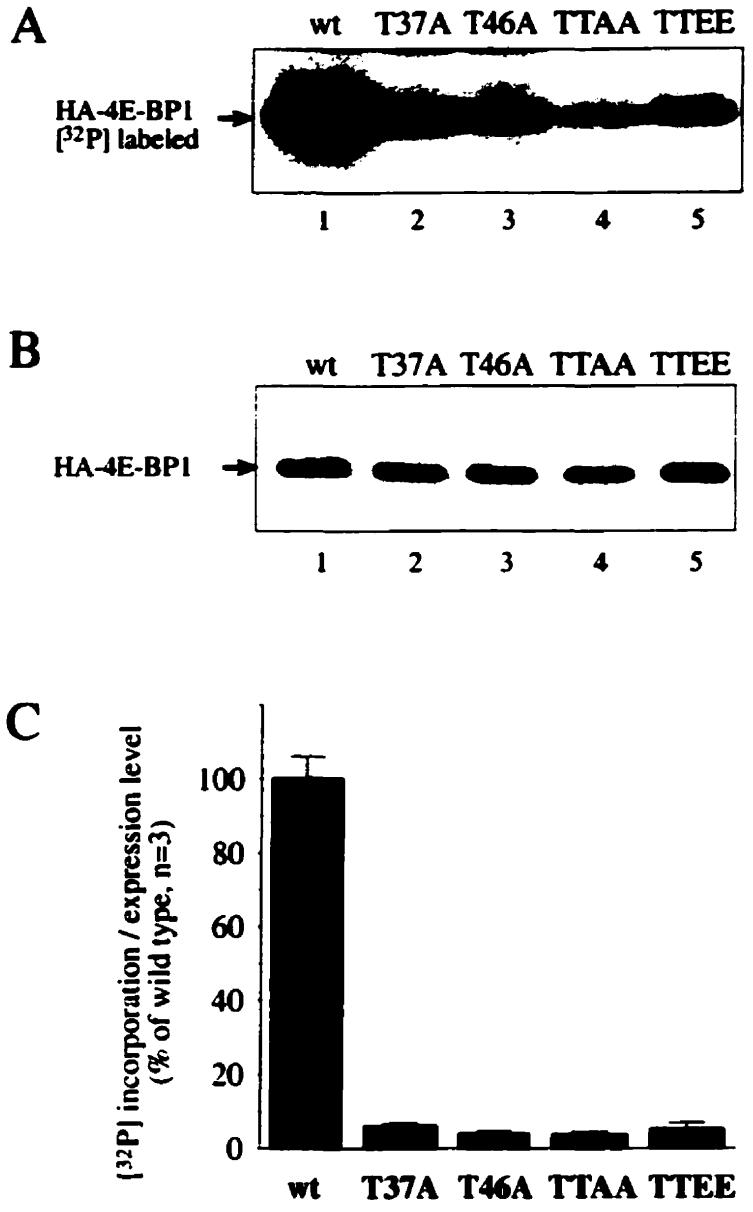


Figure 4.10. Mutation of Thr37 or Thr46 drastically reduces 4E-BP1 phosphorylation in vivo. 4E-BP1 wild type (wt) and mutants Thr37Ala (T37A), Thr46Ala (T46A), Thr37Ala/Thr46Ala (TTAA), and Thr37Glu/Thr46Glu (TTEE) in a pcDNA3-3HA vector were transfected into 293T cells (six plates/construct). Three transfected plates were subjected to ^{32}P-labeling, and HA-tagged 4E-BP1 was immunoprecipitated using an anti-HA antibody (HA.11). Precipitated proteins were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and autoradiographed. The intensity of the HA-4E-BP1 bands was quantified by PhosphorImager. The three other plates were used to analyze the expression levels of the HA-tagged proteins by Western blotting, using anti-HA as the primary antibody and ^{125}I-coupled anti-mouse IgG as the secondary antibody. Expression level was quantified by PhosphorImager. The average of the ^{32}P incorporation into the mutant proteins was normalized to the expression level of each mutant. (A) A representative autoradiogram. (B) A representative Western blot showing the levels of the mutants; this Western blot was developed by chemiluminescence, which produces a brighter signal. (C) Incorporation of ^{32}P into each mutant, normalized to the expression level. Incorporation into the wild-type protein is set to 100%.

4.10A) and normalized to expression levels (a representative Western blot is shown in Fig. 4.10B). All of the mutants are capable of binding to eIF4E, as eIF4E was coimmunoprecipitated to approximately the same extent in all lanes (Fig. 4.10A). Coprecipitation of eIF4E with the HA-tagged proteins was confirmed by Western blotting (not shown), consistent with the results obtained with bacterially expressed 4E-BP1 mutants. Unexpectedly, mutation of either Thr37 or Thr46 to alanine caused a 10- to 20-fold decrease in ^{32}P incorporation into HA-4E-BP1 (Fig. 4.10C, cf. lanes 2 and 3 with lane 1). Mutation of both Thr37 and Thr46 to alanine almost abolished 4E-BP1 phosphorylation (cf. lane 4 and lane 1), whereas replacement of the two threonines by glutamic acids had a slightly less severe effect (lane 5). The dramatic effect observed for the mutants is much greater than would be expected by abolishing Thr37 or Thr46 phosphorylation alone, as these sites account for ~30% each of the total phosphorylation on 4E-BP1 (data not shown). The remaining phosphoamino acids represent ~40% of the total 4E-BP1 phosphorylation. Thus, mutation of the Thr37 and/or Thr46 appears to prevent phosphorylation of the other sites.

To test the hypothesis that Thr37 and Thr46 mutation prevents the phosphorylation of other sites, phosphopeptide maps were generated from the mutant and wild-type proteins. Because the mutant proteins were phosphorylated to a much lower extent than the wild-type protein (Fig. 4.10), an equivalent quantity of radioactivity was loaded on each plate. Thus, ~10-20 times less material was employed for the wild-type map than for those of the various mutants (Fig. 4.11, upper left). The characteristic pattern of 4E-BP1 phosphorylation was observed for the wild-type HA-tagged protein (Fig. 4.11A). The map generated from the Thr37Ala mutant shows a similar pattern, with the exception of the disappearance of peptides containing Thr37 (Fig. 4.11B; the other peptides migrating in this area result from partial chymotryptic cleavage of the peptide containing Thr37 and Thr46, and thus contain phospho-Thr46). Mutation of Thr46 has a more pronounced

effect on phosphorylation of the other sites. With the exception of two phosphopeptides whose intensity does not change, phosphorylation on all other sites is dramatically reduced as compared to the wild-type protein. It is noteworthy that Thr37 phosphorylation is also reduced severalfold (Fig. 4.11C) in the Thr46Ala mutant. Thus, Thr46 appears to play a key role in modulating the phosphorylation of the other sites, including Thr37. The map generated from the double mutant Thr37Ala/Thr46Ala showed, as expected, the disappearance of the phosphopeptides containing Thr37 and Thr46, but also an extensive decrease in phosphorylation of the remaining sites (Fig. 4.11D). Strikingly, however, replacement of the Thr37 and Thr46 residues by glutamic acids partially restored phosphorylation on the remaining sites, although the signals from Thr37 and Thr46 phosphorylation were absent (Fig. 4.11E). We therefore conclude that mutating threonines 37 and 46 to glutamic acid partially mimics phosphorylated Thr37 and Thr46. It should be emphasized, however, that the phosphorylation of the serum-sensitive sites is not restored to wild-type levels in the Thr37Glu/Thr46Glu mutant (in Fig. 4.11, an equal number of counts was loaded; thus, ~10 times less material from the wild-type sample was used). These results demonstrate that phosphorylation on Thr37 and Thr46 is a prerequisite for the subsequent phosphorylation of the serum-stimulated sites on 4E-BP1, as illustrated in the model in Figure 4.11F.

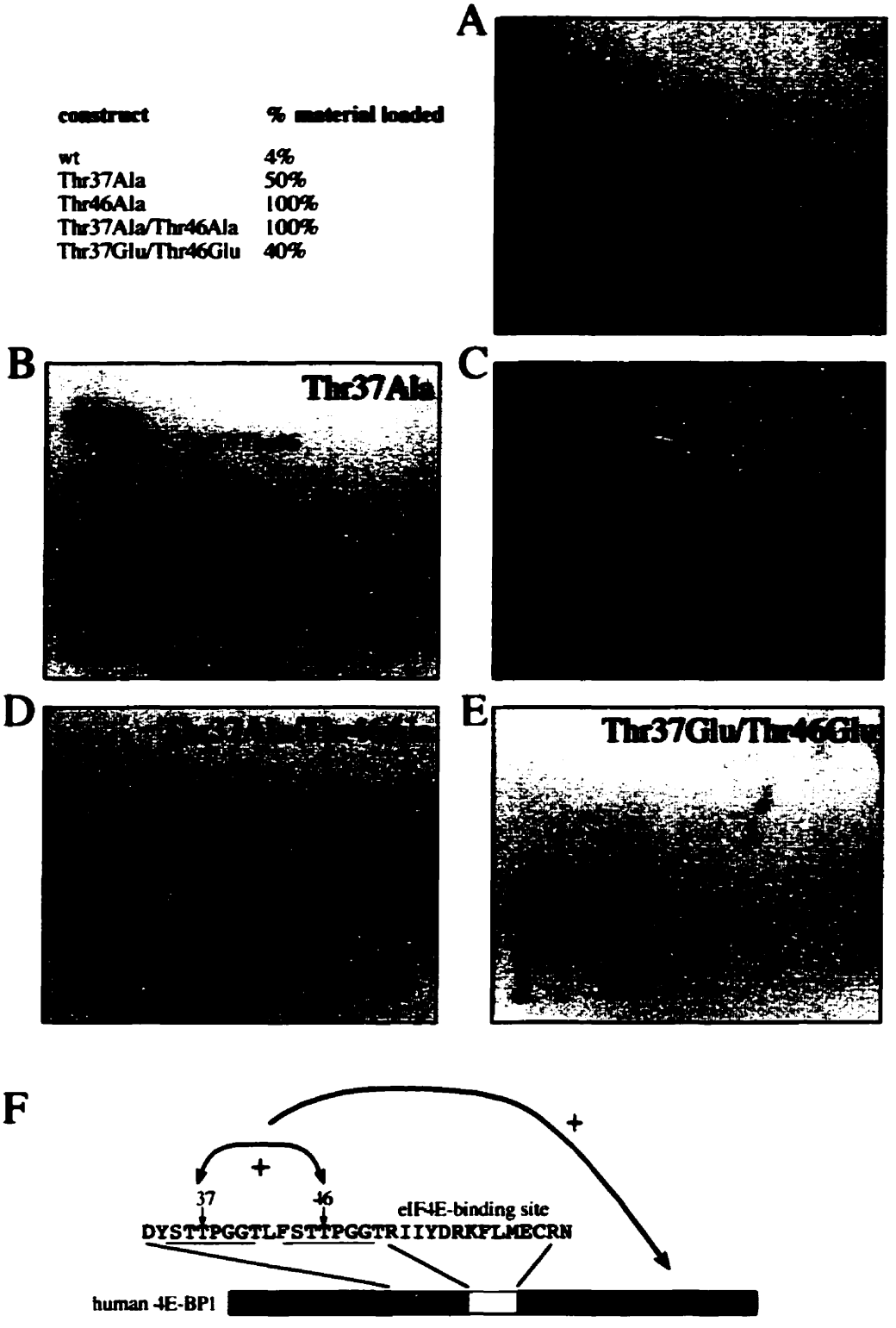


Figure 4.11. Effects of mutations of Thr-37 and/or Thr-46 on other phosphorylation sites. (A-E) Phosphopeptide maps were performed on HA-tagged 4E-BP1 proteins from Fig. 4.10. (F) Thr-37 and Thr-46 affect 4E-BP1 phosphorylation. The arrows indicate (1) a mutually positive effect of Thr-37 and Thr-46 phosphorylation, and (2) a positive effect of Thr-37 and Thr-46 phosphorylation on the phosphorylation of the serum-sensitive sites. The fraction of samples from Fig. 4.10 loaded onto each map is shown (upper left). An equivalent quantity in cpm was loaded for each mutant.

4.4 Discussion

In this report, we describe that Thr37 and Thr46 are phosphorylated by FRAP/mTOR immunoprecipitates in a 4E-BP1/eIF4E complex, and that this phosphorylation does not result in the disruption of the complex. Thr37 and Thr46 are phosphorylated to a high stoichiometry under serum-starvation conditions, and their phosphorylation state does not significantly increase following serum stimulation. ^{32}P incorporation into 4E-BP1 *in vivo* is drastically reduced when either Thr37 or Thr46 is mutated to alanine. Phosphopeptide mapping revealed that mutation of Thr37 or Thr46, which are located amino-terminal to the eIF4E-binding site, affects phosphorylation of all 4E-BP1 sites, including the serum-inducible residues. This effect is relieved to some extent by the mutation of Thr37 and Thr46 to glutamic acid, which partially mimics the phosphorylated threonines (Fig. 4.11E). Taken together, these data indicate that phosphorylation of Thr37 and Thr46 *in vivo* is a prerequisite for phosphorylation of serum sensitive 4E-BP1 sites. Several phosphorylation sites were previously reported to be serum sensitive; these include Thr37, Thr46, Ser65, Thr70, Ser83 (all lying in the consensus S/T-P (205), and Ser112 (a site preceded by acidic residues, 214). In 293 cells, as in Rat1a cells, however, we have observed that the most serum-sensitive sites are Ser65 and Thr70 (chapter 5). It remains possible that in different cell types the sensitivity of the various phosphorylation sites to extracellular stimuli differs. Based on our results, we propose a two-step model for 4E-BP1 phosphorylation (Fig. 4.12). FRAP/mTOR (or a tightly associated kinase) first phosphorylates Thr37 and Thr46 on 4E-BP1 complexed with eIF4E. This phosphorylation event appears to be relatively independent of extracellular stimuli. The second step, which is activated by extracellular stimuli, is the phosphorylation of the sites located carboxy-terminal to the eIF4E-binding site, including Ser65 and Thr70 (chapter 5). The second phosphorylation step results in release from eIF4E and stimulation of translation.

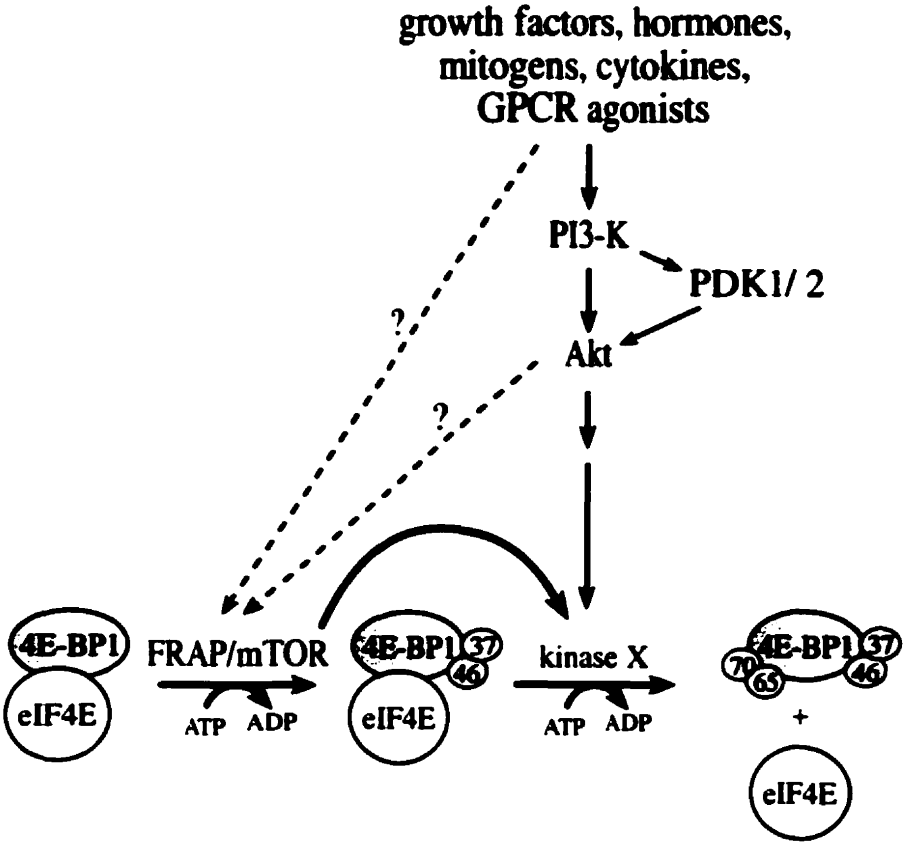


Figure 4.12. A two-step model for the phosphorylation of 4E-BP1. FRAP/mTOR phosphorylates 4E-BP1 (in a 4E-BP1/eIF4E complex) on Thr37 and Thr46. Phosphorylation of Thr37 and Thr46 is is required for phosphorylation by an unidentified serum-sensitive kinase (acting downstream of Akt/PKB) to phosphorylate 4E-BP1 on the serum-sensitive sites. The latter phosphorylation triggers the release of 4E-BP1 from eIF4E. A direct link between Akt/PKB and FRAP/mTOR remains to be proven. GPCR = G-protein coupled receptor.

Phosphorylation of proteins on multiple residues often occurs in a sequential manner. In some instances, the recognition site of a kinase contains a phosphorylated residue. This is the case for glycogen synthase kinase-3 (GSK-3), which phosphorylates serines or threonines lying in the consensus SXXX^{P}S, where ^{P}S is a phosphoserine (355). Other kinases, including casein kinase I, also exhibit a preference for phosphorylated amino acids in the vicinity of their target site (356). Priming phosphorylation is, however, not restricted to the kinase recognition site. For example, phosphorylation may induce a conformational change in the substrate to render a site more easily accessible to a kinase or create a docking site for another protein, similar to the SH2/phosphotyrosine interaction. In this regard, 14-3-3 proteins, which recognize phosphoserines, serve as chaperones, inhibitors, or adaptors to modulate the function of their binding partners (357, 358).

Many kinases are able to phosphorylate 4E-BP1 *in vitro*, including several different MAP kinases (ERK2, JNK, p38), protein kinase C, and casein kinase II (359; A.-C.G., unpublished). Other PIK family members, such as ATM and ATR, can also phosphorylate 4E-BP1 (231, 360, 361). 4E-BP1 is mostly unstructured in solution (199). Thus, it is not surprising that it is a good kinase substrate. Upon binding to eIF4E, however, a small region of localized structure is induced in the 4E-BP1/eIF4E contact region (54, 199, 200). This induced fit appears to mask some phosphorylation sites, as ERK2 is unable to phosphorylate 4E-BP1 in a 4E-BP1/eIF4E complex. As previously mentioned, a 4E-BP1/eIF4E complex is most likely the true physiological substrate. Therefore, using a complex of 4E-BP1/eIF4E in *in vitro* kinase assays is more likely to identify a relevant *in vivo* kinase. Contrary to ERK2, FRAP/mTOR phosphorylates 4E-BP1 to the same extent, and on the same sites, regardless of whether 4E-BP1 is free in solution or bound to eIF4E. The cocrystal structure of eIF4E complexed with a 4E-BP1 peptide (amino acids 51-67; amino acids 65-67 were not visible in the density map) was

determined (54). 4E-BP1 binds to a phylogenetically conserved region on the convex surface of eIF4E. Similar results were obtained by nuclear magnetic resonance (NMR), using both the full-length protein as well as a fragment of 4E-BP1. Although chemical shifts induced by the peptide and the full-length 4E-BP1 were largely overlapping, the full-length 4E-BP1 induced shifts in additional eIF4E residues (32). This effect appears to be due to the amino-terminal extension of full-length 4E-BP1, which may include the Thr46 phosphorylation site. Acidic residues flank the region on eIF4E in contact with the 4E-BP1 peptide (Glu-140 and Asp-143 are predicted to be close to 4E-BP1 Thr46; Glu-70 is predicted to be in the vicinity of Ser65; 54). Thus, it is conceivable that the mechanism leading to 4E-BP1 release from eIF4E involves electrostatic repulsion between the negatively charged phosphates on 4E-BP1 and the acidic residues on eIF4E (54). In this regard, phosphorylation of 4E-BP1 at Thr37 and Thr46 could theoretically decrease the affinity of 4E-BP1 for eIF4E, although this was not detected in the m^7GDP-agarose-binding experiments (which are only qualitative; a better stoichiometry of 4E-BP1 phosphorylation by FRAP/mTOR is required to perform quantitative measurements of affinity). Alternatively, it is possible that phosphorylation of Thr37 and Thr46 alters the conformation of the 4E-BP1/eIF4E complex to allow access to the other phosphorylation sites on 4E-BP1. A third possibility is that phosphorylation of 4E-BP1 by FRAP/mTOR creates a docking site for a different kinase or for an adaptor molecule that recruits a kinase.

The two sites phosphorylated by FRAP/mTOR *in vitro* are the least rapamycin sensitive *in vivo*. Also, under conditions of serum starvation, Thr37 and Thr46 are phosphorylated to a high stoichiometry, relative to the other sites. Furthermore, in cells treated with rapamycin and subsequently stimulated with serum, phosphorylation at these sites is only slightly affected (124, 215, 223). Only when starved cells were treated with rapamycin was a rapid reduction in the phosphorylation of Thr37 and Thr46 observed. Several

hypotheses, which are not mutually exclusive, could explain this observation: (1) FRAP is inhibited by rapamycin, even in the presence of serum, but another rapamycin-insensitive/serum-sensitive kinase (for example a MAP kinase) is responsible for phosphorylating 4E-BP1 under these conditions; (2) Some residual FRAP kinase activity is retained in the presence of rapamycin but is inhibited in the absence of serum; (3) A phosphatase active in the absence of serum, but inactive in the presence of serum, is responsible for the effect. In the latter case, if the phosphatase is rapidly inactivated following serum stimulation, then even a low FRAP/mTOR kinase activity could be sufficient to induce 4E-BP1 phosphorylation. However, when cells are serum deprived, the phosphatase activity may predominate. In the absence of rapamycin, FRAP/mTOR is active and this is sufficient to phosphorylate Thr37 and Thr46. When rapamycin is added in the absence of serum, however, the balance may change: The phosphatase could remain active, and the activity of FRAP/mTOR is inhibited, so a net decrease in the phosphorylation of Thr37 and Thr46 is observed.

Thr37 and Thr46 phosphorylation is only moderately increased (1.3- to 1.7-fold) following serum stimulation. This modest serum sensitivity is comparable to that reported for FRAP/mTOR activation by insulin (354, 362). There is no evidence at present that Thr37 and Thr46 phosphorylation occurs downstream of Akt/PKB, as opposed to the phosphorylation of the serum-sensitive sites (124). The induction of phosphorylation by serum on the sites carboxy-terminal to the eIF4E-binding motif is far more pronounced (see Fig. 4.6), although an exact quantification was not possible in this study. The most prominent serum-induced sites have been mapped to Ser65 and Thr70 (chapter 5). Because the serum-responsive sites on 4E-BP1 are also the most rapamycin sensitive, it is highly likely that FRAP/mTOR is involved in their phosphorylation, although probably not directly. It remains possible that the rapamycin-induced dephosphorylation of Thr37 and Thr46 prevents the subsequent phosphorylation of the

serum-sensitive sites following serum stimulation. This is, however, unlikely because phosphorylation on Thr37 and Thr46 is restored with rapid kinetics to almost maximal levels following addition of serum, whereas the serum-sensitive sites remain dephosphorylated. It is also important to mention that the relatively high level of phosphorylated Thr37 and Thr46 in the rapamycin-treated, serum-stimulated cells (Fig. 4.9) is not sufficient to induce phosphorylation on the serum-sensitive sites. Thus, FRAP/mTOR likely impacts on the phosphorylation of the serum-sensitive sites either by stimulating their phosphorylation following serum stimulation or by inducing their dephosphorylation in the presence of rapamycin. In this regard, an attractive hypothesis as to the function of FRAP/mTOR is that it serves as a gatekeeper, somewhat similar to the mechanisms controlling the cell cycle at the restriction point. FRAP/mTOR could alter the phosphorylation of the serum-sensitive sites of 4E-BP1 through the modulation of the activity of a kinase or a phosphatase. In *Saccharomyces cerevisiae*, a nutrient-induced, rapamycin-sensitive association between the phosphatases PP2A or Sit4 (the yeast homolog of protein phosphatase 6) and a protein termed TAP42 was reported. This association was postulated to modulate the activity or substrate specificity of the phosphatases, or to target them to specific substrates. Because of the rapamycin-sensitive nature of this interaction, it was postulated that the phosphatases and TAP42 were downstream components in the TOR signaling pathway. In mammals, a homolog of TAP42, α4, associates with mammalian PP2A, as well as with protein phosphatases 4 and 6 (363). However, regulation of the association and activity of these proteins by FRAP/mTOR remains controversial. A recent report indicates that PP2A can partially dephosphorylate a FRAP/mTOR-phosphorylated 4E-BP1 substrate *in vitro* and that the dephosphorylation can be prevented by preincubation of TAP42 with PP2A (364). Thus, it is possible that phosphatases play an active role in FRAP/mTOR signaling to 4E-BP1.

Our data are consistent with FRAP/mTOR being a physiological 4E-BP1 kinase, as was suggested previously (339, 352, 353). However, there are discrepancies between our results and the data reported previously. Brunn et al. (352) found that FRAP/mTOR phosphorylates five sites (Thr37, Thr46, Ser65, Thr70, and Ser83) on 4E-BP1. Using the same antibody utilized by Brunn et al. (as well as baculovirus-expressed FRAP/mTOR), we obtained significant and reproducible phosphorylation only on Thr37 and Thr46. We also detected other phosphopeptides in some of our kinase assays, but phosphorylation of these phosphopeptides was not reproducible. Further, these phosphopeptides were labeled only to a very low stoichiometry as compared to Thr37 and Thr46, and, as mentioned previously, washing the FRAP/mTOR immunoprecipitate with a stringent buffer decreased their phosphorylation even further. We believe that the differences between our results and those of Brunn et al. are probably due to the washing conditions of the immunoprecipitate and times of incubation (see Materials and Methods). It is possible that a kinase that is tightly associated with FRAP/mTOR is responsible for the phosphorylation reported for the other residues on 4E-BP1. Our data are largely in agreement with those of Burnett et al. (353), which defined Thr37 and Thr46 as the two sites phosphorylated by FRAP/mTOR on 4E-BP1. However, Burnett et al. (353) reported that, *in vitro*, phosphorylation of 4E-BP1 at Thr37 and Thr46 by FRAP/mTOR decreases binding to eIF4E. We did not observe such a decrease, as we clearly detected Thr37 and Thr46 in the fractions bound to eIF4E. However, no quantitative measurement of the eIF4E/4E-BP1 interaction was performed, so it remains possible that the strength of this interaction is decreased by phosphorylation on Thr37 and Thr46. Burnett et al. (353) also reported that mutants of Thr37 and Thr46 expressed in 293 cells bind constitutively to eIF4E. We obtained the same result (A.-C. Gingras, unpubl.), but explain it by the fact that Thr37 and Thr46 are necessary for the phosphorylation of the serum-inducible phosphorylation sites, which lead to 4E-BP1 dissociation from eIF4E.

It is of the utmost importance to determine which kinases phosphorylate the serum-sensitive sites of 4E-BP1. It is highly likely that Ser65 and Thr70, the most serum-sensitive sites, are phosphorylated by a proline-directed kinase. However, because of the lack of sensitivity to the MEK inhibitor PD98059, it is unlikely that ERKs are involved in mediating the phosphorylation on the serum-sensitive sites. Other MAP kinase family members (such as JNK and p38), which can phosphorylate 4E-BP1 *in vitro*, are not activated by the same stimuli that elicit 4E-BP1 phosphorylation. Other proline-directed kinases, such as the cyclin-dependent kinases, are not likely to be involved in phosphorylating 4E-BP1 *in vivo*, because they are mostly nuclear, whereas 4E-BP1 is present mainly in the cytoplasm (A.-C.G., unpublished; 365). One approach to identify these kinases, based on our results, is to use a 4E-BP1/eIF4E complex prephosphorylated at Thr37 and Thr46 as a substrate in a biochemical purification scheme.

4.5 Materials and methods

4.5.1 Plasmids and antibodies. The human 4E-BP1 coding sequence cloned into the cytomegalovirus-based vector pACTAG-2 was used as a template for PCR mutagenesis, which was used to mutate Thr37 and Thr46 to either Ala or Glu. To generate double mutants Thr37Ala/Thr46Ala and Thr37Glu/Thr46Glu, either Thr46Ala or Thr46Glu cloned into pACTAG-2 was used as template to mutate Thr37. Similarly, the deletion of amino acids 34-50 and the deletion in the eIF4E-binding site (amino acids 54-63) were generated by PCR mutagenesis. The products were inserted in-frame into pACTAG-2 and sequenced in their entirety. Alternatively, the mutated sequences were inserted in-frame in the vector pGEX-6p1 (Pharmacia) for protein expression in bacteria. GST-2T-4E-BP1 wild-type was described previously (195). It contains almost the entire coding region of human 4E-BP1 (lacking the first 6 amino acids) fused in-frame in the vector pGEX-2T (Pharmacia; contains a thrombin cleavage site). Murine 4E-BP1 wild type was cloned in-frame into pQE-30 vector (Qiagen), which contains an amino-terminal 6-histidine tag (His). PCR mutagenesis was utilized to mutate Thr-36 (corresponding to human Thr37) or Thr-45 (corresponding to human Thr46) to alanines. The constructs were sequenced in their entirety. The rabbit polyclonal antisera 11208 and 11209 against human 4E-BP1 have been described previously (124, 211). Antiserum 11208 was used for Western blotting analysis and 11209 for immunoprecipitation. The antibody to FRAP/mTOR was described previously (339). The anti-HA mouse monoclonal antibody HA.11 was purchased from BAbCO. The phosphospecific antibodies directed against Thr37 and Thr46 sites in 4E-BP1 were produced by immunizing New Zealand white rabbits with the following synthetic peptides coupled to keyhole limpet hemocyanin: Thr37(P), PGDYSTT*PGGTLFC, and Thr46(P), GTLFSTT*PGGTRIIC. Enzyme-linked immunosorbent assay (ELISA), using the phosphopeptide and corresponding nonphosphopeptide, was used to identify the best-responding rabbits. IgG was purified using protein-A-Sepharose. Antibodies reactive with the nonphosphopeptide were

removed by adsorption to a nonphosphopeptide affinity column. Antibodies that flowed through this column were next passed over a column of immobilized phosphopeptide; after washing, antibodies were eluted at low pH and dialyzed. The resulting antibodies were characterized by ELISA against the phosphopeptide and nonphosphopeptide to determine the extent of phosphospecificity and by Western blots against whole-cell extracts and purified 4E-BP1 proteins. The antibody directed against phospho-Thr37, however, cross-reacts with phospho-Thr46, because of the high sequence identity surrounding the two sites. Similarly, the anti-phospho-Thr46 antibody cross-reacts with phosphorylated Thr37.

4.5.2 Protein expression and purification. An amino-terminal deletant (NΔ27) of eIF4E, which behaves essentially as the wild-type protein, was bacterially expressed and purified by m^7GDP chromatography (31). Glutathione S-transferase (GST)-fused 4E-BP1 proteins were expressed and purified from DH10β *Escherichia coli* essentially according to the manufacturer's (Pharmacia) instructions. Specifically, bacteria were grown to an OD of 0.8 and induced for 2 hr at 37°C with 0.5 mM IPTG. Bacteria were pelleted, resuspended in PBS containing 1 mM PMSF, and lysed by sonication. After centrifugation (Sorvall SS34 for 1 hr at 15,000 rpm), the cleared lysate was incubated for 15 min at 20°C with glutathione-Sepharose beads (1 ml beads per liter of starting culture). Beads were pelleted and rinsed three times with PBS containing 1 mM PMSF and 1% Triton X-100. The elution was performed in 20 mM Tris-HCl (pH 8.5) containing 10 mM reduced glutathione. Proteins were dialyzed against 20 mM HEPES-KOH (pH 7.4), 74 mM KCl, and 1 mM DTT, and quantified by SDS-PAGE and Coomassie staining, as compared to a BSA standard. Alternatively, the construct pGEX-2T-4E-BP1 wild type was expressed and bound to the glutathione beads as above and digested with thrombin on the beads, according to the manufacturer's instructions. The cleaved 4E-BP1 protein was then purified by reverse-phase HPLC on a C4 column, as

described (54). Histidine-tagged 4E-BP1 proteins were expressed and purified from M15pREP4 cells according to the manufacturer's (Qiagen) instructions, eluted with imidazole, and dialyzed against 20 mM HEPES-KOH (pH 7.4), 74 mM KCl, and 1 mM DTT. Epitope-tagged FRAP was purified from two-liter baculovirus infections using Sf9 host cells (Invitrogen) grown in 10-liter fermentors for infection. Infection conditions for large-scale cultures ranged from 24-48 hr. The baculovirus construct, lysis conditions, and chromatography methods were described previously (338).

4.5.3 Cell culture and transient transfections. Human embryonic kidney 293 cells (ATCC CRL 1573) and human embryonic kidney 293T cells (overexpressing the SV40 large T antigen; ATCC CRL 11268) were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% FCS. For transient transfection of 293T cells, 1 x 10^6 cells were plated onto a 100-mm dish 24 hr prior to transfection of 15 μg of pACTAG-2-4E-BP1 DNA, using a modified calcium phosphate technique (365). Cells were either harvested or labeled 48 hr post-transfection.

4.5.4 Extract preparation and Western blotting. Cells were rinsed twice with cold buffer A (20 mM Tris-HCl at pH 7.5, 100 mM KCl, 20 mM β-glycerolphosphate, 1 mM DTT, 0.25 mM Na3VO4, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 10 nM okadaic acid, 1 mM PMSF) and scraped into a minimal volume of the same buffer. Lysis was performed by three freeze-thaw cycles. Cell debris was pelleted by centrifugation, and the protein concentration in the supernatant was measured using the Bio-Rad assay. For analysis of endogenous 4E-BP1, 75 μg of total cell extract was incubated at 100°C for 7 min to enrich for 4E-BP1, which is heat stable (195, 209). Samples were then incubated on ice for 5 min, and precipitated material was removed by centrifugation (microcentrifuge, 13,000 rpm for 5 min). Laemmli sample buffer was added to the supernatant, which was then subjected to SDS-15% PAGE. For analysis of transfected

HA-4E-BP1, cells were lysed by three freeze-thaw cycles, and 50 μg of protein was analyzed by SDS-15% PAGE. For qualitative analysis, Western blotting and chemiluminescence detection were performed as described, using either the anti-4E-BP1 11208 antiserum (1:1500) or the phosphospecific anti-Thr37/Thr46 antibody (1:1000). For quantitative analysis of the endogenous proteins, membranes were first incubated with the anti-4E-BP1 11208 antiserum (1:1500) or the phosphospecific anti-Thr37/Thr46 antibody (1:1000), followed by incubation with ^{125}I-coupled protein A. For the quantitation of overexpressed HA-tagged 4E-BP1, the anti-HA (HA.11) monoclonal antibody (0.5 μg/ml) was utilized.

4.5.5 Metabolic labeling and immunoprecipitation. ^{32}P-Labeling was performed essentially as described (124). In small scale experiments, 100- or 150-mm dishes of 293 cells or 293T cells were ^{32}P-labeled and processed under the same conditions described in Gingras et al. (124), but using nitrocellulose membranes instead of PVDF membranes for the transfer. For large-scale experiments designed to identify the phosphorylation sites, ten 150-mm plates of confluent 293 cells (~2 x 10^8 cells) were starved for 30 hr and incubated for 3.5 hr in phosphate-free DMEM (GIBCO) containing 1 mCi/ml of [^{32}P]orthophosphate (DuPont NEN; 3000 mCi/mmole; total volume of 7.5 ml). Dialyzed FCS (15%; GIBCO) was then added for 40 min, and the cells were rinsed and lysed as (124). In parallel, 200 x 150-mm plates of confluent 293 cells (~4 x 10^9 cells) were starved for 33 hr and stimulated for 40 min with 15% FCS (GIBCO). Cells were rinsed and extracts were prepared in the same manner as for the ^{32}P-labeled extracts. The extract (at this point, the cold and hot extracts were processed separately) was precleared by incubation with protein A beads (50 μl of packed beads per 108 cells) with end-over-end rotation at 4°C for 2 hr. The supernatant was transferred to a fresh tube, together with 25 μl of 11209 antisera bound to 25 μl of packed protein A-Sepharose beads per 10^8 cells, and incubated end-over-end for 4 hr at 4°C. Beads were spun and washed three

times (with 100 times the bead volume) in lysis buffer, two times in RIPA buffer, and two times in LiCl solution (200 mM LiCl, 1 mM DTT). Immunoprecipitates from the ^{32}P-labeled and cold samples were eluted in Laemmli sample buffer and the elutions were combined. Immunoprecipitated material was subjected to SDS-15% PAGE and transferred to 0.2-μm pore size nitrocellulose (Schleicher & Schuell), which were dried and autoradiographed. Radioactive bands corresponding to 4E-BP1 were excised and Cerenkov counted.

4.5.6 Phosphopeptide maps and phosphoaminoacid analysis. Tryptic/chymotryptic digestion of 4E-BP1 immobilized on the nitrocellulose membranes was performed essentially as described (124, 351). For the large-scale experiment, the sample was divided into three tubes for digestion and mapping. The digest was performed using a 200:1 mixture of N-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin and chymotrypsin (1 μg per sample, Worthington) for 10 hr, followed by the addition of 0.5 μg of enzyme mix three times for 1 hr each. The sample was then lyophilized (Speed-vac, Savant), resuspended in 500 μl of water, lyophilized again, resuspended in 250 μl of water, lyophilized a third time, resuspended in 250 μl pH 1.9 buffer [2.5% (vol/vol) formic acid, 7.8% (vol/vol) glacial acetic acid], and lyophilized a fourth time. For two-dimensional phosphopeptide mapping, the first dimension (electrophoresis) was performed in pH 1.9 buffer using an HTLE 7000 apparatus (CBS Scientific); the second dimension was performed in phosphochromatography buffer [37.5% (vol/vol) n-butanol, 25% (vol/vol) pyridine, 7.5% (vol/vol) glacial acetic acid]. Plastic-backed cellulose-coated TLC plates (Kodak; 20 x 20 cm) were employed. The plates were dried and subjected to autoradiography. To determine which phosphoamino acid is phosphorylated in the spots of interest, the cellulose was scraped off. Peptides were eluted from the cellulose with pH 1.9 buffer, using a spin filter (Bio101). The material was lyophilized, resuspended in 100 μl 6N HCl and incubated at 110°C for 60 min in a screw-cap tube.

The sample was lyophilized and rinsed several times with water (debris was removed by centrifugation before the last lyophilization). Two-dimensional phosphoamino acid analysis mapping was performed essentially as described (351).

4.5.7 Mass spectrometry. Spots containing radioactivity were scraped from the cellulose plates and prepared for analysis by LC-MS/MS as described (366). The system used was adapted from previous reports (367). In brief, a capillary HPLC column (50 μm i.d., 12 cm long) packed in-house with C18 material was coupled via a homemade μESI interface to a triple quadrupole mass spectrometer (TSQ 7000; Finnigan Mat, San Jose, CA). A pressurisable bomb was used to load 5 μl from a solution containing the phosphopeptide onto the capillary column. The column was then reattached to the HPLC gradient delivery system (Michrom, Auburn, CA). A gradient for column elution was developed over 30 min, and a precolumn flow splitter reduced the flow from 50 μl/min to ~150 nl/min. The eluted peptides were ionized by electrospray ionization, detected, and the specific peptide ions were automatically selected and fragmented by a triple quadrupole mass spectrometer (367). To achieve this, the mass spectrometer switched between the MS mode (for peptide mass identification) and the MS/MS mode (for peptide characterization and sequencing). The selected peptides were fragmented by a process called collision-induced dissociation (CID) to generate a tandem mass spectrum (MS/MS spectrum or CID mass spectrum), which contained the sequence information for a single peptide. Individual CID mass spectra were then searched with the computer program Sequest (368) against the known sequence of 4E-BP1. The search was modified to allow for the potential increase in mass to serine, threonine, and tyrosine by phosphorylation (369). All phosphopeptides matched by computer searching algorithms were manually verified.

4.5.8 Kinase assays. FRAP/mTOR immunoprecipitation from rat brain was carried out essentially as described (339) with the following modification: The immunoprecipitated material was washed three times with lysis buffer, three times with lysis buffer containing 400 mM KCl, and twice with kinase buffer (339). FRAP/mTOR kinase assays were performed essentially as described (339) with either FRAP/mTOR immunoprecipitated from rat (339) or with baculovirus-expressed, flag-tagged FRAP/mTOR. FRAP/mTOR kinase reactions were incubated for 20-40 min at 30°C, with gentle agitation. The ERK2 assay was performed for 10 min using recombinant ERK2 (NEB), according to the manufacturer's instructions.

4.5.9 Chromatography on m^7GDP-agarose. 4E-BP1 and eIF4E proteins were incubated for 1 hr with m^7GDP coupled to agarose adipic resin in a total volume of 50 μl (5-30 μl of packed beads per reaction, beads were prepared according to Edery et al. (177). Beads were then spun in a microcentrifuge (3000 rpm, 30 sec), washed 3 times with 20 volumes of buffer containing 75 mM KCl, 20 mM HEPES-KOH (pH 7.4), and resuspended in Laemmli sample buffer. Samples were analyzed by SDS-PAGE and subjected to autoradiography.

4.6 Acknowledgments

This paper was co-authored by Anne-Claude Gingras, Steven P. Gygi, Brian Raught, Roberto D. Polakiewicz, Robert T. Abraham, Merl F. Hoekstra, Ruedi Aebersold, and Nahum Sonenberg. S.P.G. and B.R. contributed equally to this work. We thank Joe Marcotrigiano for providing the purified eIF4E NΔ27 protein and the purified cleaved GST-4E-BP1 proteins, Eric J. Brown and Stuart L. Schreiber for the baculovirus-flag FRAP construct, Chantal Binda and Colin Lister for excellent technical assistance, Yanick Matteau for help with tissue culture, and Hiroaki Imataka for the pcDNA3-3HA vector. We thank William B.C. Merrick, Eric J. Brown, Michel L. Tremblay, Francis Poulin, Stéphane Pyronnet and Mathieu Miron for helpful discussions and comments on the manuscript. This work was supported by grants from the National Cancer Institute of Canada and the Howard Hughes Medical Institute (HHMI) to N.S. and from the National Science Foundation Science and Technology Center for Molecular Biotechnology to R.A. N.S. is a Medical Research Council (MRC) of Canada Distinguished Scientist and a HHMI International Scholar. A.-C.G. and B.R. were supported by a doctoral award and a post-doctoral fellowship, respectively, from the MRC of Canada. S.P.G. is supported by a National Institutes of Health grant T32HG00035-3 and a grant from Oxford Glycosciences.

CHAPTER 5

HIERARCHICAL PHOSPHORYLATION OF THE TRANSLATION INHIBITOR 4E-BP1

5.1 Introduction and perspectives

We have defined in the previous chapter two subsets of phosphorylation sites in HEK 293 cells for 4E-BP1: one which is relatively serum-resistant and consists of Thr37 and Thr46, and one which is extremely serum-sensitive. We also reported that phosphorylation of Thr37/Thr46 is required for the phosphorylation of additional, unknown serum-sensitive sites. Mutation of Thr37 and/or Thr46 to alanine residues prevents phosphorylation of the serum-sensitive sites, but substitution of Thr37 and Thr46 by glutamic acid residues partially restores the phosphorylation of the serum-sensitive sites (215). We thus suggested that phosphorylation of Thr37 and Thr46 serves as a priming event, allowing for the succeeding serum-induced phosphorylation of the remaining 4E-BP1 sites. Phosphorylation of Ser65 was also subsequently reported by other investigators to be dependent on prior phosphorylation of Thr37, Thr46 and Thr70 (370, 371). However, at variance with our data, these studies suggested that phosphorylation of Thr70 does not depend on prior phosphorylation of Thr37 and Thr46 (370, 371). The number and identity of the phosphorylation sites required to effect the release of 4E-BP1 from eIF4E also remained in question. Thr37 and Thr46 phosphorylation was reported both to have little or no effect on eIF4E binding (215, 222, 372), or (at least for Thr46) to effect a significant reduction in eIF4E binding affinity (353, 370, 373). Significant discrepancies also exist regarding the effect of Ser65 phosphorylation on eIF4E binding (204, 205, 222, 370, 373). Since previous studies relied on the use of transfected, overexpressed mutant proteins (which may not faithfully mimic the behavior of the endogenous protein), we directly analyzed the order of phosphate addition on the endogenous 4E-BP1 protein. Here, using phosphopeptide mapping and mass spectrometry, we identify Ser65 and Thr70 as the principle serum-responsive, rapamycin-sensitive 4E-BP1 phosphorylation sites in HEK 293 cells. Further, using a novel combination of two-dimensional isoelectric-focusing/SDS-PAGE followed by Western blotting with phosphospecific antibodies, we unambiguously

delimit the order of phosphate addition to endogenous 4E-BP1; phosphate groups are first added to Thr37 and Thr46. This priming step is followed by phosphorylation of Thr70 and, finally, of Ser65. We also demonstrate that phosphorylation of Ser65 alone, or phosphorylation of both Ser65 and Thr70, does not disrupt 4E-BP1 binding to eIF4E. Thus, multiple phosphorylation events (most likely via different kinases) are required to release 4E-BP1 from eIF4E.

5.2 Abstract

In most instances, translation is regulated at the initiation phase, when a ribosome is recruited to the 5' end of an mRNA. The eIF4E-binding proteins (4E-BPs) interdict translation initiation by binding to the translation factor eIF4E, and preventing recruitment of the translation machinery to mRNA. The 4E-BPs inhibit translation in a reversible manner. Hypophosphorylated 4E-BPs interact avidly with eIF4E, whereas 4E-BP hyperphosphorylation, elicited by stimulation of cells with hormones, cytokines, or growth factors, results in an abrogation of eIF4E binding activity. We previously reported that phosphorylation of 4E-BP1 on Thr37 and Thr46 is relatively insensitive to serum-deprivation and rapamycin treatment, and that phosphorylation of these residues is required for subsequent phosphorylation of a set of unidentified serum-responsive sites. Here, using mass spectrometry, we identify the serum-responsive, rapamycin-sensitive sites as Ser65 and Thr70. Utilizing a novel combination of two-dimensional isoelectric focusing/SDS-PAGE and Western blotting with phosphospecific antibodies, we also establish the order of 4E-BP1 phosphorylation *in vivo*; phosphorylation of Thr37/Thr46 is followed by Thr70 phosphorylation, and Ser65 is phosphorylated last. Finally, we demonstrate that phosphorylation of Ser65 and Thr70 alone is insufficient to mediate eIF4E release, indicating that a combination of phosphorylation events is necessary to dissociate 4E-BP1 from eIF4E.

5.3 Results

5.3.1 Two sets of 4E-BP1 phosphopeptides with different sensitivities to kinase inhibitors

Using two-dimensional phosphopeptide mapping combined with mass spectrometry, we previously identified two 4E-BP1 phosphorylation sites, Thr37 and Thr46 (chapter 4). These residues are phosphorylated to a high stoichiometry in the absence of serum, and the phosphorylation state of Thr37 and Thr46 is only moderately increased following serum-stimulation of HEK 293 cells (Fig.5.1; 215). A second group of phosphopeptides (designated ***1-4*** in Fig. 5.1A) are detected only at very low levels in the absence of serum (Fig. 5.1B), but are present at high levels after serum addition (Fig. 5.1A). To study the signaling pathway(s) mediating these phosphorylation events, the sensitivity of the two groups of phosphopeptides to treatment with various kinase inhibitors (the PI3K inhibitors LY294002 and wortmannin, the FRAP/mTOR inhibitor rapamycin, and the MEK inhibitor PD098059) was investigated. The response of phosphopeptides ***1-4*** to serum addition is drastically blunted when PI3K or FRAP/mTOR signaling are inhibited by LY294002, wortmannin, or rapamycin (Fig. 5.1 C-E). The ERK inhibitor PD098059 did not affect the phosphorylation of any of the peptides (Fig. 5.1F). Thus, PI3K and FRAP/mTOR signaling are required for the phosphorylation of peptides ***1-4*** in response to serum stimulation. The phosphorylation state of phosphopeptide ***5*** is not consistent from experiment to experiment. In some cases, this phosphopeptide cannot be detected even in the presence of serum (see e.g. Fig. 5.2D), and it is not reproducibly responsive to serum treatment or sensitive to kinase inhibitor treatment (124, 215; see below).

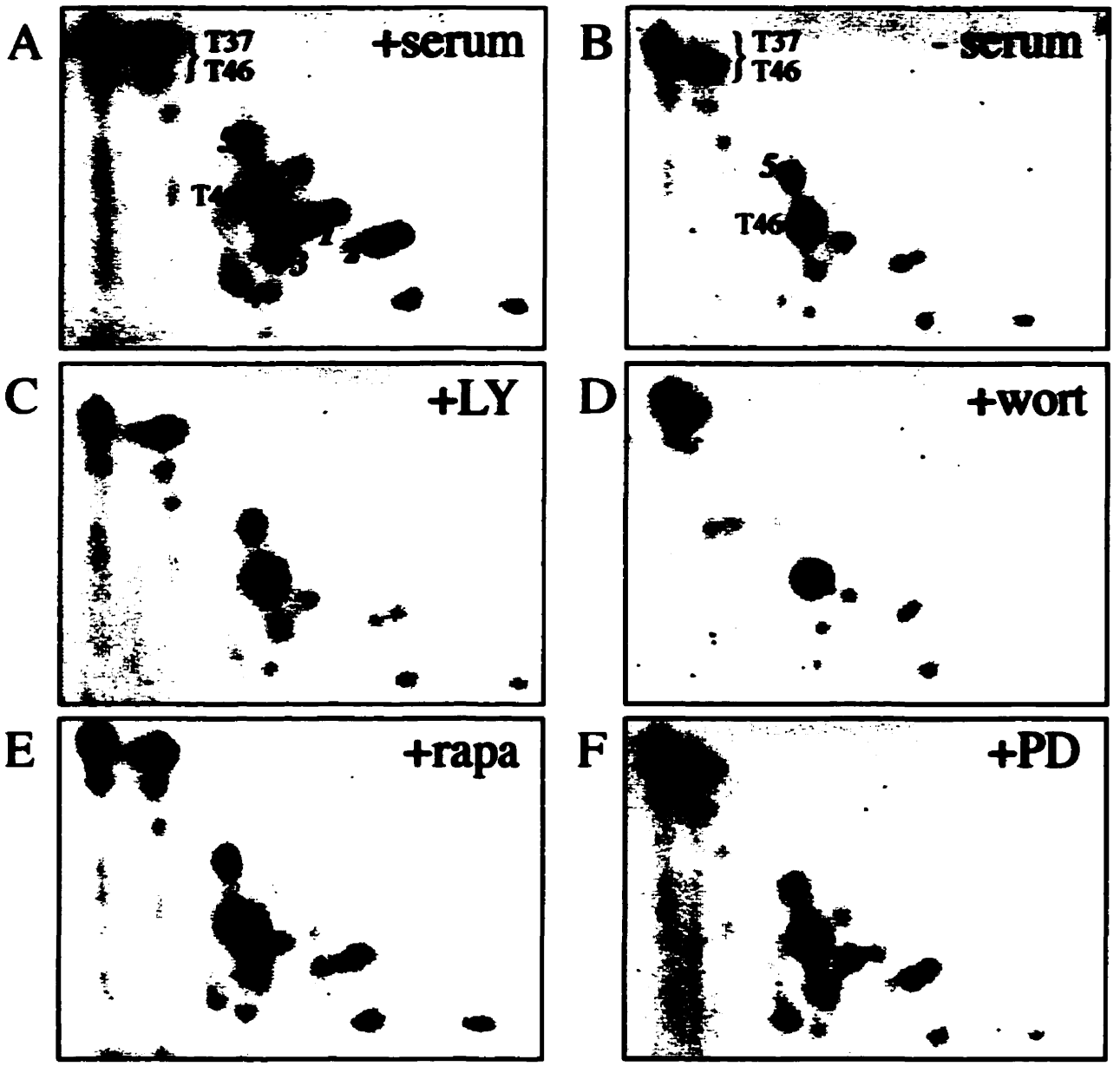


Figure 5.1. Sensitivity of 4E-BP1 phosphopeptides to serum deprivation and kinase inhibitor treatment. HEK 293 cells were starved of serum for 30 hr. ^{32}P-labeling was performed for 3.5 hr. Kinase inhibitors were then added (30 min), followed by stimulation for 30 min with 10% dialyzed FBS. Phosphopeptide maps were prepared (see Material and Methods). Previously identified phosphopeptides containing Thr37 and/or Thr46 are indicated (T37, T46). Phosphopeptides exhibiting sensitivity to serum-deprivation or kinase inhibitors are labeled *1-5*. The various treatments are indicated in the top right corner of each panel: serum-stimulation (+serum, panel A), no stimulation (-serum, B), pre-treatment with LY294002 (+LY, C), wortmannin (+wort, D), rapamycin (+rapa, E), or PD098059 (+PD, F). Panels A and B, which served here as controls, were published previously (see chapter 4).

5.3.2 Identification of the PI3K- and FRAP/mTOR-dependent phosphorylation sites

We next employed a combination of phosphopeptide mapping and mass spectrometry to identify phosphopeptides ***1-5***. Several hundred 150mm plates of HEK 293 cells (at 90% confluence) were deprived of serum for 30 hrs. Ten dishes of serum-starved cells were metabolically labeled with [^{32}P] orthophosphate. All of the cells were then stimulated with 10% serum for 30 minutes, and lysed. 4E-BP1 was immunoprecipitated from both the unlabeled and radiolabeled lysates. The radiolabeled and unlabeled immunoprecipitated materials were mixed, and subjected to SDS-15% PAGE. After transfer to a nitrocellulose membrane and autoradiography, the membrane fragment harboring 4E-BP1 was excised and incubated with a mixture of trypsin:chymotrypsin (200:1). Digested peptides were washed extensively and subjected to two-dimensional phosphopeptide mapping. Phosphopeptides ***1-5*** (Fig. 5.1A, 5.2B) were isolated from the maps, and identified by capillary liquid chromatography-electrospray ionization tandem mass spectrometry (LC-MS/MS). The sequence of the phosphopeptides and identities of the phosphorylated residues are indicated in Fig. 5.2A. Phosphopeptides ***1-4*** contain phosphorylated Ser65 and/or Thr70. These two residues are located immediately downstream of the eIF4E-binding site (Fig. 5.2C). Phosphopeptide ***5*** contains phosphorylated Ser83. Phosphoaminoacid analysis (PAA) of each of the phosphopeptides was conducted in parallel. The phosphoserine (S) and/or phosphothreonine (T) content of each peptide is indicated (Fig. 5.2A). The identity of the phosphopeptides containing phosphorylated Ser65 and Thr70 was further confirmed by two-dimensional phosphopeptide mapping of epitope-tagged wild-type 4E-BP1 (Fig. 5.2D), and Ser65Ala (Fig. 5.2E) or Thr70Ala (Fig. 5.2F) mutant proteins expressed in HEK 293T cells. Substitution of Ser65 with an alanine residue resulted in a loss of phosphopeptides ***3*** and ***4***, while the Thr70 to alanine mutation resulted in a loss of phosphopeptides ***1***, ***2*** and ***4***.

A

spot #	Sequence	location	PAA	site
1	(K)TPPR	70-73	T	T70
2	(R)NSPVTKTPPR	64-73	T	T70
3	(R)NSPVTK	64-69	S	S65
4	(R)NSPVTKTPPR	64-73	S/T	S65/T70
5	(R)DLPTIPGVTSPSSDEPM*EASQSHLR	74-98	S	S83

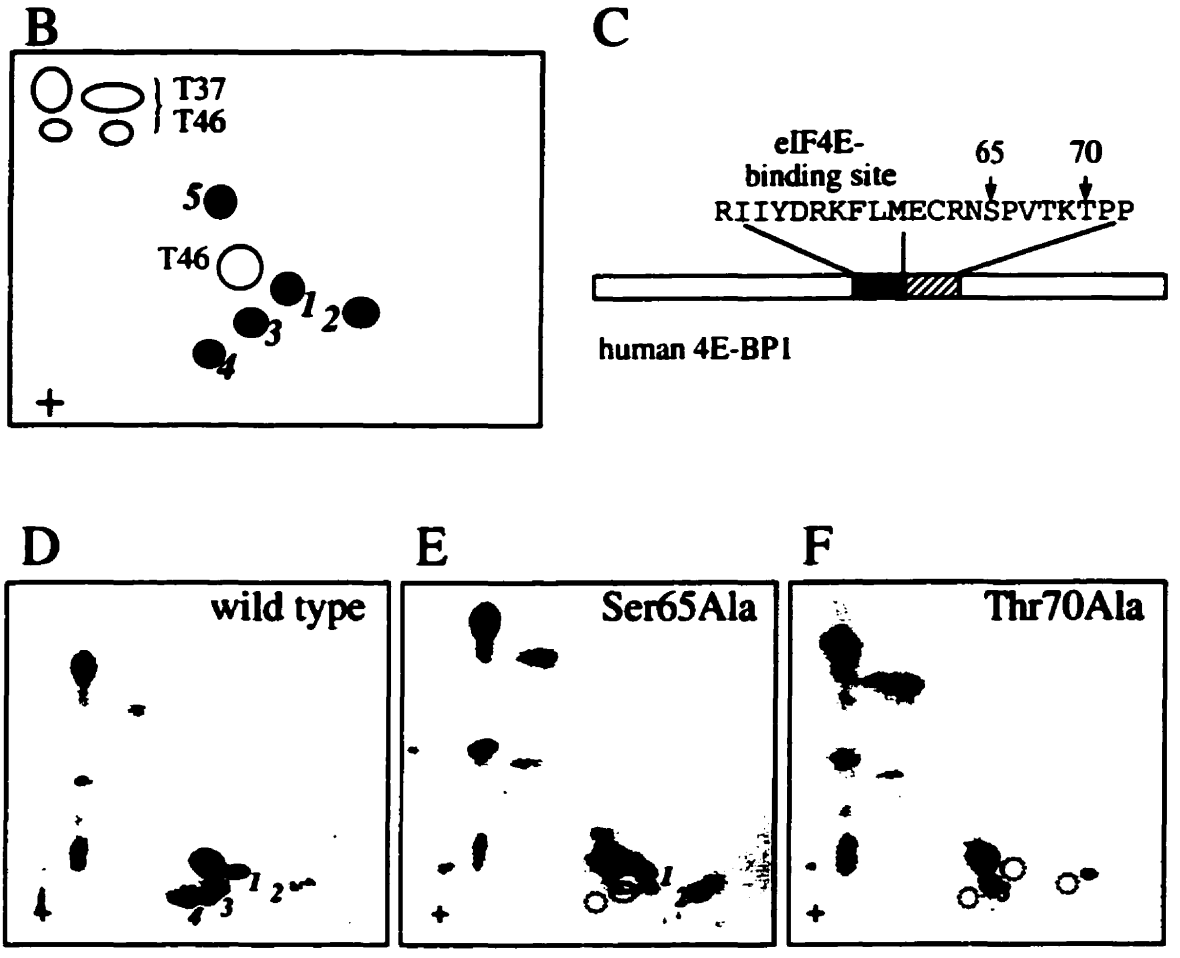


Figure 5.2. Identification of Ser65 and Thr70 as the serum-responsive, kinase inhibitor-sensitive phosphorylation sites. 4E-BP1 was immunoprecipitated from 2 x 10^8 serum-stimulated cells (1/20 labeled *in vivo* with [^{32}P] orthophosphate), and subjected to phosphopeptide mapping. Peptides ***1-5*** (see Fig. 1A) were isolated from the plates and identified by LC-MS/MS. (A) Determination of the sequence of phosphopeptides ***1-5***, and of the identity of the phosphorylated residues by mass spectrometry. The phosphorylated residue is underlined; * indicates oxidized methionine. Trypsin cleavage sites are indicated in parentheses. Phosphoaminoacid analysis (PAA) was performed in parallel. (B) Schema of the phosphopeptides identified by two-dimensional phosphopeptide mapping. (C) Positions of Ser65 and Thr70 in 4E-BP1, relative to the eIF4E-binding site. (D-F) Confirmation of the identity of the phosphopeptides containing Ser65 and Thr70. HEK 293T cells expressing HA-tagged 4E-BP1 proteins were subjected to metabolic ^{32}P-labeling, and two-dimensional phosphopeptide mapping of the HA-4E-BP1 proteins was performed. Broken circles indicate the absence of phosphopeptides, as compared to the wild type protein.

5.3.3 Phosphospecific antibodies directed against Ser65 and Thr70

To further investigate the regulation of Ser65 and Thr70 phosphorylation, phosphospecific antibodies directed against these sites were produced. To confirm the specificity of these reagents, HA-tagged wild type 4E-BP1 protein, or 4E-BP1 proteins harboring alanine substitutions at Ser65 or Thr70 were expressed in HEK 293T cells, and immunoblotting was performed using an antiserum directed against the entire 4E-BP1 protein (Fig. 5.3A and B, top panels), an antibody directed against phospho-Ser65 (Fig. 5.3A, bottom panel), or an antibody directed against phospho-Thr70 (Fig. 5.3B, bottom panel). The HA-4E-BP1 wt and mutant proteins were expressed to similar levels (Fig. 5.3A and B, lanes 1 and 2). However, the antibody directed against phospho-Ser65 did not detect the HA-4E-BP1 Ser65Ala protein (Fig. 5.3A, lane 2, bottom panel). Similarly, the antibody directed against phospho-Thr70 did not react with the HA-4E-BP1 Thr70Ala mutant protein (Fig. 5.3B, lane 2). Extracts from 293 cells deprived of serum for 30 hours (Fig. 5.3A and B, lanes 3), or deprived of serum for 30 hours then replenished with serum for 30 minutes (Fig. 5.3A and B, lanes 4), were also subjected to immunoblotting with these antisera. In both cases, only a very weak signal was detected in the starved cell extracts (lanes 3), whereas strong signals were observed after serum-stimulation (lanes 4). Thus, our phosphospecific antibodies were demonstrated to be specific for the appropriate phosphorylated residues, and confirm that phosphorylation of Ser65 and Thr70 is sensitive to serum-stimulation. These antibodies were also utilized to confirm the heightened sensitivity to kinase inhibitors of phosphorylation at Ser65 and Thr70 (data not shown).

5.3.4 Order of phosphate addition on the endogenous 4E-BP1 protein

The endogenous 4E-BP1 protein was next analyzed using a combination of two-dimensional isoelectric focusing (IEF)/SDS-PAGE and Western blotting. 4E-BP1 from serum-stimulated HEK 293 cells migrates as six isoforms in this system (Fig. 5.4B). (In

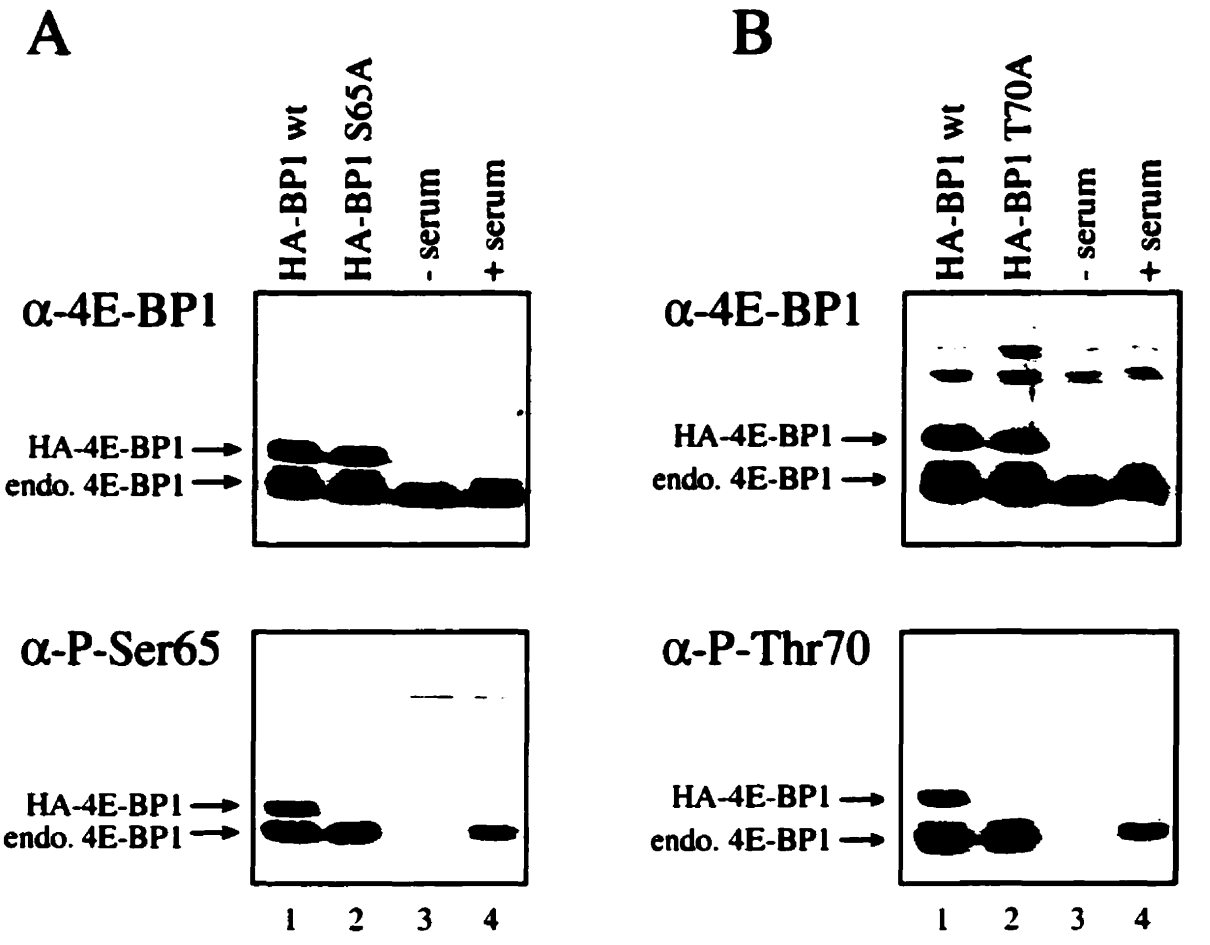


Figure 5.3. Phosphospecific antibodies directed against Ser65 and Thr70 confirm the serum sensitivity of phosphorylation at these sites. (A) Western blotting using an antibody against total 4E-BP1 (top panel), or a phosphospecific antibody directed against Ser65 (bottom) were performed on extracts from HEK 293T cells transfected with HA-4E-BP1 wt (lane 1) or HA-4E-BP1 Ser65Ala (lane 2), or extracts from HEK 293 cells which were serum-starved (lane 3), or serum-starved, and then serum-stimulated for 30 minutes (lane 4). (B) Western blotting was performed as in (A), but using an HA-4E-BP1 Thr70Ala protein in lane 2, and a phosphospecific antibody directed against Thr70 in the bottom panel. The positions of the endogenous 4E-BP1 (BP1), and the transfected proteins (HA-BP1) are indicated.

some experiments, a seventh more acidic isoform is also detected). In cells deprived of serum, only two predominant isoforms are detected (***a*** and ***c***), which migrate toward the basic region of the gel (Fig. 5.4A). Similarly, only isoforms ***a*** and ***c*** are detected in 4E-BP1 from cells pre-treated with rapamycin or LY294002 prior to serum-stimulation (data not shown). Isoform ***a*** is unphosphorylated, because it does not incorporate ^{32}P (data not shown). Isoforms ***b-f*** incorporate ^{32}P (and are thus predicted to be phosphorylated on one to five residues, respectively).

Our previous phosphopeptide mapping experiments (124, 215; chapters 3-4; Fig. 5.1) suggested that isoform ***c*** (predicted to be the diphosphorylated species), which is present in serum-starved cells, contains both phosphorylated Thr37 and Thr46. Interestingly, isoform ***b*** (presumably the monophosphorylated protein) is a minor species, both in serum-stimulated cells and in serum-starved or rapamycin-treated cells. This is likely because Thr37 and Thr46 are regulated coordinately by FRAP/mTOR, and is consistent with our phosphopeptide mapping data (124, 215; chapters 3-4; Fig. 5.1), in which equimolar amounts of phospho-Thr37 and phospho-Thr46 are detected. Also, when Thr46 is mutated to alanine, phosphorylation of Thr37 is dramatically decreased (chapter 4), suggesting that the phosphorylation state of these two sites is intimately linked.

To identify the phosphorylated residues in each of the isoelectric variants, the IEF/SDS-PAGE technique was combined with the use of phosphospecific antibodies. To ensure proper identification of the isoforms, each blot incubated with phosphospecific antibodies was subsequently re-probed with antisera raised against the entire 4E-BP1 protein (data not shown). An antibody directed against phospho-Thr37 and phospho-Thr46 recognizes isoforms ***b-e*** (all of the phosphorylated isoforms, but not the unphosphorylated ***a*** isoform; Fig. 5.4C). An antibody directed against phospho-Thr70 reacts avidly with isoforms ***d-f*** (but only poorly with isoforms ***b*** and ***c***; Fig. 5.4D. It should be noted that since these

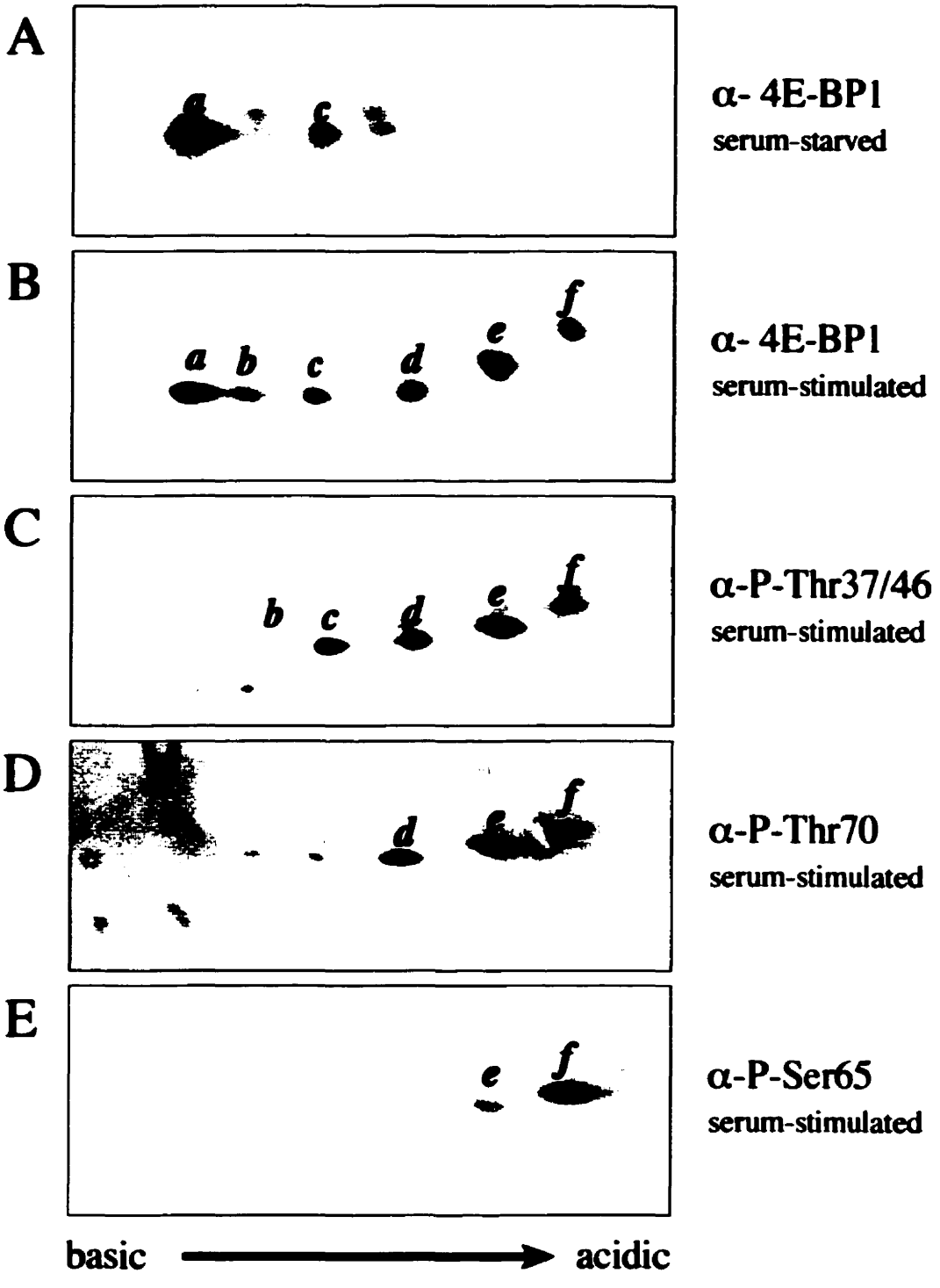


Figure 5.4. Phosphorylation of endogenous 4E-BP1 in vivo is an ordered process, culminating in the phosphorylation of Ser65. Two-dimensional IEF/SDS-PAGE (see Materials and Methods) was performed on lysates from serum-starved (A), or serum-stimulated (B-E) HEK 293 cells. Western blotting was then performed using antisera directed against the entire 4E-BP1 protein (A and B), or a series of phosphospecific antibodies raised against 4E-BP1 phosphorylated on Thr37/Thr46 (C), Thr70 (D), or Ser65 (E). Western blots C-E were subsequently re-probed with the antisera raised against the entire 4E-BP1 protein to confirm the identity of the isoforms

blots represent a population of endogenous 4E-BP1 molecules, it is reasonable to assume that even in serum-stimulated cells some isoforms may be in the process of being dephosphorylated. Thus, the very weak reactivity of isoforms ***b*** and ***c*** to the phospho-Thr70 antibody could indicate an ongoing low level of dephosphorylation). The antibody specific to phosphorylated Ser65 weakly recognizes isoform ***e***, but reacts strongly with isoform ***f*** (Fig. 5.4E). These data thus indicate that; 1) Thr37 and Thr46 are phosphorylated in the absence of prior phosphorylation; 2) Thr70 is phosphorylated predominantly only in isoforms already phosphorylated on Thr37 and Thr46, and; 3) Ser65 is phosphorylated only in molecules already phosphorylated on Thr37, Thr46 and Thr70. Thus, analysis with phosphospecific antibodies indicate that the order of phosphate addition on endogenous 4E-BP1 *in vivo* is Thr37/Thr46, Thr70, Ser65. The nature of the fifth phosphorylated isoform detected in our IEF/SDS-PAGE technique remains elusive, but could be Ser83, or possibly Ser112 (a site reported to be phosphorylated in adipocytes; 214).

5.3.5 Mutational analysis of 4E-BP1 confirms the order of phosphate addition

To further substantiate the conclusions of the IEF/SDS-PAGE experiments, we applied this technique to a series of HA-tagged 4E-BP1 proteins expressed in HEK 293T cells. As expected, the HA-tagged wt 4E-BP1 protein resolves into six major isoforms (Fig. 5.5A). Consistent with previous data (215; chapter 4), mutation of Thr37 and Thr46 to alanine residues results in a striking accumulation of the unphosphorylated isoform ***a*** (Fig. 5.5B). No phosphorylation of Ser65 or Thr70 is detected in this mutant, as determined by Western blotting with phosphospecific antibodies (data not shown). Mutation of Thr70 to alanine results in an almost complete disappearance of isoforms ***e*** and ***f*** (Fig. 5.5C). The phosphorylation state of Ser65, as determined with phosphospecific antibodies, is reduced in the HA-4E-BP1 Thr70Ala mutant (although the extent of this reduction varies from experiment to experiment; data not shown). Mutation

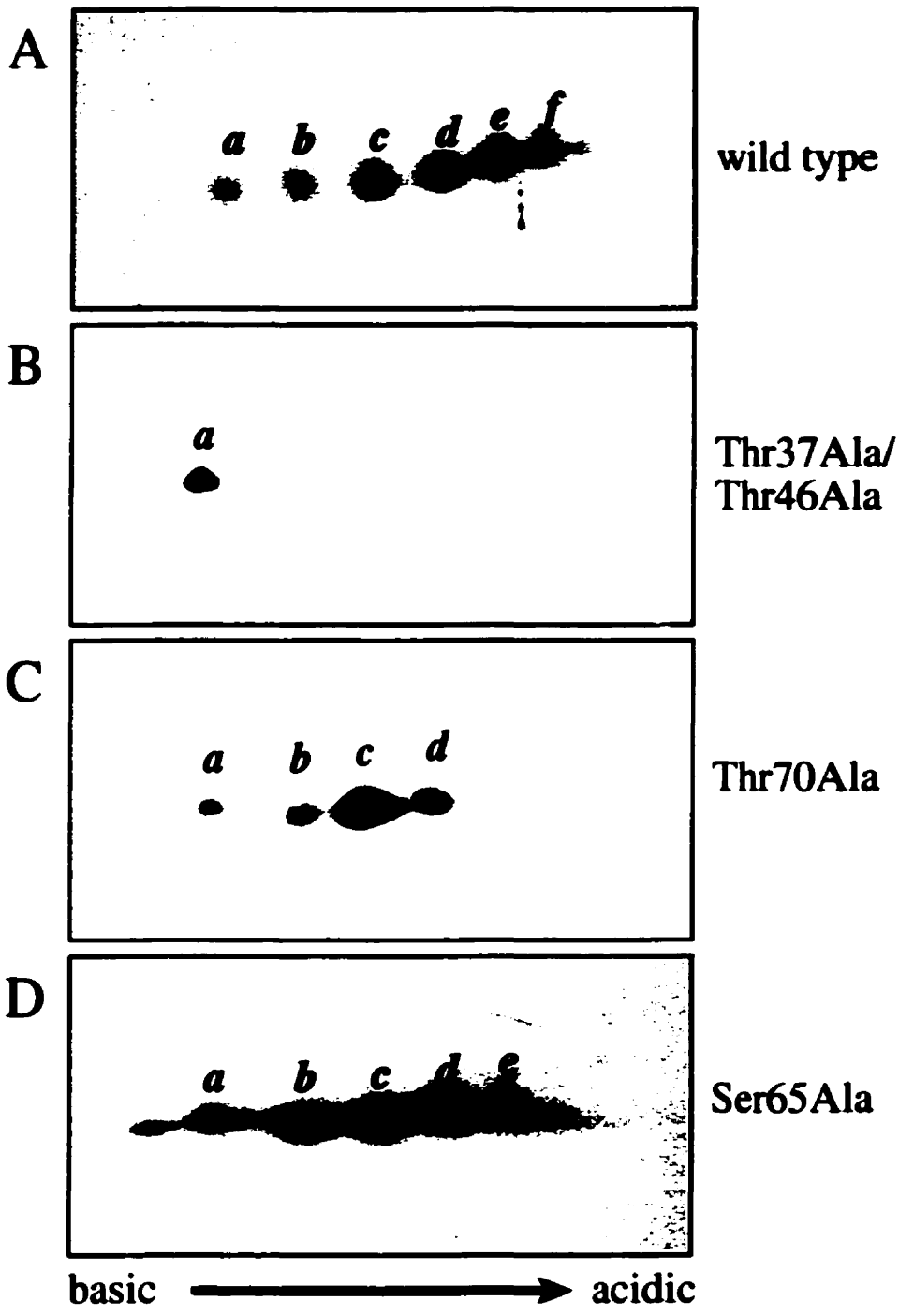


Figure 5.5. Effects of mutating individual phosphorylation sites on 4E-BP1 phosphorylation. HA-tagged 4E-BP1 proteins were expressed in HEK 293T cells. 48 hr after transfection, lysates were prepared and analysed by two-dimensional IEF/SDS-PAGE, as above. Western blots were prepared using an antibody directed against the HA tag.

of Ser65 to alanine resulted in the disappearance only of isoform *f* (Fig. 5.5D), confirming that Ser65 phosphorylation is not required for phosphorylation of the other sites. Phosphorylated Thr37/Thr46 and Thr70 are detected to similar levels in the wt and Ser65Ala mutant protein (data not shown). In sum, analysis of a panel of phosphorylation site mutant proteins confirms our phosphospecific antibody results, regarding the order of phosphate addition to the endogenous 4E-BP1 protein. These data also further demonstrate that phosphorylation of Thr37 and Thr46 is necessary for phosphorylation of the remaining sites. Phosphorylation of Thr70 is not required for Thr37 and Thr46 phosphorylation, but does modulate the phosphorylation of Ser65. Consistent with it being added last, the presence or absence of a phosphate group on Ser65 does not influence the phosphorylation of the other sites. Also consistent with these data is the fact that the tryptic peptide encompassing amino acids 64-73 (Fig. 5.1A, 5.2A-B) was found by mass spectrometry to contain either phosphorylated Thr70 alone, or phosphorylated Thr70 and Ser65, but not phosphorylated Ser65 alone.

5.3.5 Phosphorylation of Ser65 alone is insufficient to mediate release from eIF4E

Our data demonstrate that the phosphorylation of 4E-BP1 is an ordered process culminating in the phosphorylation of Ser65. However, the role of the various phosphorylation events in the regulation of binding to eIF4E remained unclear. 4E-BP1 phosphorylated on Thr37 and Thr46 retains the ability to interact with eIF4E (215; chapter 4). Therefore, it was pertinent to determine whether phosphorylation of Ser65 alone (or a combination of Ser65 and Thr70) is sufficient to elicit eIF4E release. The cocrystal structure of eIF4E complexed with a peptide derived from 4E-BP1 (amino acids 51-67; 54) suggests that Ser65 phosphorylation could engender an electrostatic repulsion with acidic amino acids (such as Glu70) on the dorsal surface of eIF4E (54). Further, 4E-BP1 phosphorylated on Ser65 is not bound to eIF4E *in vivo* (data not shown). However, this cannot be taken as proof that isoforms phosphorylated only on this site are unable to

bind to eIF4E, since we have demonstrated that 4E-BP1 molecules which are phosphorylated on Ser65 are also always phosphorylated on several other residues.

To determine whether Ser65 phosphorylation alone prevents binding to eIF4E, wild-type and mutant (Δ4E, Thr37Ala/Thr46Ala, Ser65Ala, and Thr70Ala) recombinant 4E-BP1 proteins were incubated with recombinant ERK2 in the presence of [γ^{32}P]ATP. Except for the Ser65Ala mutant (which was phosphorylated to <10% of the wt protein), the other proteins were phosphorylated to similar extents (not shown). Thus, the principal phosphorylation site by ERK2 under these conditions is Ser65 (also see below). Phosphorylated proteins were incubated with an equimolar amount of eIF4E, recovered on an m^{7}GDP-agarose ("cap-affinity") column (which retains eIF4E and associated proteins), and analyzed by SDS-PAGE followed by autoradioagraphy (Fig. 5.6A, top, "cap-column bound"). The unbound fraction was analyzed in parallel (Fig. 5.6A, bottom, "unbound"). The phosphorylated 4E-BP1 Δ4E mutant, which is unable to interact with eIF4E, was found exclusively in the unbound fraction, confirming the specificity of this assay (Fig. 5.6A, lane 2; compare top and bottom panels). Similarly, phosphorylated wt 4E-BP1 was not co-precipitated with an eIF4E mutant protein (Trp73Ala) deficient for 4E-BP1 binding (lane 6). ERK2-phosphorylated wt 4E-BP1 interacted efficiently with wt eIF4E (lane 1). 4E-BP1 proteins harboring mutations at Thr37 and Thr46 (TTAA) or at Thr70 (Thr70Ala), were also efficiently phosphorylated by ERK2, and interacted efficiently with wt eIF4E (lanes 3 and 5). To determine whether Ser65 is phosphorylated in the isoforms bound to eIF4E, wt 4E-BP1 labeled *in vitro* (Fig. 5.6B), and the same protein isolated after binding to eIF4E, were subjected to phosphopeptide mapping (Fig. 5.6C; protein from Fig. 5.6A, lane 1, top panel). Mapping was also performed on the 4E-BP1 Ser65Ala mutant protein (Fig. 5.6D). The identity of the *in vitro*-radiolabeled phosphopeptides was determined by mixing these samples with *in vivo*-labeled endogenous 4E-BP1 (data not shown). These data confirm that Ser65 is the major site

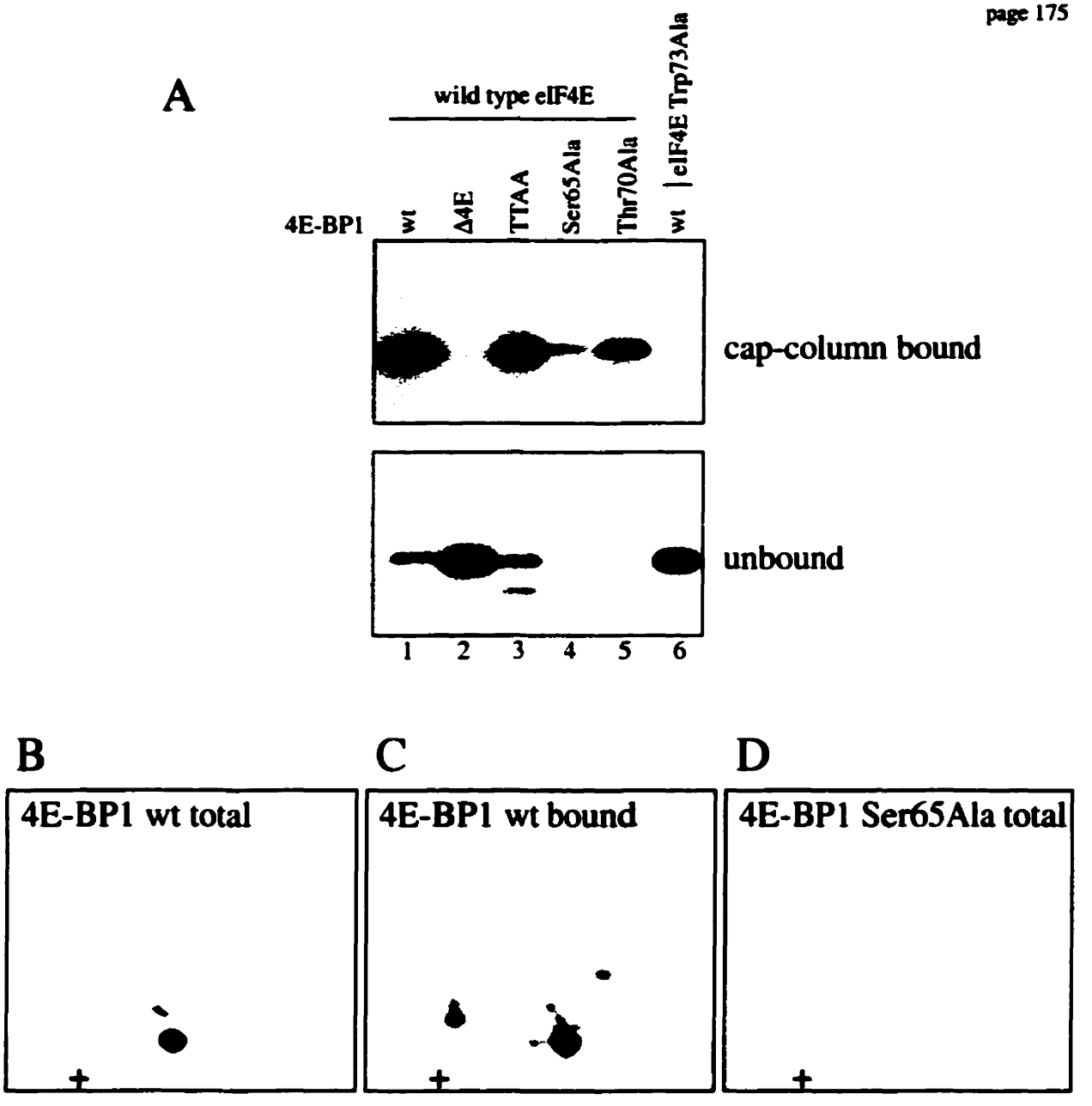


Figure 5.6. Phosphorylation of Ser65 alone is insufficient to effect release from eIF4E. Bacterially-expressed wild-type and mutant GST-4E-BP1 proteins were phosphorylated for 15 min with recombinant ERK2 in the presence of γ^{32}P-ATP. Labeled 4E-BP1 proteins were incubated for 1 hr with equimolar concentrations of recombinant GST-eIF4E proteins. (A) Complexes were recovered on an m^{7}GDP-affinity column ("cap-column bound"), and analyzed by SDS-PAGE and autoradiography. Fractions not retained on the cap column ("unbound") were analyzed similarly. Δ4E is a 4E-BP1 mutant deleted in the binding site for eIF4E, and TTAA is the double point mutant Thr37Ala/Thr46Ala. Trp73Ala is an eIF4E mutant unable to bind to 4E-BP1. (B-D) Phosphopeptide mapping of ERK2-phosphorylated 4E-BP1. (B) Phosphopeptide map of 4E-BP1 phosphorylated *in vitro* by ERK2. (C) Phosphopeptide map of 4E-BP1 phosphorylated *in vitro* by ERK2, and recovered on a cap column. (D) Phosphopeptide map of the Ser65Ala mutant phosphorylated *in vitro*.

phosphorylated *in vitro* by ERK2 (Fig. 5.6B), and, more importantly, demonstrate that phosphorylated Ser65 is clearly found in the fraction of 4E-BP1 bound to eIF4E (Fig. 5.6C). Thus, phosphorylation of Ser65 alone is insufficient to abrogate eIF4E binding.

5.3.7 Phosphorylation of both Ser65 and Thr70 is not sufficient to effect eIF4E release

Since serum-stimulation increases the phosphorylation state of Ser65 and Thr70, it remained possible that phosphorylation of both of these residues could be sufficient to mediate eIF4E release. The study of the effects of Thr70 phosphorylation has been hampered by the absence of a kinase that can specifically phosphorylate this site to a high stoichiometry *in vitro*. We therefore synthesized peptides encompassing the eIF4E-binding site (42, 54), which were unphosphorylated (aa 51-67, see Materials and Methods), monophosphorylated at Ser65 (aa 51-67), or diphosphorylated at Ser65 and Thr70 (aa 51-75). The interaction between the 4E-BP1 peptides and eIF4E was determined by fluorescence quenching (Fig. 5.7). Binding of the 4E-BP1 peptides to the convex dorsal surface of eIF4E leads to quenching of the intrinsic eIF4E fluorescence, mainly due to the interaction between eIF4E Trp73 (the source of the fluorescent signal) and three amino acid side chains in the peptides (54). The unphosphorylated peptide exhibits a K_d of 105.6 +/- 4.4 nM for eIF4E. This value is comparable to that obtained previously for the same peptide (50nM) by isothermic calorimetry (54). Consistent with the results presented in Fig. 5.6, phosphorylation of Ser65 does not disrupt eIF4E binding, and only reduces the eIF4E binding affinity by two-fold (Fig. 7; K_d = 210 +/- 18nM). Finally, phosphorylation of both Ser65 and Thr70 was also not sufficient to abolish eIF4E binding, and did not alter the affinity of the 4E-BP1 peptide for eIF4E, as compared to the monophosphorylated peptide (K_d = 175 +/- 11nM). Thus, at least in the context of these phosphopeptides, phosphorylation of both Ser65 and Thr70 is insufficient to abrogate eIF4E binding, suggesting that phosphorylation of additional sites, most likely the "priming" sites Thr37 and Thr46, is required to effect eIF4E release.

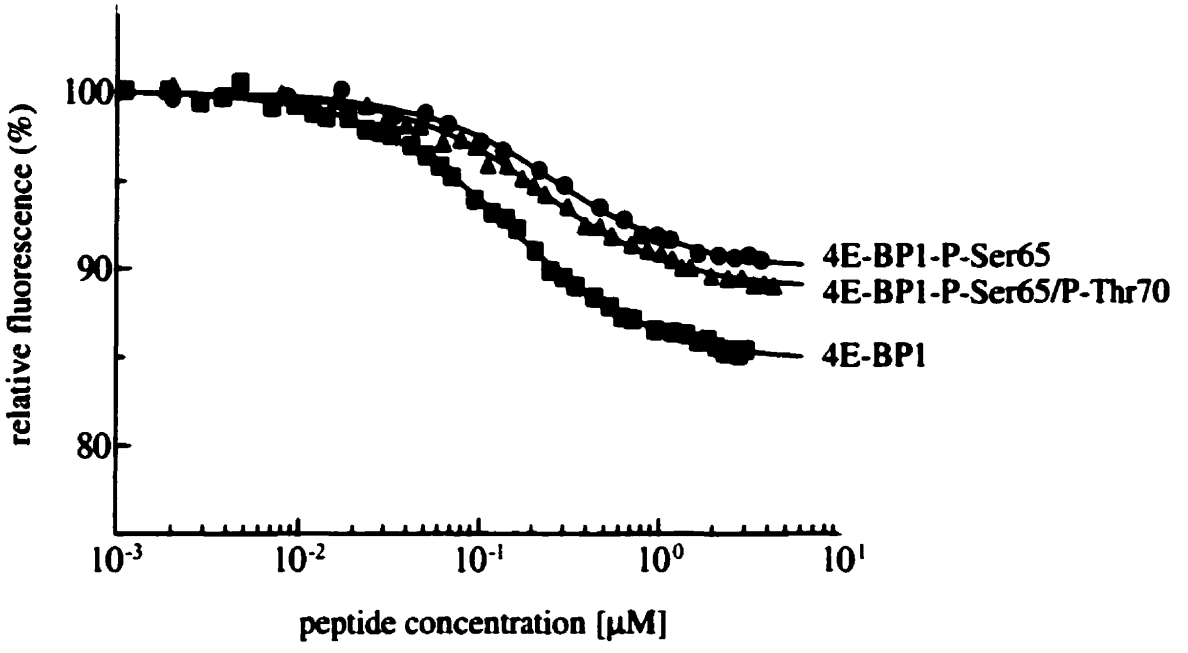


Figure 5.7. 4E-BP1 peptides phosphorylated on Ser65 and Thr70 interact with eIF4E. Increasing concentrations of an unphosphorylated 4E-BP1 peptide (residues 51-67; ■), a monophosphorylated peptide (Ser65; ●), or a diphosphorylated peptide (Ser65 and Thr70; ▲) were incubated with eIF4E, and the binding affinity measured using fluorescence quenching of eIF4E. The eIF4E concentration in the experiment shown was 0.1 M. The peptide concentration range at which the saturation stage is achieved determines the binding constant, and is essentially equal for each of the three peptides.

5.4 Discussion

Using phosphopeptide mapping, mass spectrometry, and a novel combination of two-dimensional isoelectric focusing/SDS-PAGE combined with Western blotting with phosphospecific antibodies directed against various 4E-BP1 phosphorylation sites, we have determined the order of phosphate addition to endogenous 4E-BP1; Thr37 and Thr46 phosphorylation is followed by phosphorylation of Thr70 and, finally, of Ser65. The results presented here are in agreement with some previously published data, in which phosphorylation of Ser65 was demonstrated to be affected by mutation of Thr37, Thr46 or Thr70 (215, 370, 371). However, our results are at variance with other data presented in the same studies. For example, Mothe-Satney et al. report that phosphorylation of Thr70 is independent of prior phosphorylation of Thr37 and Thr46 (370, 371). It is important to note that the latter studies relied mainly on the analysis of transfected, overexpressed mutant proteins, which may not faithfully reproduce the behavior of the endogenous protein. Overexpression can lead to mislocalization, or an alteration in upstream signaling events. Further, since 4E-BP1 is an unstructured peptide (54, 199, 200), aberrant phosphorylation (i.e. phosphorylation of residues not normally phosphorylated in the endogenous protein) may occur upon expression of large quantities of the protein. In this regard, we have observed phosphorylation of Ser65 in some experiments involving transfection of a Thr70Ala mutant protein (e.g. Fig. 5.2F), while our results with the endogenous protein clearly demonstrate that Ser65 is phosphorylated predominantly only in molecules already phosphorylated on Thr70. In addition, most previous studies have analyzed the phosphorylation of 4E-BP1 via mobility shift in the one-dimensional SDS-PAGE system; this method does not allow for the analysis of individual phosphorylation sites, since phosphorylation of some of the residues elicits little (if any) shift in electrophoretic mobility. In the current study, we have demonstrated directly that phosphorylation of the endogenous 4E-BP1 occurs in an ordered fashion.

Our data also clearly indicate that phosphorylation of both Thr70 and Ser65 is not sufficient to disengage a 4E-BP1 peptide from eIF4E. These results are at variance with reports which have characterized the 4E-BP1/eIF4E interaction utilizing recombinant 4E-BP1 proteins phosphorylated *in vitro* with ERK, and harboring alanine substitutions at Thr37, Thr46, Thr70 and Ser83 (222, 370, 373). Using the Far-Western technique (a qualitative method to detect interactions between a nitrocellulose membrane-bound protein and a radiolabeled protein in solution), phosphorylation of either Thr46 or Ser65 was reported to result in a decrease in eIF4E binding (370, 373). Also, while this manuscript was in preparation, Karim et al. reported that phosphorylation of Ser65 alone leads to a 100-fold decrease in the affinity of 4E-BP1 for eIF4E, as measured using surface plasmon resonance. These authors also report that phosphorylation of Ser65 reduces eIF4E binding activity in a pull-down assay (222). This is at variance with our data, which were obtained using both full-length proteins and synthetic phosphopeptides. When we performed co-precipitation experiments with the wild-type 4E-BP1 protein phosphorylated by ERK2, we detected only a single major phosphopeptide in the 4E-BP1 fraction bound to eIF4E, which was demonstrated to be phosphorylated on Ser65. Thus, Ser65 phosphorylation alone clearly does not lead to a dissociation of 4E-BP1 from eIF4E, under conditions in which the *in vivo* phosphorylated 4E-BP1 is unable to bind to eIF4E (e.g. 124). This conclusion was further substantiated by using peptides monophosphorylated at Ser65 or diphosphorylated at Ser65 and Thr70.

It should be noted that the use of phosphopeptides in this type of study has several advantages over the use of recombinant proteins phosphorylated *in vitro* by various kinases: 1) much improved reproducibility from experiment to experiment; 2) phosphorylation only of the desired residue; 3) 100% stoichiometry of phosphorylation; and 4) the possibility of generating large quantities of the phosphopeptide for quantitative measurements. The use of synthetic peptides has also allowed for better control of

disulfide bond formation caused by a cysteine residue (Cys62) located in the vicinity of the eIF4E binding site. Under non-reducing conditions, oxidation of Cys62 induces dimer formation (both in the context of the peptides and in the full-length protein). This is especially problematic for the unphosphorylated peptide, which forms dimers more readily than the phosphorylated peptides, presumably because the phosphate groups exert some degree of repulsion to partially prevent dimerization. Remarkably, peptide dimerization was observed to enhance eIF4E apparent binding affinity by up to 2-3 orders of magnitude (for the unphosphorylated peptide; A.N., unpublished). Thus, performing affinity measurements without protection against Cys62 oxidation can give rise to spurious results.

The identity of the kinase(s) responsible for phosphorylating 4E-BP1 *in vivo* is not known. FRAP/mTOR immunoprecipitates can phosphorylate 4E-BP1 *in vitro*, on Thr37 and Thr46 (215, 353). However, a FRAP/mTOR "activating" antibody was reported to stimulate the phosphorylation of Ser65 and Thr70 *in vitro*, suggesting that FRAP/mTOR could be responsible for phosphorylating all of the 4E-BP1 phosphorylation sites (339, 352, 371). It was also reported that a kinase activity specific to Ser65 and Thr70 could be liberated from a FRAP/mTOR immunoprecipitate, suggesting that a second kinase is present in FRAP/mTOR immunoprecipitates (372). Thus, whether FRAP/mTOR phosphorylates 4E-BP1 on all or some of these sites remains to be determined. Nevertheless, FRAP/mTOR signaling clearly plays a critical regulatory role in the phosphorylation of Ser65 and Thr70, because these residues display a higher degree of rapamycin sensitivity than Thr37 and Thr46 (124). Treatment of cells with the phosphatase inhibitor calyculin prevents 4E-BP1 dephosphorylation in response to rapamycin treatment (374). Thus, it is a distinct possibility that a PP2A-type phosphatase directed toward Ser65 and Thr70 is activated following rapamycin treatment, and FRAP/mTOR activity is required for repression of this phosphatase.

The phosphorylation of several different proteins has been demonstrated to occur in an ordered fashion, but the order of addition on the various phosphorylated residues on the endogenous proteins has only been determined for a few (e.g. the ribosomal S6 protein and the ribosomal protein S6 kinase 1). The combination of techniques used here (two-dimensional IEF/SDS-PAGE, followed by Western blotting with phosphospecific antibodies) has enabled us to define an order of addition of the phosphate groups onto endogenous 4E-BP1 following serum stimulation of HEK 293 cells. Thus, combined with mass spectrometry, IEF/SDS-PAGE and phosphospecific antibodies can be a useful technique to characterize complex, multi-step phosphorylation events on any protein of interest.

5.5 Materials and Methods

5.5.1 Construction and expression of 4E-BP1 mutant proteins. The human 4E-BP1 cDNA was used as a template for PCR mutagenesis to mutate Ser65, Thr70 and Ser83 to Ala, Glu or Asp. Mutated sequences were inserted in-frame into both the cytomegalovirus-based vector pACTAG-2 (which contains an N-terminal 3HA tag fusion, for expression in mammalian cells), and pGEX-6p1 (which contains an N-terminal GST tag, for bacterial expression; Pharmacia). The murine wt eIF4E coding sequence and a mutant Trp73Ala were also inserted in-frame into pGEX-6p1. Inserts were sequenced in their entirety. Other constructs were previously published (215). Transient transfection of pACTAG-2-4E-BP1 into HEK 293 (ATCC CRL 1573) or HEK 293T cells (ATCC CRL 11268) was performed using Lipofectamine (Life Technologies), according to the manufacturer's instructions. GST fusion proteins were expressed in BL21 bacteria, purified as described (215), and quantified by Bradford assay and Coomassie staining after SDS-PAGE.

5.5.2 eIF4E purification. For fluorescence measurements, murine eIF4E (residues 28-217 and residues 33-217) was expressed in *E. Coli* (177), purified from inclusion bodies, folded in a one-step dialysis from 6M guanidinum hydrochloride, and subjected to anion exchange chromatography on a MonoS column. Protein solutions were buffer exchanged with Ultrafree-15 ml filters with a Biomax 5 kDa NMWL membrane (Millipore). Immediately before the spectroscopic measurements, the protein sample was filtered through an Ultrafree-0.5 ml Biomax 100 kDa NMWL (Millipore).

5.5.3 Isoelectric focusing/SDS-PAGE. HEK 293 or HEK 293T cells were rinsed twice with cold PBS, scraped, and pelleted. After removal of the PBS, cells were resuspended in 200μl (per 100mm plate) of freshly prepared extraction buffer (20mM Hepes-KOH at pH7.5, 100mM KCl, 20mM ß-glycerolphosphate, 1mM DTT, 0.25mM Na_3VO_4, 10mM

NaF, 1mM EDTA, 1mM EGTA, 10nM okadaic acid, and 1mM PMSF, supplemented with a protease inhibitor cocktail from Boehringer Mannheim). Cells were lysed by three freeze-thaw cycles, debris were pelleted by centrifugation, and the protein concentration in the supernatant was measured by Bradford assay (Biorad). This lysate was diluted directly into Laemmli sample buffer for SDS-PAGE and Western blotting. For two-dimensional isoelectric focusing (IEF)/SDS-PAGE, the lysate (20μl containing ~200μg protein) was mixed with 27mg urea and 7μl IEF sample buffer (17% v/v pharmalytes pH 3-10, 4% v/v pharmalytes 2-5.5, 14%v/v ß-mercaptoethanol, 35% w/v CHAPS, 1.8% w/v SDS), then incubated at room temperature until the urea was dissolved.

Two-dimensional isoelectric focusing/SDS-PAGE was performed using the Protean II system (Biorad). Briefly, the first dimension gel (55% w/v urea, 4.257% w/v acrylamide, 0.243% w/v bis-acrylamide, 1.5% w/v CHAPS, 0.5% v/v NP-40, 4% v/v Pharmalytes 3-10, 1% v/v Pharmalytes 2.5-5) was polymerized in 17cm x 1.5mm (inside diameter) glass tubes to 3 cm from the top. After rinsing the surface of the gel, the sample was applied and overlaid with upper chamber buffer (20mM NaOH). The lower (10mM H_3PO_4) and upper chambers were filled with buffer, and the IEF dimension was run at 200V for 2hr, 500V for 5hr, and 800V for 16 hr at room temperature. Gels were extruded from the capillary tubes, equilibrated in Laemmli sample buffer for 1 hr, and subjected to SDS-15%PAGE (16cm configuration, 1.5mm thickness) for the second dimension. Proteins were electro-transferred to nitrocellulose membranes, and analyzed by Western blotting.

Western blotting was performed as described (215). The phosphospecific antibodies to Ser65 and Thr70 were generated at Cell Signaling Technology (Beverly, MA). The other primary antibodies used for Western blotting in this study were: the anti-HA mouse monoclonal antibody HA.11 (BAbCO), the rabbit polyclonal antisera 11208 directed

against 4E-BP1 (211), and the phosphospecific antibody directed against Thr37 and Thr46 (215).

5.5.4 Phosphopeptide mapping of 4E-BP1 and identification of the phosphorylated residues. The procedure utilized for phosphopeptide mapping was essentially as described (124, 184, 215). Briefly, HEK 293 or HEK 293T cells were labeled *in vivo* with [^{32}P] orthophosphate, and 4E-BP1 was immunoprecipitated with the anti-4E-BP1 11209 antisera, or with an anti-HA antibody. Immunoprecipitated 4E-BP1 was subjected to SDS-15%PAGE, and transferred to 0.2μm pore size nitrocellulose membranes (Schleicher & Schuell). Membranes were subjected to autoradiography, and the membrane region harboring 4E-BP1 was excised and subjected to tryptic:chymotryptic (200:1) digestion. Digested products were applied to plastic-backed cellulose-coated thin layer chromatography plates (for Fig. 5.1; Kodak), or to glass-backed cellulose-coated plates (Fig. 5.2 and 5.6; Merck). Following electrophoresis and liquid chromatography, plates were dried and subjected to autoradiography. Phosphopeptides were eluted from the plates, and analyzed by LC-MS/MS, as described (215, 366, 367, 375). Phosphoaminoacid analysis was also performed on phosphopeptides eluted from the plates, as described (215, 351).

5.5.5 Kinase assays and chromatography on m^{7}GDP-agarose. Phosphorylation of 4E-BP1 with recombinant ERK2 (New England Biolabs) was performed for 15 min at 30 °C, in the presence of γ^{32}P-ATP, according to the manufacturer's instructions. Chromatography on m^{7}GDP-agarose was performed as described (215). Proteins retained on m^{7}GDP-agarose were washed 3 times with 20 volumes of buffer (20mM Hepes-KOH and 75mM KCl), and analyzed by SDS-PAGE and autoradiography.

5.5.6 Phosphopeptide synthesis and purification. A peptide corresponding to residues 51-67 of mammalian 4E-BP1, RIIYDRKFLMECRNSPV, was synthesized by Boc protocols for solid phase peptide synthesis, and cleaved using a standard HF procedure (377). The phosphopeptide synthesis was performed manually on a Wang resin (Novabiochem) using Fmoc protocols for solid phase peptide synthesis. Phosphoserine and phosphothreonine were directly incorporated during the synthesis as the corresponding preformed mono-protected N-α-Fmoc-O-benzyl phosphoaminoacid derivatives, purchased from Novabiochem (378). The phosphopeptides were cleaved using a trifluoroacetic acid:triisopropylsilane:water mixture (TFA:TIS:H_2O, 95:2.5:2.5). After cleavage, crude peptides were purified to homogeneity by semi-preparative HPLC, and characterized by amino acid analysis (see below) and MALDI-TOF or ESI spectrometry (51-67 4E-BP1 peptide, predicted mass 2141.8, measured mass 2141; P-Ser65 phosphopeptide, predicted mass 2220.1, measured mass 2221.0; P-Ser65/P-Thr70 diphosphopeptide, predicted mass 3180.55, measured mass 3181.0). Peptide concentrations were determined by amino acid analysis after acid gas-phase hydrolysis. Analyses were performed three times for each peptide via conversion of the amino acids to phenylothiocarbamyl derivatives according to standard protocols, using HPLC (Waters, Millipore), and a PicoTag 3.9x150 mm column (Waters, Millipore). All the peptides lacked secondary structure in solution, as evidenced by the presence of minima at ~200nm recorded on CD spectra (AVIV model 202 spectropolarimeter).

5.5.7 Affinity measurements by fluorescence quenching. Measurements of the affinity of the 4E-BP1 peptides for eIF4E were determined by fluorescence quenching, essentially as described (379). Before the experiment, eIF4E was saturated with a 50-fold excess of m^7GTP. Titration experiments were performed in 50 mM Hepes - KOH pH 7.20, 100 mM KCl, 1 mM dithiothreitol (DTT) and 0.5 mM disodium ethylenediaminetetraacetate (Na_2EDTA). pH was measured within ± 0.01 unit on a

Beckman Φ300 pH meter. 1 µl aliquots of increasing concentrations (1 µM to 5 mM) of each peptide were added to 1400 µl of eIF4E solution. Solutions of 4E-BP1 peptides were strictly controlled using HPLC and MS for lack of disulfide dimer formation, which can influence the binding affinities (data not shown). The concentration of eIF4E was determined from absorption. Absorption and fluorescence spectra were recorded on Lambda 20 UV/VIS and LS-50B instruments (Perkin-Elmer). The sample was thermostated within ±0.2°C and the temperature was controlled inside the cuvette with a thermocouple. An excitation wavelength of 290 nm (slit 2.5 nm, auto cut-off filter) and an emission wavelength of 350 nm (slit 2.5 to 4 nm, 290 nm cut-off filter) were used, with a correction for the photomultiplier sensitivity. These conditions ensured the observation of only the tryptophan (Trp73) emission, such that the emission of Tyr54 in 4E-BP1 was eliminated. Measurements were also run with eIF4E at several concentrations, from 0.05 to 1.0 µM. Fluorescence intensity was monitored by time-synchronized measurements at a single wavelength, with an integration time of 30 sec and a gap of 30 sec for adding the peptide, with magnetic stirring. During the time required for addition of the peptide, the UV xenon flash lamp was switched off, to avoid photobleaching of the sample. A curve for the fluorescence intensity as a function of the total ligand concentration was fitted to the experimental data points by means of a non-linear, least-squares method, using the program ORIGIN 6.0 (Microcal Software) (379). The dissociation constants were calculated as a weighted average of at least 3 independent series.

5.6 Acknowledgments

This paper was co-authored by Anne-Claude Gingras, Brian Raught, Steven P. Gygi, Anna Niedzwiecka, Mathieu Miron, Stephen K. Burley, Roberto D. Polakiewicz, Aleksandra Wyslouch-Cieszynska, Ruedi Aebersold and Nahum Sonenberg. A-CG and BR contributed equally to the manuscript. We thank Wojciech Augustyniak, Mariusz Tasior, Colin Lister and Fabienne Vozy for excellent assistance, and Dr. Pawel Mak, Institute of Molecular Biology, Jagiellonian University, Krakow, Poland, for amino acid analysis of the peptides. We thank Drs. Roger Duncan, John Silvius, Joseph Marcotrigiano and members of the Sonenberg laboratory for useful discussions. We are grateful to Francis Poulin and Drs. Jie Chen and Thomas Neufeld and for critical reading of the manuscript. This work was supported by grants from the National Cancer Institute of Canada and the Howard Hughes Medical Institute to NS, from the Human Science Frontiers Program to NS and SKB, from the National Institutes of Health (HG00041) to SPG and from the NSF Science and Technology Center for Molecular Biotechnology to RA. Work conducted at Warsaw University was supported by the Polish Committee for Scientific Research (KBN) 6 P04A 055 17. A-CG is supported by a Doctoral Award from the Canadian Institutes of Health Research (CIHR), BR by a postdoctoral fellowship from the CIHR, and MM by a Cancer Research Society studentship. NS is a CIHR Distinguished Investigator and a Howard Hughes Medical Institute International Scholar.

CHAPTER 6

GENERAL DISCUSSION

The work presented in this thesis described that the phosphorylation of human 4E-BP1 is regulated by the kinase activities of PI3K, Akt/PKB and FRAP/mTOR. Furthermore, I demonstrated that phosphorylation of 4E-BP1 is an ordered mechanism. Phosphorylation first occurs on two threonine residues (Thr37 and Thr46), followed by phosphorylation of Thr70 and, finally, of Ser65. Thr37 and Thr46 are phosphorylated by FRAP/mTOR immunoprecipitates, yet *in vivo* these two sites are relatively resistant to rapamycin treatment. Phosphorylation of Ser65 and Thr70 is extremely sensitive to rapamycin. In this discussion, I analyze in more depth the mechanism of activation of FRAP/mTOR, and I provide clues to explain this apparent discrepancy. I will also discuss the importance of phosphatases in the regulation of 4E-BP1 phosphorylation, and the relevance of the phosphorylation on the different sites in mediating release from eIF4E. I will conclude by a more general discussion about the pleiotropic roles of FRAP/mTOR in nutrient signaling, and about the roles (putative or proven) of FRAP/mTOR, PI3 kinases, and translation factors in cell growth, development, and tumorigenicity.

6.1 Thr37/Thr46 phosphorylation and FRAP/mTOR kinase activity

6.1.1 Why is phosphorylation of Thr37 and Thr46 insensitive to rapamycin?

As described in chapter 4, FRAP/mTOR, despite its homology to PI3Ks, functions as a protein kinase. Endogenous FRAP/mTOR, or FRAP/mTOR expressed in insect cells, autophosphorylates on Ser2481 (338, 380). FRAP/mTOR immunoprecipitates can phosphorylate several other protein targets *in vitro*, including S6K1, 4E-BP1 and 4E-BP2 (reviewed in 106, 381). Protein kinase activity is required for FRAP/mTOR function. Mutations at positions corresponding to those that abolish the lipid kinase activity of PI3Ks prevent FRAP/mTOR autophosphorylation, and abrogate its ability to signal to S6K1 and 4E-BP1 (e.g. 338, 339).

While rapamycin complexed with FKBP12 potently inhibits downstream signaling by FRAP/mTOR *in vivo*, whether this complex inhibits the kinase activity of FRAP/mTOR

remains a controversial issue. Rapamycin in combination with FKBP12 does inhibit FRAP/mTOR autokinase activity *in vitro*, but a much higher concentration of rapamycin than is required *in vivo* is necessary to observe this effect (380; and references therein). Addition of rapamycin to cells in culture fails to inhibit FRAP/mTOR autophosphorylation *in vivo* at Ser2481, as determined by the use of a phosphospecific antibody directed against this site (380), and little or no difference in the kinase activity of FRAP/mTOR immunoprecipitates is detected after rapamycin treatment (380; and references therein). Finally, whereas a mutation in the kinase domain of the yeast Tor2p is lethal, rapamycin treatment leads only to G1 arrest. If rapamycin were to inhibit Tor2p kinase activity, rapamycin treatment and mutation of the Tor2p kinase domain would be expected to evoke equivalent phenotypes. Taken together, these data argue against a direct inhibition of the kinase activity of Tor and FRAP/mTOR by rapamycin. Therefore, if FRAP/mTOR is the *in vivo* kinase phosphorylating Thr37 and Thr46, it is not surprising that the phosphorylation at these sites is relatively resistant to rapamycin treatment (chapters 3-5).

How can this apparent lack of effect of rapamycin on FRAP/mTOR kinase activity be reconciled with the effects of rapamycin ? FRAP/mTOR is a large molecular weight protein (289 kDa) which possesses several ill-defined domains. The entire N-terminal portion of the protein consists of HEAT repeats, a motif involved in protein-protein interactions (see section 1.8.6.5, and 382). It is thus believed that the Tor proteins serve as signaling scaffolds. The effects of rapamycin on FRAP/mTOR signaling could therefore be effected through modulation of the scaffolding activities of FRAP/mTOR, rather than of its protein kinase activity *per se*. This is addressed in more detail below.

6.1.2 Is FRAP/mTOR downstream of PI3K in a linear signaling pathway ?

As discussed throughout this thesis, both TOR and PI3K signaling are required for the inactivation of 4E-BP1. We ordered the events (from the membrane to 4E-BP1 phosphorylation). The PI3K signal is upstream of the Akt/PKB signal, which occurs upstream of the FRAP/mTOR signal. However, whether TOR activity is directly regulated by PI3K and Akt/PKB in a linear pathway is unknown. Initial doubts about a linear pathway were cast following study of S6K1 phosphorylation. S6K1 activity is inhibited by wortmannin and LY294002, but also with rapamycin, indicating that both PI3K and FRAP/mTOR signaling are required for its activation (reviewed in 383). Intriguingly, the PI3K and FRAP/mTOR inputs to S6K1 can be separated. Deletion of an N-terminal fragment confers rapamycin resistance, yet this truncation mutant remains sensitive to wortmannin treatment. Deletion of both the N- and C-termini of S6K1 confers resistance to rapamycin and wortmannin (186, 384). These data argue against a linear pathway comprised of PI3K and FRAP/mTOR, but instead suggest that two separate inputs are required for full activation of S6K1, such that a disruption in either of the two signaling pathways can abrogate S6K1 activity. The S6K1 studies thus suggested that FRAP/mTOR does not lie directly downstream of PI3K.

The roles of growth factors and various components of the PI3K pathway in the regulation of FRAP/mTOR activity were next investigated directly. Following growth factor treatment, or in response to expression of a constitutively active Akt/PKB molecule, FRAP/mTOR immunoprecipitates display only a modest increase (or no change at all) in kinase activity *in vitro* (354, 362, 385). In fact, FRAP/mTOR kinase activity in immunoprecipitates is constitutively active, even when isolated from cells deprived of growth factors. This is in contrast to the dramatic increase in S6K1 activity (or 4E-BP1 phosphorylation at Ser65 and Thr70) observed *in vivo* following stimulation of the same cells by the same growth factors (106, 381). It could be argued that the

FRAP/mTOR immunoprecipitation and *in vitro* kinase assay do not accurately reflect the *in vivo* situation (due, for example, to the loss of an inhibitor or to an artificial activation of the kinase during immunoprecipitation). However, overexpression of a membrane-targeted Akt/PKB protein only moderately increases mTOR autophosphorylation *in vivo*, as assessed with the S2481 phosphospecific antibody (354, 380, 385).

The existence of a direct link between Akt/PKB and FRAP/mTOR was also investigated. Phosphospecific antibodies directed against putative Akt/PKB phosphorylation sites in FRAP/mTOR were utilized to monitor the influence of the PI3K pathway on FRAP/mTOR phosphorylation. One such site, Ser2448, is phosphorylated *in vivo*. Addition of insulin or interleukin-3 increased Ser2448 phosphorylation in a PI3K- and Akt/PKB-dependent manner (385, 386). However, the relevance of this phosphorylation to FRAP/mTOR activity is not clear, because a FRAP/mTOR mutant protein possessing an alanine substitution at this site is able to fully activate S6K1 following growth factor stimulation (385).

Overall, these data seem to indicate that FRAP/mTOR does not lie downstream of PI3K in a conventional linear signaling pathway. This, again, agrees well with our data concerning the phosphorylation of 4E-BP1 Thr37 and Thr46. Both sites are only mildly affected following serum-deprivation, LY294002 or wortmannin treatment (which decreases PI3K and Akt/PKB activities), and are only modestly increased following serum or insulin addition, or expression of MyrAkt (chapters 3-5).

Taken together, these data suggest that FRAP/mTOR activity is not directly regulated by PI3K signaling. PI3K and FRAP/mTOR are both clearly required, however, for activation of S6K1, 4E-BPs and other downstream targets. How these signals may be integrated remains unknown, but a role for FRAP/mTOR (and Tor) in nutrient sensing is

gaining widespread acceptance. This model suggests that Tor/FRAP/mTOR relays a signal to downstream targets only in the presence of sufficient nutrients, and thus serves as a nutrient-dependent "gatekeeper".

6.1.3 FRAP/mTOR signals to translation in response to nutrient availability

Inactivation of the TOR proteins, or rapamycin treatment, mimics nutrient deprivation in yeast, *Drosophila* and mammalian cells (246, 249, 387, 388). Thus, a current working model for TOR signaling proposes that these kinases relay a permissive signal to downstream targets only in the presence of sufficient nutrients to fuel protein synthesis. In some cases, the TOR proteins appear to function in a co-regulatory capacity with other conventional, linear signaling pathways (such as the PI3K pathway). In this way, a passive nutrient sufficiency signal may be combined with stimulatory signaling from a second pathway to coordinate cellular processes that require the uptake of nutrients. The absence of either signal is predicted to prohibit activation of downstream targets.

In mammals, amino acid levels modulate the activity of several proteins involved in translation, including the 4E-BPs and S6K1 (reviewed in 125). Amino acid deprivation leads to a reduction in 4E-BP1 and S6K1 phosphorylation (e.g. 154). Amino acid re-addition to amino acid-deprived cells partially restores 4E-BP1 phosphorylation, and synergizes with insulin, IGF I or serum to elicit complete phosphorylation (e.g. 144, 154, 389). Leucine is critical for 4E-BP1 and S6K1 phosphorylation, and leucine alone (and, to a lesser extent, other branched-chain amino acids) can partially stimulate the phosphorylation of 4E-BP1 and S6K1 in amino acid-deprived cells. The specificity of leucine suggests that a leucine receptor may play an important role in amino acid sensing (reviewed in 125).

A role for FRAP/mTOR in amino acid signaling was suggested by the observations that a rapamycin-resistant FRAP/mTOR protein confers resistance upon S6K1 to amino acid deprivation (390), and a rapamycin-resistant S6K1 protein is insensitive to amino acid withdrawal (154). Amino acid deprivation does not affect PI3K or Akt/PKB activity, nor does it inhibit the activation of these proteins by serum or insulin treatment (154, 391-394). Therefore, amino acid modulation of S6K1 and 4E-BP1 phosphorylation does not involve PI3K or Akt/PKB. Amino acid-induced 4E-BP1 and S6K1 phosphorylation is sensitive to wortmannin treatment (389, 393, 395), but at higher concentrations than those required to inhibit phosphorylation of 4E-BP1 and S6K1 after insulin stimulation (394). This is consistent with the concentration of wortmannin required for FRAP/mTOR inhibition (230, 231; see above). Thus, several lines of evidence suggest that FRAP/mTOR may play an important nutrient checkpoint role, allowing propagation of intracellular signals to the translational apparatus only when sufficient amino acids are present. (Fig. 3)

The connection between Tor and nutrient signaling in yeast has been well documented. Treatment of *S. cerevisiae* with rapamycin mimics the effects of starvation, and elicits activation or repression of the same subset of genes modulated following amino acid deprivation (396-398, 399; section 6.5).

6.1.4 Is FRAP/mTOR a mediator of "Translational Homeostasis"?

Treatment of cells with translational inhibitors such as anisomycin or cycloheximide leads to hyperphosphorylation of both 4E-BP1 and S6K1 (e.g. 128, 400). By contrast, in a murine fibroblast cell line transformed by eIF4E overexpression (401), in which general translation rates are increased (402), both 4E-BP1 and S6K1 are maintained in a hypophosphorylated state (403). Inducible overexpression of eIF4E also leads to 4E-BP1 and S6K1 dephosphorylation, indicating that this effect is due to eIF4E (or increased

translation) itself, and is not, in this case, a secondary effect of the transformation process (403). Interestingly, 4E-BP1 is also hypophosphorylated in several murine mammary tumor cell lines, in contrast to non-tumorigenic parental cell strains (404). These data are consistent with a model in which the signaling pathways modulating 4E-BP1 and S6K1 activity respond to an unscheduled change in translation initiation. It is also conceivable that transformed cells may acquire the ability to bypass this translational inhibition mechanism.

6.2 Phosphatase activity and 4E-BP1 phosphorylation

6.2.1 Rapamycin-sensitivity of Ser65 and Thr70 phosphorylation

The phosphorylation of the two most rapamycin-sensitive sites, Ser65 and Thr70, is under the control of PI3K and Akt/PKB activity (chapter 3), and is dramatically increased by the presence of serum or insulin (chapters 3-5). FRAP/mTOR, albeit unable to directly phosphorylate these sites, is critical to maintain them in a hyperphosphorylated state following growth factor or hormone stimulation. Indeed, the inhibitory effects of rapamycin on 4E-BP1 phosphorylation at Ser65 and Thr70 can be prevented by co-expression of a rapamycin-resistant FRAP/mTOR (A-C.G and B. Raught, unpublished). It may be that FRAP/mTOR activity is required for the activation of a kinase responsible for the phosphorylation of these sites. Alternatively, it is possible that FRAP/mTOR activity controls a phosphatase activity, which would be responsible for dephosphorylating these sites in the presence of rapamycin. The latter hypothesis is attractive, and agrees well with our observations concerning the phosphorylation of Ser65 and Thr70. We and others have observed a phosphatase activity directed against Ser65, which co-precipitates with FRAP/mTOR. This phosphatase activity is inhibitable by calyculin A, okadaic acid, or microcystin (375; RT Abraham, pers. comm.; A.-C.G. and B. Raught, unpublished). Furthermore, in HEK 293 cells, the rapamycin sensitivity of phosphorylation at Ser65 and Thr70 is drastically reduced by pre-treatment of cells with

calyculin A, tautomycin or okadaic acid (B. Raught, A.-C.G. and N.S., in prep). This observation prompted us to analyze the relative turnover rates of phosphate on individual 4E-BP1 sites, in the presence or absence of rapamycin. This was performed by [^{32}P] labeling of serum-stimulated HEK 293 cells for several hours (steady state), followed by a chase (for several time points) with cold phosphate, in the presence or absence of rapamycin. 4E-BP1 was immunoprecipitated at each of the time points and phosphopeptide maps were performed, as described in chapters 3-5. This experiment allows us to specifically study the rate of removal of the phosphate groups (and hence phosphatase activity) without having to take into account the re-addition of the phosphate (i.e. kinase activity). While the phosphorylation of Thr37 and Thr46 is unchanged by the presence of rapamycin (at times up to 60 minutes), the phosphorylation at Ser65 and Thr70 is dramatically reduced (B. Raught, A.-C.G. and N.S., in prep). At t = 30 minutes, no phosphopeptides containing Ser65 or Thr70 could be detected in the rapamycin-treated samples, while in the absence of rapamycin, phosphorylation on these sites is almost the same as t=0 (B. Raught, A.-C.G. and N.S., in prep). Taken together, these data indicate that, upon treatment of cells with rapamycin, a phosphatase activity directed against 4E-BP1 Ser65 and Thr70 becomes activated. Because of the pattern of inhibition of this phosphatase activity (low concentrations of okadaic acid, calyculin A or microcystin), the phosphatase involved is most likely PP2A or a PP2A-type phosphatase (e.g. PP4 or PP6; see below). Thus, in the absence of rapamycin, FRAP/mTOR appears to repress a phosphatase directed against Ser65 and Thr70. The role of FRAP/mTOR in regulating the phosphorylation of Thr37, Thr46, Ser65 and Thr70 is depicted in Fig. 6.1.

6.2.2 How does FRAP/mTOR regulate phosphatase activity ?

PP2A and PP2A-like phosphatases have been linked to the Tor pathway in yeast for several years. Genetic screening in *S. cerevisiae* has identified the PP2A-like phosphatase Sit4p, two PP2A regulatory subunits (*CDC55* and *TPD3*), and a

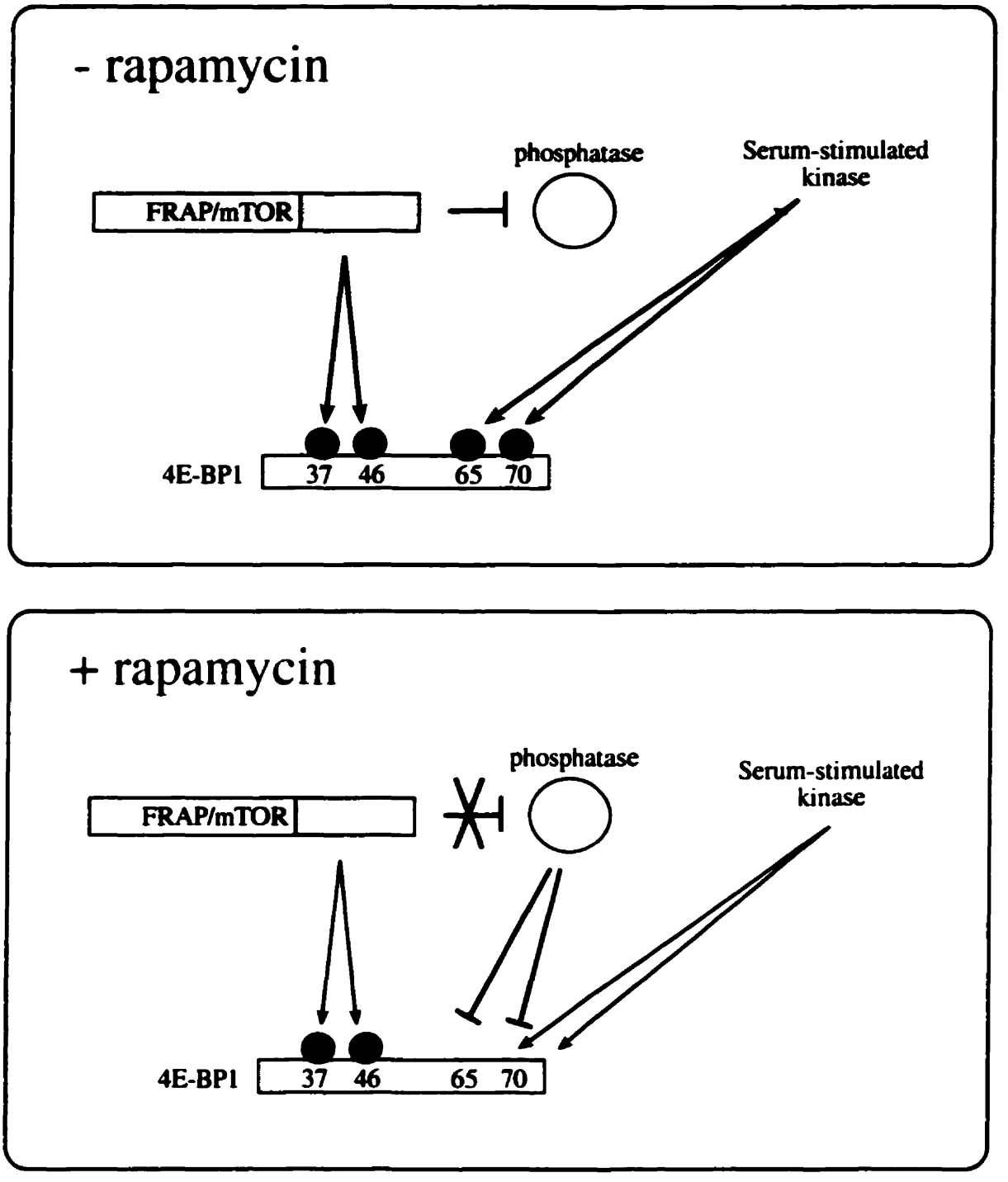


Fig. 6.1. FRAP/mTOR differentially regulates the phosphorylation of 4E-BP1 at Thr37/Thr46 and Ser65/Thr70. A protein kinase activity present in the FRAP/mTOR immunoprecipitates phosphorylates 4E-BP1 at Thr37 and Thr46. This kinase activity is only mildly affected by rapamycin treatment. FRAP/mTOR also regulates the phosphorylation of Ser65 and Thr70 through repression of a phosphatase directed against these sites. In the presence of rapamycin, the phosphatase is de-repressed and dephosphorylates Ser65 and Thr70.

phosphatase-associated protein (Tap42p), as part of a rapamycin-sensitive signaling pathway (246, 405). Tap42p interacts directly with the catalytic subunits of PP2A and Sit4p. *S. cerevisiae* expressing a temperature-sensitive Tap42 mutant protein exhibit a dramatic defect in translation initiation at the non-permissive temperature (405). Thus, Tap42p is thought to repress PP2A (or Sit4p) activity (also see 398, 406).

Phosphorylation of Tap42p regulates its interaction with phosphatases. While phosphorylated Tap42p competes with the phosphatase adapter (A) subunit for binding to the catalytic subunit, dephosphorylated Tap42p does not efficiently compete for binding (407). Tap42p phosphorylation is modulated by Tor signaling. The Tap42p-PP2A association *in vivo* is disrupted by nutrient deprivation or rapamycin treatment (405, 407). Further, a yeast Tor2p immunoprecipitate can phosphorylate Tap42p *in vitro* (407), and Tap42p phosphorylation is rendered rapamycin resistant in yeast strains expressing a rapamycin-resistant Tor1 protein (407).

Tap42 orthologs are found in *Arabidopsis* (408), *Drosophila*, (GenBank accession number AAF53289), and mammalian cells (409, 410). The B cell receptor binding protein α4 (a.k.a Ig binding protein 1, IGBP1) is the mammalian ortholog of Tap42p (409, 410). An interaction between α4 and phosphatases is also conserved in mammals; α4 binds directly to the catalytic subunits of PP2A (411, 412), PP4 and PP6 (363, 413). Like Tap42p, α4 is also a phosphoprotein, and the α4-PP2A interaction was reported to be abrogated by rapamycin treatment (although this finding remains somewhat controversial; 411, 412). These observations suggest that Tap42p/α4 phosphorylation, and PP2A binding, are regulated by TOR signaling, and that an inhibition in TOR signaling leads to Tap42p/α4 dephosphorylation, dissociation of the Tap42p/α4-phosphatase complex, and phosphatase de-repression (Fig. 6.2). Although this model is very attractive, it must be noted that the mechanism by which α4 or Tap42p modulate the

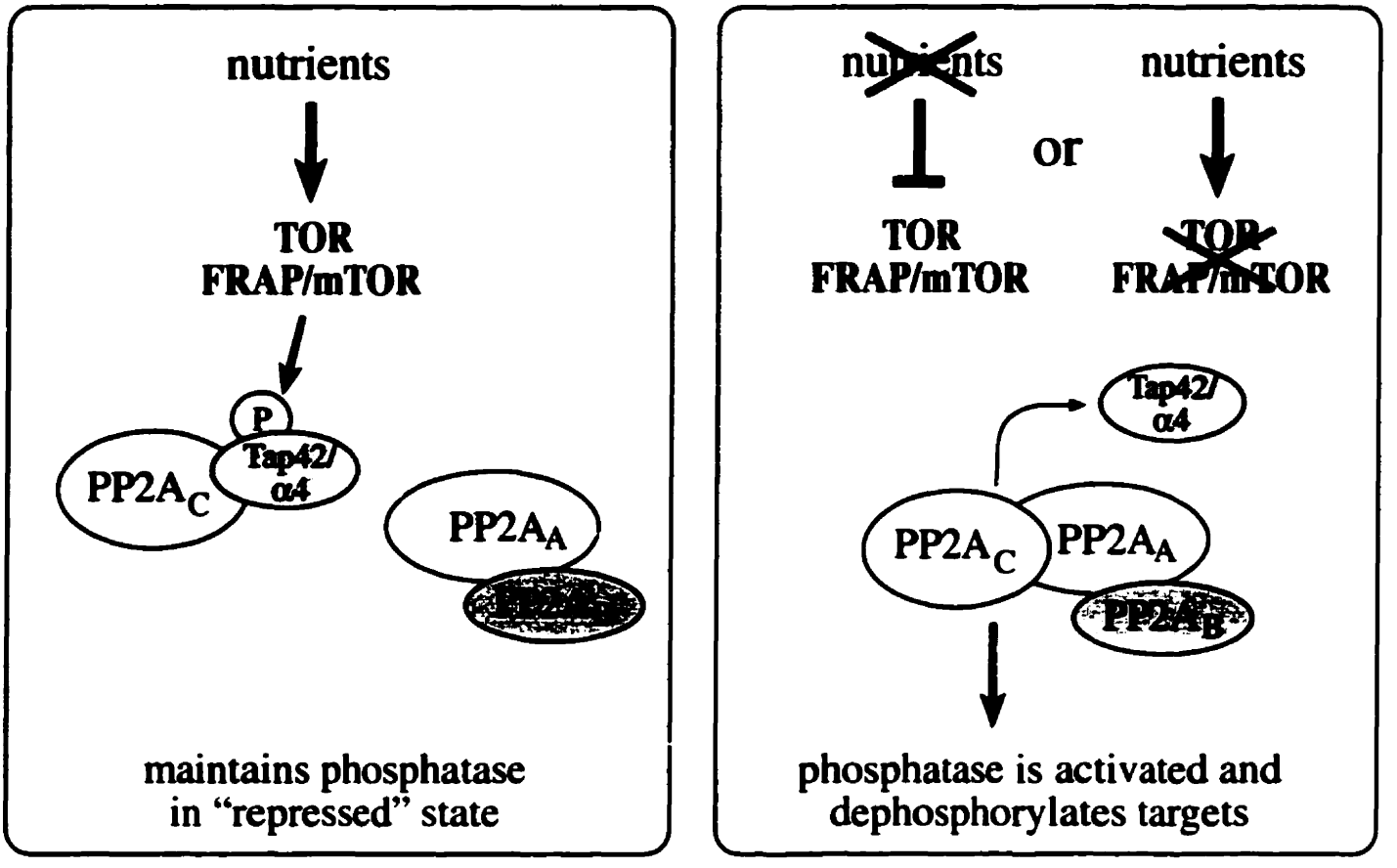


Figure 6.2. A putative mechanism for Tor (FRAP/mTOR) signaling in response to nutrient levels. In conditions of nutrient availability, Tor (or FRAP/mTOR) maintains Tap42p (or α4) in a phosphorylated state. Phosphorylation activates a direct interaction with the catalytic (C) subunit of PP2A. Interaction of Tap42p (or α4) with PP2A$_C$ prevents association with the adaptor (A) and regulatory (B) subunits, and thus inhibits dephosphorylation of downstream PP2A targets. In the absence of nutrients, or in cells treated with rapamycin, FRAP/mTOR does not signal to Tap42p (or α4), resulting in dephosphorylation of Tap42p (or α4), and loss of PP2A binding activity. PP2A is then free to associate with the A and B subunits, and to dephosphorylate downstream targets. Since loss of TOR or dTOR mimics nutrient starvation, Tor (FRAP/mTOR) likely signals constitutively in the presence of nutrients, whereas in the absence of nutrients it is inactive (resulting in no signal). For clarity, only the association of Tap42p (α4) with the phosphatase PP2A is indicated; it can also associate with other phosphatases (see text for details).

activity of their binding partners is not well understood; α4 or Tap42p binding has been reported to both increase and decrease phosphatase activity, or to alter substrate specificity (364, 412). Interestingly, it has been shown *in vitro* that incubation of PP2A and α4 resulted in a phosphatase unable to dephosphorylate a 4E-BP1 substrate (364). It remains to be tested whether α4 signals to 4E-BP1 *in vivo*.

6.2.3 Dephosphorylation of Thr37 and Thr46 under stress conditions.

Thr37 and Thr46 are found in their phosphorylated state even in the presence of rapamycin or PI3K inhibitors, or in the absence of serum (with the exception of the treatment of serum-starved cells with rapamycin; chapter 4). However, we now know that certain stress conditions can elicit dephosphorylation of these two sites. For example, EMCV infection induces a complete dephosphorylation of 4E-BP1, including on Thr37 and Thr46 (A.-C.G. and Y. Svitkin, unpublished). In addition, osmotic shock (treatment with high concentrations of sorbitol), heat shock or UV irradiation result in a rapid dephosphorylation of all of the 4E-BP1 sites, including Thr37 and Thr46 (B. Raught and A.-C.G., unpublished). On a two-dimensional IEF/SDS-PAGE, this correlates with the collapse of all 4E-BP1 isoforms into a single spot (B. Raught and A.-C.G., unpublished). This unphosphorylated 4E-BP1 isoform binds more tightly to eIF4E as compared to an intermediately phosphorylated isoform (see, for example, chapter 2), indicating that these stresses lead to a "super"-activation of 4E-BP1. A phosphatase appears to be involved in this effect, as dephosphorylation of 4E-BP1 induced following sorbitol treatment can be prevented by pre-incubation with okadaic acid or calyculin A (B. Raught and A.-C.G., unpublished). In this case, overexpression of a rapamycin-resistant FRAP/mTOR is insufficient to protect 4E-BP1 from dephosphorylation. Stress leads to the activation of several signaling pathways, two of which are the JNK and the p38MAPK pathway. The JNK and the p38 MAPK pathways are involved in the regulation of the activity of several downstream target proteins, including eIF4E. The

use of a chemical inhibitor of p38MAPK (SB203580), and constitutively active or dominant-negative components of these pathways, indicated a lack of correlation between the dephosphorylation of 4E-BP1 and the activation of these pathways (B. Raught and A.-C.G., unpublished). Thus, the mechanism leading to dephosphorylation (and super-activation) of 4E-BP1 after stress remains unknown. The effects of dephosphorylation of Thr37 and Thr46 in binding to eIF4E are depicted in Fig. 6.3.

6.2.4 Identification of the phosphatase activities involved in dephosphorylation of 4E-BP1

As mentioned in section 6.2.1, we found that a phosphatase activity inhibitable by microcystin, calyculin A or okadaic acid is present in the FRAP/mTOR immunoprecipitates. It would be interesting to identify the phosphatase activity present in the FRAP/mTOR immunoprecipitates, as it is a very good candidate for mediating 4E-BP1 dephosphorylation. Western blotting with antibodies to the PP2A catalytic subunit, or to the PP2A-type phosphatases (e.g. PP4 and PP6) could be performed on FRAP/mTOR immunoprecipitates isolated after treatment of cells with rapamycin or wortmannin, or following amino acid or serum deprivation. If none of the known phosphatases is found in the FRAP/mTOR immunoprecipitates, a biochemica purification of the phosphatase activity from FRAP/mTOR immunoprecipitates could be attempted (each fraction could be tested for its ability to remove [^{32}P] from an *in vitro*-labeled 4E-BP1 substrate). A purification scheme could involve the use of a microcystin-Sepharose pull-down, since the phosphatase activity directed against 4E-BP1 is inhibited by microcystin (e.g. 414). Microcystin-bound proteins could be fractionated by SDS-PAGE and silver-stainable bands subjected to mass spectrometry.

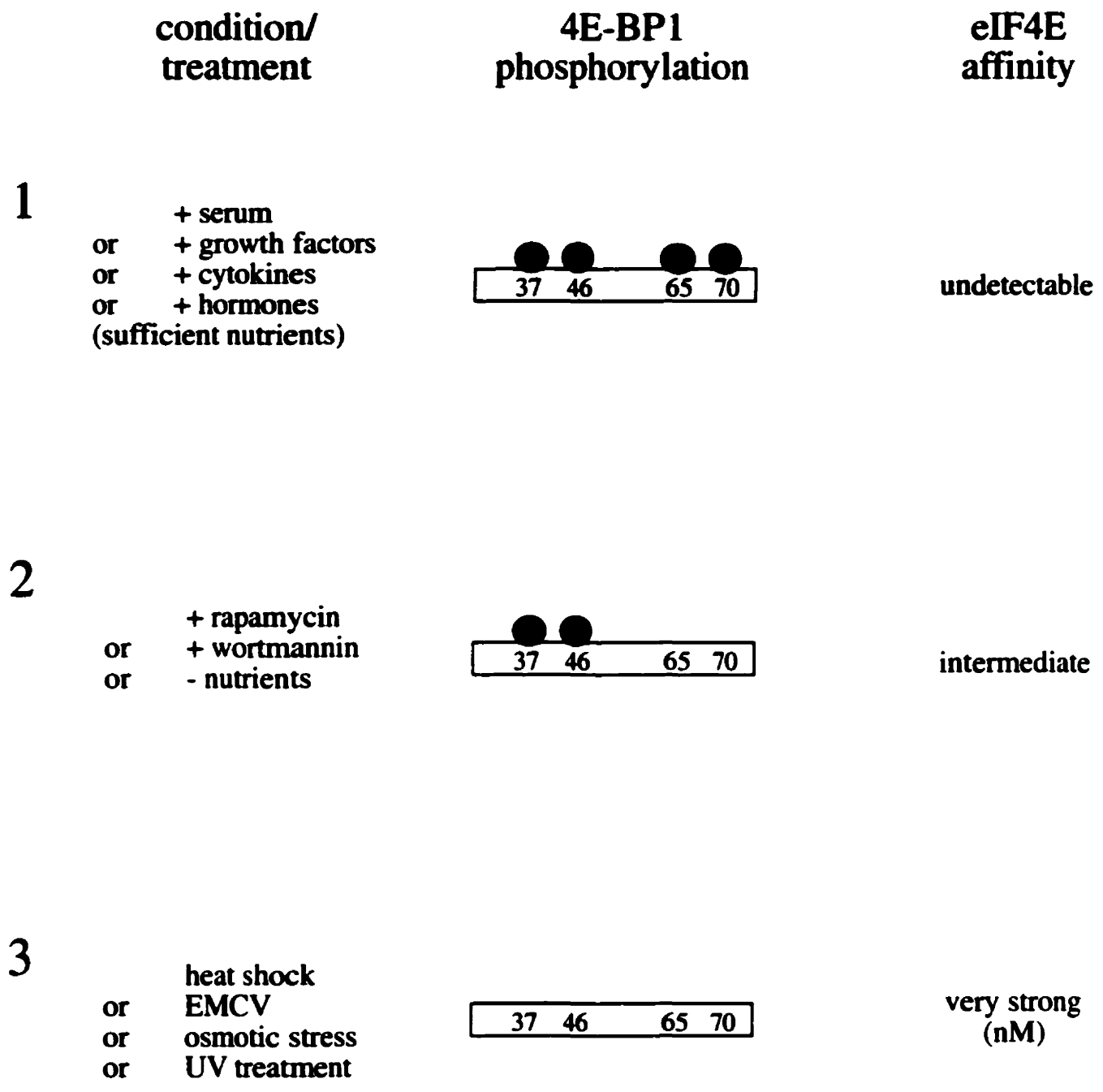


Figure 6.3. Two sets of phosphorylation sites on 4E-BP1 are differentially affected by serum starvation and stress conditions. Serum deprivation induces dephosphorylation of Ser65 and Thr70. This results in an increased affinity for eIF4E. Stress conditions further induce the dephosphorylation of Thr37 and Thr46, resulting in a super-activated 4E-BP1.

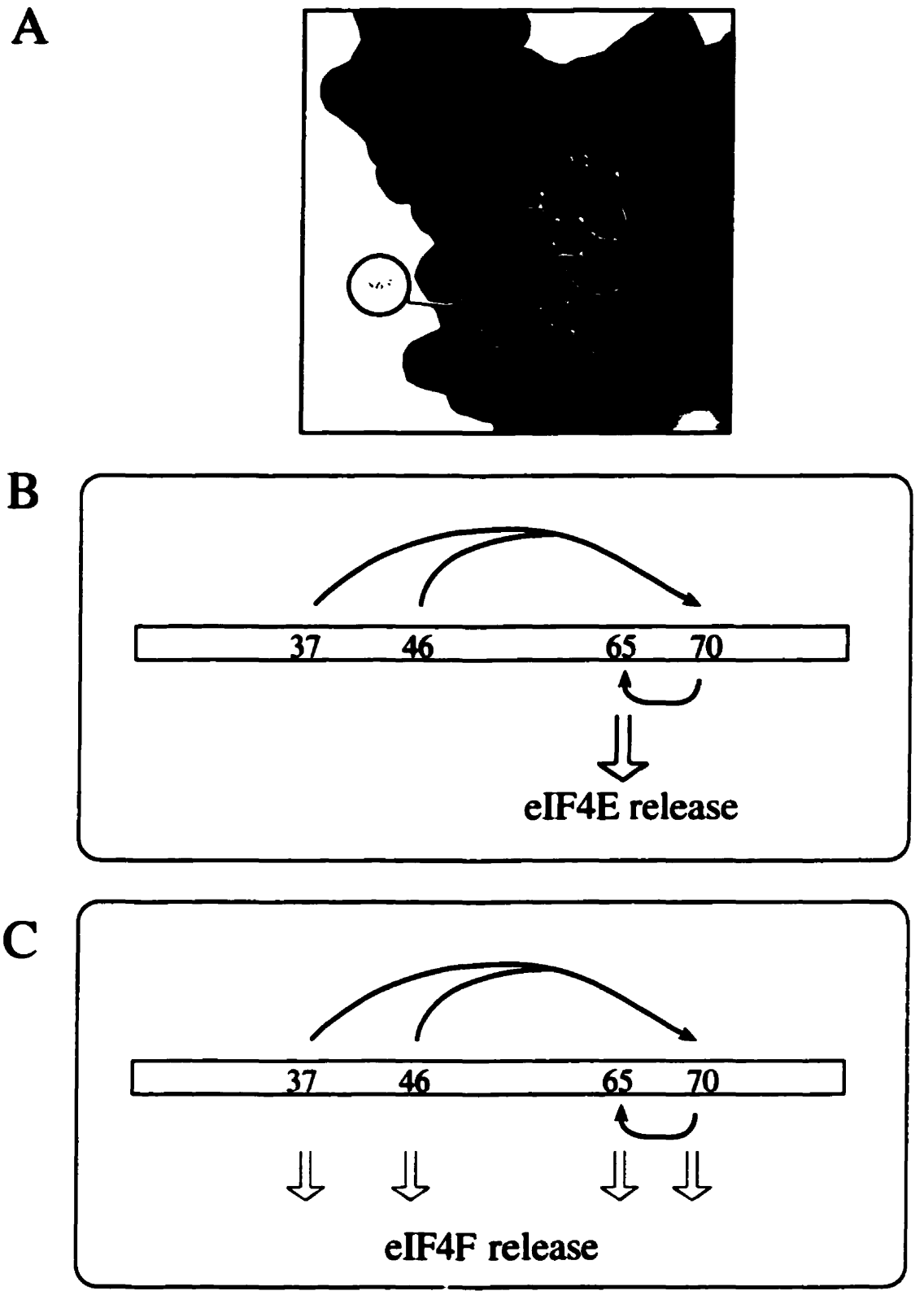


Figure 6.4. Phosphorylation of 4E-BP1 culminates at Ser65. A) Location of Ser65 on the co-crystal structure of eIF4E and 4E-BP1. B) "Priming only" model to explain to importance of phosphorylation at Thr37, Thr46 and Thr70. C) "Priming + binding" model for the function of phosphorylation at Thr37, Thr46 and Thr70.

5). Thus, it appears that a combination of phosphorylation events (likely involving phosphorylation of all the sites) is necessary to mediate release.

These studies have been hampered by the absence of a phosphorylated substrate to perform thermodynamic measurements. For example, in chapter 4 and in figure 6 of chapter 5, we could only perform qualitative measurements of the binding, measuring the association of 4E-BP1 and eIF4E by pull-down assays. While this method is sensitive enough to detect major changes in affinity, it probably would not be able to detect more subtle changes. In addition, all the studies performed to date (except for our figure 7, chapter 5) were performed using an *in vitro* phosphorylated recombinant protein substrate. In most cases, the stoichiometry of phosphorylation at the various sites is unknown. This is especially troublesome in studies, such as that of Karim et al (222), which attempt to quantify (using surface plasmon resonance) the effect of phosphorylation at individual 4E-BP1 sites, using *in vitro* phosphorylated mutant proteins.

What is needed is a method that will generate a homogeneous population of proteins, phosphorylated to a >90% stoichiometry, and only on the desired sites. This phosphorylated substrate should then be assessed quantitatively for binding to eIF4E. In a first attempt to perform such an experiment, we have employed phosphopeptides containing the eIF4E-binding sites, and adjacent sequences C-terminal to this motif (including Ser65 and Thr70). The affinity of these phosphopeptides for eIF4E was measured, and it was found that phosphorylation at Ser65 (and in tandem at Ser65 and Thr70) decreased the affinity for eIF4E, but only by a factor 2 (chapter 5, figure 7). We are in the process of producing, through a protein-ligation approach, longer phosphopeptides (phosphorylated in various combinations) for affinity measurements.

6.3.3 Why is there priming?

Another unresolved issue is how phosphorylation at Thr37 and Thr46 primes the phosphorylation on the other sites, and how the phosphorylation at Thr70 facilitates phosphorylation of Ser65. Very few examples of "priming" phosphorylation have been reported to date for *in vivo* substrates. Most of them concern kinase substrates that possess several serine or threonine residues which are targets for phosphorylation by the same kinase, and separated by only a few amino acids. Glycogen synthase kinase-3 (GSK-3), for example, recognizes serines or threonines lying in the consensus SXXX^{P}S, where ^{P}S is a phosphoserine (355). Thus, after the first phosphorylation event (itself primed by another kinase, or through recognition of an acidic residue), GSK-3 can sequentially phosphorylate several serine or threonine residues in the substrate, as is the case for glycogen synthase. GSK-3 is not unique in this respect: other kinases, such as casein kinase I, also exhibit a preference for phosphorylated amino acids near their target site (356). This type of priming phosphorylation, which can be reproduced *in vitro*, is the best understood of all priming mechanisms. It is unknown at present whether such a mechanism could be responsible for the priming of Ser65 phosphorylation by phosphorylation at Thr70 (SPVTK^{P}T). It is however unlikely that this mechanism could explain the priming of Thr70 by Thr37 and Thr46, since these residues are probably outside of the kinase recognition site.

Other priming mechanisms could include inducing a conformational change in the substrate to render a site more easily accessible to a kinase. While 4E-BP1 is unstructured in solution, it is possible that phosphorylation at Thr37 and Thr46 could alter the conformation of the 4E-BP1/eIF4E complex. Alternatively, the first phosphorylation event could generate a docking site for a protein with affinity for phosphorylated residues. If the bound protein is an adaptor, such as 14-3-3 proteins, this could potentially recruit another kinase to the substrate, to phosphorylate the other sites

(357, 358). It is also possible that a yet unidentified kinase exhibits an affinity for the phosphorylated residues. Another possibility is that phosphorylation would generate a recognition site for an enzyme, such as Pin1, which specifically binds to Ser/Thr-Pro sites, and isomerizes the proline, resulting in the alteration of their conformation, and or their activity (reviewed in 382, 415). The isomerization would probably be expected in this case to alter the conformation of the 4E-BP1/eIF4E complex. It is unknown which of these possibilities (or any other) applies to the priming of 4E-BP1 phosphorylation. While conformational changes are very difficult to assess without an enzymatic activity readout, or large amounts of the proteins phosphorylated on the appropriate sites (see section 6.3.2), it may be possible to identify proteins that specifically bind to 4E-BP1 (or rather to the 4E-BP1/eIF4E complex) when 4E-BP1 is doubly phosphorylated on Thr37 and Thr46. Such a condition is attainable, for example, in serum starved cells (see for example, chapter 5, figure 4).

6.4 Kinases directly phosphorylating 4E-BP1

6.4.1. Kinase(s) phosphorylating Thr37 and Thr46

It remains unclear whether FRAP/mTOR itself is responsible for phosphorylation of 4E-BP1 at Thr37 and Thr46, or whether a kinase that is tightly associated to FRAP/mTOR may mediate 4E-BP1 phosphorylation. In all experiments performed thus far, FRAP/mTOR was isolated through immunoprecipitation from mammalian or insect cells, and might contain contaminants. In fact, upon washing of a FRAP/mTOR immunoprecipitate with SDS, 4E-BP1 kinase activity is lost (A.-C.G., unpublished). Thus, it could be that FRAP/mTOR is inactivated by SDS (this has been reported to be the case for other enzymes) or the kinase responsible for 4E-BP1 phosphorylation is not FRAP/mTOR, but an associated kinase which is lost in the wash. Since FRAP/mTOR immunoprecipitates contain several additional proteins, as detected by silver staining (A.-C.G., unpublished), the presence of contaminating kinase activity has not been ruled out.

This could be accomplished through washing the immunoprecipitates with increasing salt concentrations (100-1000 mM KCl; the 4E-BP1 kinase activity is still associated with the beads in 500 mM KCl), or with different non-ionic and ionic detergents. Wash fractions could be assessed for the presence of 4E-BP1-kinase activity (with the phosphospecific antibodies to Thr37 and Thr46) and for the presence of FRAP/mTOR. Positive fractions could be further purified on ATP-sepharose, a substrate to which many kinases can bind, and purified proteins could be subjected to mass spectrometric sequence determination.

6.4.2. Kinase(s) phosphorylating Ser65 and Thr70

Although MAP kinases readily phosphorylate 4E-BP1 S/T-P sites (and especially Ser65) *in vitro*, their involvement in *in vivo* phosphorylation has been ruled out, and the physiological kinases for these sites remain unknown. One important problem that we have encountered in our attempts to identify the kinase(s) phosphorylating the 4E-BP1 S/T-P sites is that MAP kinases readily phosphorylate 4E-BP1 when free in solution (i.e. not bound to eIF4E). Because of their abundance and stability, MAP kinases are very likely to contaminate the kinase fractions. We suspect that this is the reason initial attempts to purify a Ser65 kinase identified ERK2 as the kinase (221). Because Thr37 and Thr46 phosphorylation prime the phosphorylation of Ser65 and Thr70, the ideal substrate for a 4E-BP1 S/T-P kinase is a complex of 4E-BP1/eIF4E phosphorylated at Thr37 and Thr46. The production of such a substrate has been problematic: the stoichiometry of phosphorylation with FRAP/mTOR *in vitro* is low and varies from experiment to experiment (A.-C.G., unpublished). I have also mutated these residues to glutamic acids, but these acidic residues act only as a poor mimic of the phosphorylation sites (chapter 4). We are currently attempting to generate, via phosphopeptide synthesis and protein ligation, a full length 4E-BP1 protein, doubly phosphorylated on Thr37 and Thr46 to serve as a substrate (in a complex with eIF4E) for the identification of kinases phosphorylating 4E-BP1 at Thr70 and Ser65. Because of the role of Thr70

phosphorylation in facilitating Ser65 phosphorylation, another substrate, pre-phosphorylated on Thr37, Thr46 and Thr70 may be required to identify a Ser65 kinase. Once a proper substrate is obtained, the kinase purification could be performed with standard chromatography methods, followed by affinity purification and mass spectrometry identification, as described above.

6.5 TOR as a master switch for catabolism versus anabolism

I have discussed in detail the role of FRAP/mTOR in mediating phosphorylation of the translational inhibitors 4E-BP1 and 4E-BP2. There are several additional translational targets for FRAP/mTOR: S6K1 and S6K2 (section 1.8.2), which phosphorylate the ribosomal S6 protein and the translation initiation factor eIF4B (section 1.7.4), eIF4GI (section 1.7.2), and a translation elongation factor, eEF2 (416; Fig. 6.5). Many other translation factors are also known to be phosphoproteins (5), but the pathways modulating the phosphorylation state of these factors have not been studied. Additional proteins involved in translation control may thus also be downstream of FRAP/mTOR.

It has been suggested that the primary function of the FRAP/mTOR (or Tor) pathway is the regulation of translation through modulation of translation factor activity (e.g. 400). However, TOR signaling has also been demonstrated to coordinate the activity of various anabolic and catabolic pathways in response to nutrient quality in yeast (Fig. 6.6). In particular, TOR signaling controls the abundance of the translation machinery, modulates the transcription of genes involved in amino acid biosynthesis, and regulates the activity of amino acid permeases. In both yeast and mammalian cells TOR signaling regulates autophagy. The involvement of the Tor (FRAP/mTOR) proteins in each of these process will be briefly reviewed below.

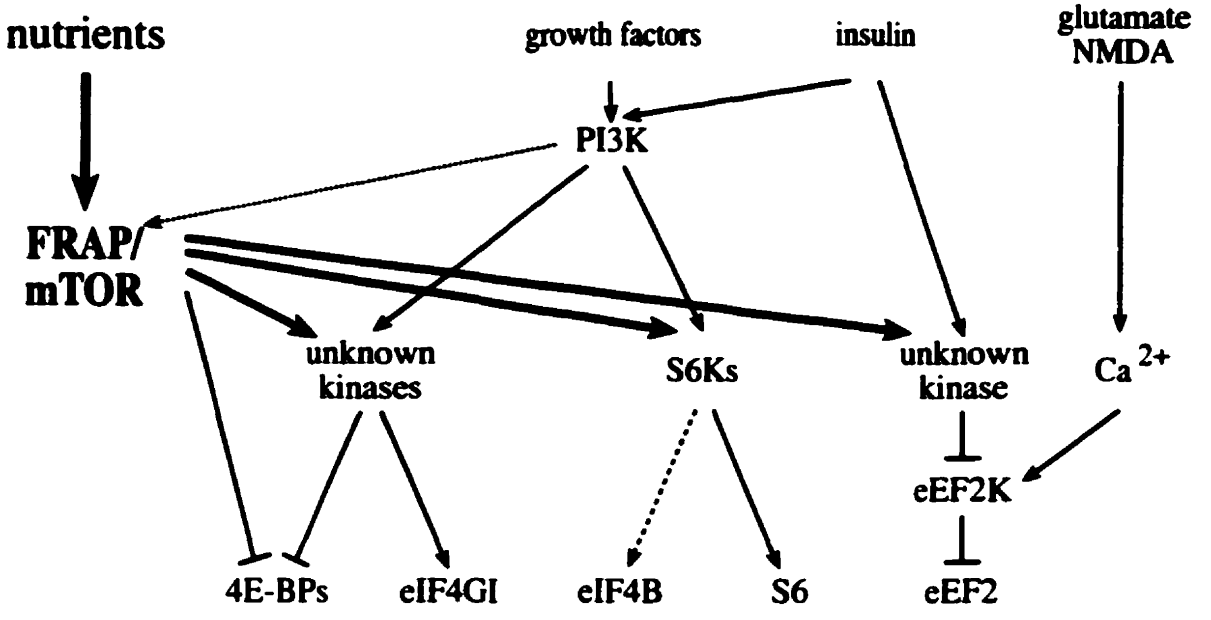


Figure 6.5. Signaling to eukaryotic translation initiation and elongation factors. FRAP/mTOR signaling, in combination with the PI3K pathway, activates the translation of rapamycin-sensitive mRNAs. In the presence of sufficient nutrients to fuel protein synthesis, mTOR and PI3K signaling activate the S6Ks, and one or more unknown kinases, to effect phosphorylation of the ribosomal S6 protein, eIF4B, eIF4GI and the 4E-BPs. In response to agents that raise intracellular Ca^{2+}, a specific Ca^{2+}/CaM-dependent kinase effects the phosphorylation of eEF2 to inhibit elongation. mTOR signaling has been reported to inhibit eEF2 phosphorylation (possibly via inhibition of the eEF2 kinase), and thus, to increase elongation rates. Phosphatases have been implicated in the dephosphorylation of several translation effectors, but are not depicted in this figure.

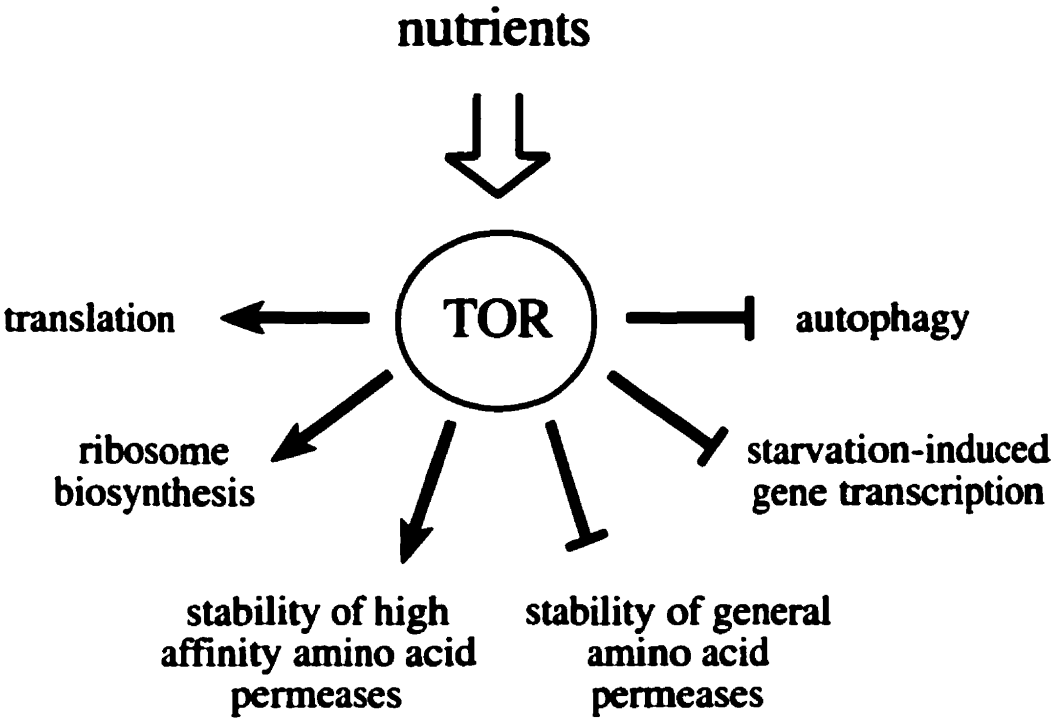


Figure 6.6 The Tor proteins regulate the balance between protein synthesis and protein degradation. TOR signaling is active in the presence of sufficient nutrients to fuel protein synthesis. The TOR signal allows for the translation of mRNAs coding for components of the translation machinery, ribosome biosynthesis, and the stabilization of high affinity amino acid permeases. At the same time, TOR signaling destabilizes general amino acid permeases, inhibits autophagy, and represses the transcription of a subset of genes required for amino acid biosynthesis.

6.5.1 TOR regulates the abundance of the translation machinery

In addition to its effect on the phosphorylation state of proteins involved in translational control, TOR signaling regulates the abundance of the components of the translation machinery (Fig. 6.6), at both the transcriptional and translational levels. The number of ribosomes in a given cell can vary dramatically, according to growth conditions (reviewed in 417). Actively growing cells require numerous ribosomes (e.g. logarithmically dividing yeast cells produce 2000 ribosomes/minute), and ribosome synthesis represents a major energy expenditure for the cell (417). In *S. cerevisiae*, ribosome biosynthesis requires the transcription of over 100 different genes, involving all 3 RNA polymerases (417). In response to nutrient availability, TOR signaling in *S. cerevisiae* regulates the transcription of rRNA by Pol I and Pol III (418, 419), and the transcription of ribosomal protein mRNAs by Pol II (396-398, 418). TOR signaling has also been implicated in the processing of the ribosomal 35S precursor rRNA (418). When nutrients are limiting, ribosome production is curtailed (or a cell may even begin to degrade ribosomes, in a scavenging process termed autophagy; see below). The abundance of several yeast translation factors was also demonstrated to be regulated by the TOR pathway (418). Transcriptional modulation in *S. cerevisiae* is responsible for a decrease in the mRNA levels of initiation and elongation factors following rapamycin treatment, although the extent of this transcriptional inhibition is less than that observed for the ribosomal proteins (418).

Through the S6Ks, mTOR signaling regulates the translation of ribosomal protein mRNAs in mammalian cells (420, 421). In *Drosophila* and mammalian cells, translation of the elongation factor mRNAs, and mRNAs coding for other proteins involved in translation, such as the poly(A) binding protein, is also regulated by the presence of the 5'TOP element (reviewed in 21). Thus, the TOR pathway simultaneously regulates the

abundance and activity of the translation machinery in both unicellular and multicellular organisms.

6.5.2 Nutrient-sensitive transcriptional regulation

Switching yeast cells to a poor carbon or nitrogen source induces a state of quiescence (G0). While the transcription of many genes is inhibited following a switch from a rich to a poor nitrogen or carbon source (or following rapamycin treatment), global mRNA profiling has revealed that the transcription of mRNAs coding for proteins involved in nutrient utilization, respiration, and protein degradation is actually augmented (396-399). Tor signaling modulates gene expression via cytoplasmic sequestration of several nutrient-responsive transcription factors. For example, the GATA transcription factor Gln3p is retained in the cytoplasm through an interaction with the Ure2 protein, while the zinc-finger containing transcription factors Msn2p and Msn4p are sequestered in the cytoplasm via an interaction with the 14-3-3 protein Bmh2p (reviewed in 246). Starvation abrogates Tor signaling, and results in a loss of cytoplasmic retention of Gln3p, Msn2p, and Msn4p, followed by nuclear translocation and transcription of various target genes (246, 398). Tor signals to several specific effectors (Tap42, Mks1p, Ure2p, Gln3p, and Gat1p) to elicit changes in the expression levels of enzymes involved in several different metabolic pathways (399, 422). How TOR signaling may affect the transcription rates of metabolic enzymes in multicellular organisms has not yet been elucidated.

6.5.3 Amino acid permeases

Permeases are necessary for nutrient uptake, and may be divided into two functional classes. One class is regulated in response to the available nitrogen source (e.g., the general amino acid permease Gap1p), and members of this class transport amino acids to be used as a nitrogen source. The second class mainly consists of high affinity

permeases, which specifically transport one or a small group of related amino acids to be used as building blocks for protein synthesis. In starved yeast cells, or in cells treated with rapamycin, ubiquitination and degradation of the high affinity tryptophan permease Tat2p is induced, leading to a decrease in tryptophan import (406, 423). This phenomenon is not unique to Tat2p, as a histidine permease (Hip1p) is also degraded upon nutrient deprivation or rapamycin treatment (423). In contrast, rapamycin treatment increases the abundance of the general permease Gap1p, indicating that TOR signaling inversely regulates the two classes of permeases (423). TOR regulation of permeases is mediated through the serine/threonine kinase Npr1p, whose phosphorylation is regulated by the Tor proteins and Tap42p, in a manner similar to the regulation of S6Ks and 4E-BPs in mammalian cells (406).

6.5.4 Autophagy

When nutrient levels are low, eukaryotic cells degrade cytoplasmic proteins and organelles to scavenge amino acids, in a process termed autophagy (424-426). Autophagy involves the sequestration of a portion of cytoplasm by a double (or multi-) layered membrane structure termed the autophagosome or autophagic vacuole. This structure fuses with lysosomal or endosomal membranes, resulting in the degradation of cytoplasmic components. The TOR proteins regulate autophagy. Rapamycin addition to yeast cultures or to mammalian cells in culture induces autophagy, even in a nutrient-rich medium (427, 428). Shifting a temperature-sensitive TOR2 yeast mutant to the non-permissive temperature also induces autophagy (427). In mammalian cells, autophagy is inhibited by amino acids and insulin. Activation of S6K is associated with inhibition of autophagy in rat hepatocytes, and the inhibition of autophagy by amino acids could be partially prevented by rapamycin treatment (394, 428).

In sum, the TOR proteins appear to act as master regulators of the balance between protein synthesis and degradation. In the presence of sufficient nutrients to fuel protein synthesis, TOR provides a permissive signal to translation, ribosome biosynthesis, and high affinity amino acid permeases, while repressing autophagy and the general amino acid permeases. In the absence of TOR signaling, the translation of mRNAs coding for components of the translation machinery is specifically inhibited, ribosome biosynthesis is inhibited, and autophagy is activated.

6.6 Regulation of cell growth and proliferation by PI3K and FRAP/mTOR signaling

In the fruit fly *Drosophila melanogaster*, the PI3K- Akt/PKB signaling module plays an important role in the regulation of cell growth (an increase in cell mass) and, in some cases, in the control of proliferation (429-431). Mutations in the PI3K or Akt/PKB *Drosophila* homologs reduce cell size, organ size and organism size. Ectopic overexpression of these kinases has the opposite effect (429-431). Mutations in dPTEN increase cell growth, while overexpression of dPTEN inhibits growth (432-434).

Recently, an important role has also been established for the *Drosophila* FRAP/mTOR homolog, dTOR, in the regulation of growth and proliferation (387, 435). Homozygotic dTOR mutants hatch normally, but larvae grow more slowly and are smaller than their wild type counterparts. These mutants remain viable for as long as 30 days, but eventually die without pupating. Interestingly, dTOR mutant cell clones generated by FLP/FRT-mediated mitotic recombination exhibit a pronounced decrease in cell size in several adult tissues, and in the developing wing imaginal disc (387). Furthermore, the cell cycle phasing of dTOR mutant cell clones differs from control cells, with more cells in G1 and relatively less cells in the S and G2 phases (387). This observation is consistent with the ability of rapamycin to arrest yeast and mammalian cells in G1 (246, 247).

While mutations of dPTEN result in increased cell growth (432-434), cell clones lacking both dPTEN and dTOR were indistinguishable from dTOR clones, indicating that the growth stimulatory effect of the PI3K pathway requires dTOR function (387).

As is observed in yeast, loss of dTOR function mimics amino acid withdrawal, eliciting an extended larval period accompanied by a reduction in nucleolar area (indicating decreased ribosome biosynthesis; 387, 436). In larval endoreplicative tissues (cells which do not divide, but which replicate their DNA complement), loss of dTOR phenocopies the cell cycle arrest elicited by starvation. This cell cycle arrest can be relieved by overexpression of the G1/S regulator cyclin E (387). These data provide further evidence that dTOR is involved in nutrient sensing, and suggest that in the presence of sufficient nutrients, dTOR transmits a positive signal, whereas dTOR signaling is inhibited when the amino acid supply is insufficient to support growth.

Phosphorylation of the *Drosophila* S6K and 4E-BP (a.k.a Thor; 437) homologs is also sensitive to rapamycin treatment, and thus appear to be downstream of dTOR (438, 439). Genetic studies have confirmed dS6K as a downstream effector of dTOR, because overexpression of this kinase partially rescues dTOR hypomorphic mutants, allowing them to survive to aldulthood (387). This effect is consistent with the phenotype of flies devoid of dS6K (440); while viable, dS6K mutants are significantly smaller than wild type flies due to a reduction in cell size (440). A decrease in cell size is also observed in cells overexpressing an active form of *Drosophila* 4E-BP (439).

PI3K and FRAP/mTOR signaling has also been implicated in cell growth and proliferation control in mammals. PI3K signaling regulates cell and organ size in the mouse heart (441). As in *Drosophila*, a FRAP/mTOR mouse mutant (termed flat-top) is significantly smaller than wild type animals, displays a marked developmental delay, and

dies *in utero* (442 and A. Peterson, pers. comm.). Deletion of the murine S6K1 yielded a small mouse phenotype and a growth delay, but knockout mice did not exhibit any obvious morphological differences from their wild-type counterparts (443). Importantly, not all cells are affected to the same degree by S6K1 deletion: for example, a disproportionate decrease in β-cell size in S6K-/- mice appears to cause glucose tolerance and hyperinsulinaemia (444). This is believed to be due to the extreme dependence of β-cells upon mitogens and nutrients. The analysis of the results in mice are complicated by the presence of a second S6K isoform (S6K2), which is also rapamycin-sensitive (443). As for 4E-BP1, a first knockout in mice showed no major difference between wild type and 4E-BP1-/- animals (445). However, a knockout animal generated in our laboratory revealed a lean phenotype (the white adipose tissue mass is severely reduced; 446). The study of knockout animals lacking all three 4E-BPs should provide further evidence for the role of these proteins in normal development.

In summary, a plethora of results from yeast, *Drosophila* and mice suggest that growth, defined as an increase in mass, and proliferation are points of convergence for at least two distinct sets of cues: signaling from the PI3K pathway, and the presence of sufficient nutrient levels to support growth, as sensed by Tor or FRAP/mTOR.

6.7 Dysregulation of PI3K and FRAP/mTOR signaling pathways in cell transformation and cancer

6.7.1 Transforming potential and mutations/amplification/deletion in tumors

Consistent with the critical importance of PI3K and FRAP/mTOR signaling in the control of growth and proliferation, dysregulation of the activity of several components in this pathway is associated with transformation. For example, PI3K was first discovered as a kinase activity co-purifying with the polyoma middle T antigen and the oncogene v-src (236, 447). Since these early reports, the transforming ability of several other oncogenes

has been demonstrated to correlate with their ability to bind to and activate PI3K (reviewed in 448). Oncoproteins derived from PI3K itself have also been identified. The p3k protein, isolated from the avian sarcoma virus ASV16 and derived from the catalytic subunit of PI3K, induces hemangiosarcoma in chickens and potently transforms chicken fibroblasts in culture (449). p65-PI3K, a truncation mutant of the regulatory PI3K p85 subunit, was isolated from a thymic lymphoma (450). This protein drives constitutive PI3K activation, causing lymphoproliferative disorders and autoimmunity when expressed in murine T lymphocytes (450, 451). Overexpression of the PI3K α catalytic subunit, caused by gene amplification, has been detected in ~40% of ovarian cancers (452). Consistent with an increase in PI3K activity as a causal agent, treatment of ovarian cancer cells in culture with LY294002 decreased proliferation and increased apoptosis (452, 453). Furthermore, LY294002 administration decreased the growth of ovarian tumors in athymic mice, and prevented ascites formation (453).

One of the most frequent targets for mutation in human cancer is PTEN. PTEN was first identified as a candidate tumor suppressor gene located on chromosome 10 (10q23; 454, 455). Somatic deletion or homozygous mutation of PTEN is detected in a large proportion of human cancers, and is especially frequent in glioblastomas, melanomas, endometrial carcinomas and prostate cancers (reviewed in 226). Germ-line mutation of PTEN is associated with dominantly inherited syndromes associated with cancer predisposition and developmental defects (e.g. 456, 457). Ectopic expression of PTEN inhibits the growth of tumor-derived cell lines (reviewed in 226). Disruption of a single copy of PTEN (+/-) in mice induces multiple types of tumors, confirming PTEN as a tumor suppressor, while homozygous PTEN-\- mice die *in utero* (458-460). The basal activity of Akt/PKB is elevated in PTEN-deficient tumor cell lines (as well as fibroblasts and tumors from PTEN knockout mice), consistent with a resistance of PTEN-/- fibroblasts to apoptosis (reviewed in 226).

Akt/PKB was first described as the cellular counterpart of the oncogene v-akt, a protein consisting of an N-terminal fusion of a viral Gag protein to the N-terminus of Akt, inducing constitutive membrane targeting of the protein (236). Overexpression of Akt transforms mammalian cells in culture (e.g. 461, 462), and Akt/PKB genes are amplified or overexpressed in several cancers (breast, gastric, ovarian, pancreatic, and prostate cancers; 463, 464-467). Overexpression of anti-sense Akt/PKB mRNA can inhibit tumorigenicity in a pancreatic cell line expressing high Akt/PKB levels (466).

6.7.2 Rapamycin as an anticancer drug

While no mutations in FRAP/mTOR have been detected in tumors to date, signaling through FRAP/mTOR appears to be critical for tumor growth. Interestingly, CCI-779 (a rapamycin ester formulated for intravenous use) inhibits the growth in mice of human tumor xenografts deficient in PTEN (C.L. Sawyers, pers. comm.). Lower doses of CCI-779 are required to inhibit PTEN-/- tumor growth than PTEN+/+ tumors, indicating that PTEN deletion sensitizes cells to growth arrest elicited by FRAP/mTOR inhibition. This effect is not confined to PTEN deletion, as tumors overexpressing myr-Akt (a membrane-targeted, activated allele of Akt/PKB) are also sensitized to CCI-779 (C.L. Sawyers, pers. comm.). Rapamycin administered alone results in growth inhibition (but not the shrinkage of an existing tumor), but CCI-779 treatment combined with androgen withdrawal led to tumor regression in an androgen-dependent prostate cancer xenograft lacking PTEN (C.L. Sawyers, pers. comm.). Rapamycin at low doses (1ng/ml) reverses the transformation of chicken embryo fibroblasts expressing p3k or Akt (468). CCI-779 also arrests the growth of various human tumor cells *in vivo* with different potencies (IC50 of ~1nM to >1μM, 469). In nude mouse xenografts, CCI-779 (when given for 5 consecutive days) inhibited glioblastoma tumor growth for up to two weeks after drug withdrawal, whereas normal immune function was restored as early as one day following withdrawal, indicating that an intermittent dosage of CCI-779 could be effective (469).

Thus, rapamycin (or its analogs) may be a promising therapeutic agent for the treatment of cancers resulting from a dysregulation of the PI3K pathway.

Excessive growth of Epstein-Barr virus (EBV)-transformed B lymphocytes is often the cause of life-threatening posttransplant lymphoproliferative disorders. Another rapamycin analog, SDZ RAD, which is in use to prevent graft rejection, has an antiproliferative effect on EBV-transformed B lymphocytes in culture and in a mouse model, blocking these cells in G1 and inducing apoptosis (470). Thus, rapamycin analogs may also be useful in the prevention of lymphoid hyperplasia or lymphoma in transplant patients.

In regard to the use of rapamycin either as an immunosuppressant or as an anti-cancer drug, it is important to note that it does not inhibit, but actually stimulates, the translation of mRNAs containing an IRES (Internal Ribosome Entry Site; reviewed in 23). All picornaviruses (including poliovirus, rhinovirus, and coxsackieviruses) and the hepatitis C virus possess an IRES (reviewed in 22), and replication of poliovirus in cultured cells is enhanced by rapamycin (325, 326). Since some of these viruses establish persistent infections, rapamycin treatment could activate their translation in patients. It will thus be important to assess the risks of this possible side effect.

6.8 Translational targets and cancer

Overexpression of 4E-BP1 or 4E-BP2 in cells transformed by eIF4E, Src or Ras reverts the transformed phenotype (348). In the case of Ras-transformed cells, the mechanism appears to be mediated (at least in part) through reversal of the anti-apoptotic activity of Ras (347). In fact, 4E-BP1 overexpression sensitizes Ras-transformed cells to apoptosis induced by lovastatin or camptothecin, but does not sensitize the parental cells to apoptosis (347). This phenotype is dependent on the ability of 4E-BP1 to bind to eIF4E,

as a mutant deficient for binding does not lead to apoptosis sensitization (347). Interestingly, the specificity of 4E-BP1 for sensitization of transformed cells (as compared to parental cells) to apoptosis is only observed when transfecting a regulatable 4E-BP1 protein. Overexpressing 4E-BP1 mutants (alanine substitutions) which are unable to be phosphorylated and regulated (Thr37Ala/Thr46Ala or Thr70Ala) induced apoptosis even in the parental cells (V. Polunovsky, pers. comm.). Thus, it appears that 4E-BP1 can induce apoptosis when it is able to bind strongly (or irreversibly) to eIF4E. This suggests that the Ras-transformed cells studied lack a mechanism to regulate (inactivate) the 4E-BPs. Consistent with this observation, dephosphorylated 4E-BP1 predominates in mammary tumors, or in cells transformed by eIF4E overexpression (403; section 6.1.4, 404). Whether this selective induction of apoptosis in transformed cells by wild type 4E-BP1 will have therapeutic implications is unknown.

A significant portion of the anti-tumor effects of rapamycin could be due to translational modulation, especially in light of the multiple translational targets for FRAP/mTOR. The amount of eIF4E protein is low in cells, relative to other initiation factors and ribosomes (471, 472). It was reasoned therefore that overexpressing eIF4E could lead to deregulation of cell physiology (reviewed in 113). Indeed, overexpression of eIF4E in immortalized rodent cell lines causes malignant transformation, as assessed by standard assays (growth in soft agar and formation of tumors in nude mice; 401, 473, 474). In NIH 3T3 cells, a two- to threefold increase in eIF4E is sufficient to elicit a transformed phenotype (473). eIF4GI overexpression also results in malignant transformation (475). Elevated levels of eIF4F components have been detected in a variety of tumors. For example, a large number of tumors have been found to harbor increased levels of eIF4E (e.g. breast carcinoma, primary bladder cancer, non-Hodgkin's lymphoma and head and neck squamous cell carcinomas; reviewed in 103, 104). Amplification of the eIF4GI gene has also been detected in 30% of squamous lung carcinomas (476). High levels of

eIF4AI are found in human melanoma cells (477). Overexpression of various eIF3 subunits in a broad spectrum of cancers was also reported (reviewed in 103). Further studies examining the protein levels and activity of the translation initiation factors will be necessary to better define the involvement of these factors in tumor formation.

6.9 Conclusions and future directions

As described in this chapter, numerous functions for FRAP/mTOR and the yeast Tor proteins have begun to emerge, several of which are linked to translational control. While the function of the phosphorylation of 4E-BP1 is known, the function of the phosphorylation of eIF4GI and eIF4B is much less clear, and needs to be investigated. An important question is whether phosphorylation of the various initiation factors modulates translation of different sets of mRNAs (e.g. the 5'TOP), or whether phosphorylation of all the factors simply results in a concerted translational up-regulation. How many mRNA targets FRAP/mTOR actually regulates is also unknown. While two-dimensional gels combined with protein sequencing is useful to detect relatively abundant proteins, those present in small quantities (which are often crucial for cell growth and proliferation) are undetectable. A similar caveat applies to the experiments using cDNA arrays and DNA chips after separation of mRNA into polysomes-associated versus non-associated.

While we are getting a good (albeit partial) idea of the pathways modulated by the TOR proteins, the molecular function of the TOR proteins is unknown. Key questions include: How do the TOR proteins sense nutrient sufficiency? How do they become inactivated in the absence of nutrients? Are they linked at all to the PI3K pathway? How do they relay signals to downstream targets? Since the TORs are expected to work as scaffolds, a better understanding of their function could be provided by identification of their binding partners. However, most attempts at isolating FRAP/mTOR binding partners (using two-

hybrid, far-western or pull-down approaches) have failed, due to the stickiness of the protein (S. Schreiber, pers. comm.; A.-C.G. and B. Raught, unpublished). In addition, while FRAP/mTOR is a phosphoprotein, little is known regarding the role of phosphorylation in FRAP/mTOR activity.

Most of the information that we have regarding FRAP/mTOR has been derived from studies using rapamycin. In yeast, Tor2p possesses two essential activities, only one of which is inhibited by rapamycin. Because there is only one TOR protein in mammals, it appears likely that some of its functions may be rapamycin-insensitive, and FRAP/mTOR may in fact affect more pathways in mammals than are known at present. The studies of the putative rapamycin-resistant activities are hampered by the fact that we do not have cells lacking FRAP/mTOR. We have attempted to generate MEFs from FRAP/mTOR mutant (flat-top) embryos (442), but these cells do not proliferate, and die (A.-C.G. and B. Raught, unpublished). A conditional knockout of FRAP/mTOR should help to provide more information about the function of this protein.

REFERENCES

1. Mathews, M. B., Sonenberg, N. and Hershey, J. W. B. (2000) Origins and principles of translational control. *In Translational control of gene expression.* N. Sonenberg, J. W. B. Hershey and M. B. Mathews, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 1-31.

2. Weyrich, A. S., Dixon, D. A., Pabla, R., Elstad, M. R., McIntyre, T. M., Prescott, S. M. and Zimmerman, G. A. (1998) Signal-dependent translation of a regulatory protein, Bcl-3, in activated human platelets. *Proc. Natl. Acad. Sci. U S A.* **95**: 5556-5561.

3. Wickens, M., Goodwin, E. B., Kimble, J., Strickland, S. and Hentze, M. W. (2000) Translational control of developmental decisions. *In Translational control of gene expression.* N. Sonenberg, J. W. B. Hershey and M. B. Mathews, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 295-370.

4. Casadio, A., Martin, K. C., Giustetto, M., Zhu, H., Chen, M., Bartsch, D., Bailey, C. H. and Kandel, E. R. (1999) A transient, neuron-wide form of CREB-mediated long-term facilitation can be stabilized at specific synapses by local protein synthesis. *Cell.* **99**: 221-237.

5. Hershey, J. W. B. and Merrick, W. C. (2000) Pathway and mechanism of initiation of protein synthesis. *In Translational control of gene expression.* N. Sonenberg, J. W. B. Hershey and M. B. Mathews, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 33-88.

6. Shatkin, A. J. (1976) Capping of eucaryotic mRNAs. *Cell.* **9**: 645-653.

7. Both, G. W., Banerjee, A. K. and Shatkin, A. J. (1975) Methylation-dependent translation of viral messenger RNAs in vitro. *Proc. Natl. Acad. Sci. U S A.* **72**: 1189-1193.

8. Muthukrishnan, S., Both, G. W., Furuichi, Y. and Shatkin, A. J. (1975) 5'-Terminal 7-methylguanosine in eukaryotic mRNA is required for translation. *Nature.* **255**: 33-37.

9. Varani, G. (1997) A cap for all occasions. *Structure.* **5**: 855-858.

10. Jacobson, A. (1996) Poly(A) metabolism and translation: the closed-loop model. *In Translational control.* J. W. B. Hershey, M. Mathews and N. Sonenberg, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 451-480.

11. Caponigro, G. and Parker, R. (1996) Mechanisms and control of mRNA turnover in Saccharomyces cerevisiae. *Microbiol. Rev.* **60**: 233-249.

12. Poncet, D., Aponte, C. and Cohen, J. (1993) Rotavirus protein NSP3 (NS34) is bound to the 3' end consensus sequence of viral mRNAs in infected cells. *J. Virol.* **67**: 3159-3165.

13. Sachs, A. B. (2000) Physical and functional interactions between the mRNA cap structure and the poly(A) tail. *In Translational control of gene expression.* N. Sonenberg, J. W. B. Hershey and M. B. Mathews, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 447-465.

14. Kozak, M. (1989) The scanning model for translation: an update. *J. Cell Biol.* **108**: 229-241.

15. Pelletier, J. and Sonenberg, N. (1985) Insertion mutagenesis to increase secondary structure within the 5' noncoding region of a eukaryotic mRNA reduces translational efficiency. *Cell.* **40**: 515-526.

16. Baim, S. B., Pietras, D. F., Eustice, D. C. and Sherman, F. (1985) A mutation allowing an mRNA secondary structure diminishes translation of Saccharomyces cerevisiae iso-1-cytochrome c. *Mol. Cell. Biol.* **5**: 1839-1846.

17. Cigan, A. M., Pabich, E. K. and Donahue, T. F. (1988) Mutational analysis of the HIS4 translational initiator region in Saccharomyces cerevisiae. *Mol. Cell. Biol.* **8**: 2964-2975.

18. Geballe, A. P. and Sachs, M. S. (2000) Translational control by upstream open reading frames. *In Translational control of gene expression.* N. Sonenberg, J. W. B. Hershey and M. B. Mathews, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 595-614.

19. Hentze, M. W. and Kühn, L. C. (1996) Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide and oxidative stress. *Proc. Natl. Acad. Sci. U S A.* **93**: 8175-8182.

20. Rouault, T. A. and Harford, J. B. (2000) Translational control of ferritin synthesis. *In Translational control of gene expression.* N. Sonenberg, J. W. B. Hershey and M. B. Mathews, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 655-693.

21. Meyuhas, O. and Hornstein, E. (2000) Translational control of TOP mRNAs. *In Translational control of gene expression.* N. Sonenberg, J. W. B. Hershey and M. B. Mathews, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 671-694.

22. Belsham, G. J. and Jackson, R. J. (2000) Translation initiation on picornavirus RNA. *In Translational control of gene expression.* N. Sonenberg, J. W. B. Hershey and M. B. Mathews, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 869-900.

23. Jackson, R. J. (2000) A comparative view of initiation site selection mechanisms. *In Translational control of gene expression.* N. Sonenberg, J. W. B. Hershey and M. B. Mathews, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 127-183.

24. Carter, M. S., Kuhn, K. M. and Sarnow, P. (2000) Cellular Internal Ribosome Entry Site elements and the use of cDNA microarrays in their investigation. *In Translational control of gene expression.* N. Sonenberg, J. W. B. Hershey and M. B. Mathews, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 615-635.

25. Si, K., Das, K. and Maitra, U. (1996) Characterization of multiple mRNAs that encode mammalian translation initiation factor 5 (eIF-5). *J. Biol. Chem.* **271**: 16934-16938.

26. Mathews, M. B., Sonenberg, N. and Hershey, J. W. B. (1996) Origins and targets of translational control. *In Translational control.* J. W. B. Hershey, M. Mathews and N. Sonenberg, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 1-29.

27. Sonenberg, N., Morgan, M. A., Merrick, W. C. and Shatkin, A. J. (1978) A polypeptide in eukaryotic initiation factors that crosslinks specifically to the 5'-terminal cap in mRNA. *Proc. Natl. Acad. Sci. U S A.* **75**: 4843-4847.

28. Sonenberg, N., Rupprecht, K. M., Hecht, S. M. and Shatkin, A. J. (1979) Eukaryotic mRNA cap binding protein: purification by affinity chromatography on sepharose-coupled m7GDP. *Proc. Natl. Acad. Sci. U S A.* **76**: 4345-4349.

29. Svitkin, Y. V., Ovchinnikov, L. P., Dreyfuss, G. and Sonenberg, N. (1996) General RNA binding proteins render translation cap dependent. *EMBO J.* **15**: 7147-7155.

30. Altmann, M., Muller, P. P., Pelletier, J., Sonenberg, N. and Trachsel, H. (1989) A mammalian translation initiation factor can substitute for its yeast homologue in vivo. *J. Biol. Chem.* **264**: 12145-12147.

31. Marcotrigiano, J., Gingras, A.-C., Sonenberg, N. and Burley, S. K. (1997) Cocrystal structure of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP. *Cell.* **89**: 951-961.

32. Matsuo, H., Li, H., McGuire, A. M., Fletcher, C. M., Gingras, A.-C., Sonenberg, N. and Wagner, G. (1997) Structure of translation factor eIF4E bound to m7GDP and interaction with 4E-binding protein. *Nat. Struct. Biol.* **4**: 717-724.

33. Rom, E., Kim, H. C., Gingras, A.-C., Marcotrigiano, J., Favre, D., Olsen, H., Burley, S. K. and Sonenberg, N. (1998) Cloning and characterization of 4EHP, a novel mammalian eIF4E-related cap-binding protein. *J. Biol. Chem.* **273**: 13104-13109.

34. Sonenberg, N., Burley, S. K. and Gingras, A.-C. (1998) RNA chiropractics. *Nat. Struct. Biol.* **5**: 172-174.

35. Gershon, P. D. and Moss, B. (1993) Stimulation of poly(A) tail elongation by the VP39 subunit of the vaccinia virus-encoded poly(A) polymerase. *J. Biol. Chem.* **268**: 2203-2210.

36. Barbosa, E. and Moss, B. (1978) mRNA(nucleoside-2'-)-methyltransferase from vaccinia virus. Purification and physical properties. *J. Biol. Chem.* **253**: 7692-7697.

37. Hodel, A. E., Gershon, P. D., Shi, X., Wang, S. M. and Quiocho, F. A. (1997) Specific protein recognition of an mRNA cap through its alkylated base. *Nat. Struct. Biol.* **4**: 350-354.

38. Izaurralde, E., Stepinski, J., Darzynkiewicz, E. and Mattaj, I. W. (1992) A cap binding protein that may mediate nuclear export of RNA polymerase II-transcribed RNAs. *J. Cell Biol.* **118**: 1287-1295.

39. Beelman, C. A., Stevens, A., Caponigro, G., LaGrandeur, T. E., Hatfield, L., Fortner, D. M. and Parker, R. (1996) An essential component of the decapping enzyme required for normal rates of mRNA turnover. *Nature.* **382**: 642-646.

40. Gingras, A.-C., Raught, B. and Sonenberg, N. (1999) eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu. Rev. Biochem.* **68**: 913-963.

41. Lamphear, B. J., Kirchweger, R., Skern, T. and Rhoads, R. E. (1995) Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. Implications for cap-dependent and cap-independent translational initiation. *J. Biol. Chem.* **270**: 21975-21983.

42. Mader, S., Lee, H., Pause, A. and Sonenberg, N. (1995) The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressors 4E-binding proteins. *Mol. Cell. Biol.* **15**: 4990-4997.

43. Imataka, H., Gradi, A. and Sonenberg, N. (1998) A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. *EMBO J.* **17**: 7480-7489.

44. Imataka, H. and Sonenberg, N. (1997) Human eukaryotic translation initiation factor 4G (eIF4G) possesses two separate and independent binding sites for eIF4A. *Mol. Cell. Biol.* **17**: 6940-6947.

45. Pestova, T. V., Hellen, C. U. and Shatsky, I. N. (1996) Canonical eukaryotic initiation factors determine initiation of translation by internal ribosomal entry. *Mol. Cell. Biol.* **16**: 6859-6869.

46. Morino, S., Imataka, H., Svitkin, Y. V., Pestova, T. V. and Sonenberg, N. (2000) Eukaryotic translation initiation factor 4E (eIF4E) binding site and the middle one-third of eIF4GI constitute the core domain for cap-dependent translation, and the C-terminal one-third functions as a modulatory region. *Mol. Cell. Biol.* **20**: 468-477.

47. Gradi, A., Imataka, H., Svitkin, Y. V., Rom, E., Raught, B., Morino, S. and Sonenberg, N. (1998) A novel functional human eukaryotic translation initiation factor 4G. *Mol. Cell. Biol.* **18**: 334-342.

48. Pyronnet, S., Imataka, H., Gingras, A.-C., Fukunaga, R., Hunter, T. and Sonenberg, N. (1999) Human eukaryotic translation initiation factor 4G (eIF4G) recruits MNK1 to phosphorylate eIF4E. *EMBO J.* **18**: 270-279.

49. Imataka, H., Olsen, H. S. and Sonenberg, N. (1997) A new translational regulator with homology to eukaryotic translation initiation factor 4G. *EMBO J.* **16**: 817-825.

50. Yamanaka, S., Poksay, K. S., Arnold, K. S. and Innerarity, T. L. (1997) A novel translational repressor mRNA is edited extensively in livers containing tumors caused by the transgene expression of the apoB mRNA-editing enzyme. *Genes Dev.* **11**: 321-333.

51. Sonenberg, N. and Gingras, A.-C. (1998) The mRNA 5' cap-binding protein eIF4E and control of cell growth. *Curr. Opin. Cell. Biol.* **10**: 268-275.

52. Morley, S. J., Curtis, P. S. and Pain, V. M. (1997) eIF4G: translation's mystery factor begins to yield its secrets. *RNA.* **3**: 1085-1104.

53. Raught, B. and Gingras, A.-C. (1999) eIF4E activity is regulated at multiple levels. *Int. J. Biochem. Cell Biol.* **31**: 43-57.

54. Marcotrigiano, J., Gingras, A.-C., Sonenberg, N. and Burley, S. K. (1999) Cap-dependent translation initiation in eukaryotes is regulated by a molecular mimic of eIF4G. *Mol. Cell.* **3**: 707-716.

55. Ptushkina, M., von der Haar, T., Vasilescu, S., Frank, R., Birkenhäger, R. and McCarthy, J. E. G. (1998) Cooperative modulation by eIF4G of eIF4E-binding to the mRNA 5' cap in yeast involves a site partially shared by p20. *EMBO J.* **17**: 4798-4808.

56. Ray, B. K., Lawson, T. G., Kramer, J. C., Cladaras, M. H., Grifo, J. A., Abramson, R. D., Merrick, W. C. and Thach, R. E. (1985) ATP-dependent unwinding of messenger RNA structure by eukaryotic initiation factors. *J. Biol. Chem.* **260**: 7651-7658.

57. Grifo, J. A., Abramson, R. D., Satler, C. A. and Merrick, W. C. (1984) RNA-stimulated ATPase activity of eukaryotic initiation factors. *J. Biol. Chem.* **259**: 8648-8654.

58. Rozen, F., Edery, I., Meerovitch, K., Dever, T. E., Merrick, W. C. and Sonenberg, N. (1990) Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F. *Mol. Cell. Biol.* **10**: 1134-1144.

59. Lorsch, J. R. and Herschlag, D. (1998) The DEAD box protein eIF4A. 2. A cycle of nucleotide and RNA-dependent conformational changes. *Biochemistry.* **37**: 2194-2206.

60. Lorsch, J. R. and Herschlag, D. (1998) The DEAD box protein eIF4A. 1. A minimal kinetic and thermodynamic framework reveals coupled binding of RNA and nucleotide. *Biochemistry.* **37**: 2180-2193.

61. Linder, P., Lasko, P. F., Ashburner, M., Leroy, P., Nielsen, P. J., Nishi, K., Schnier, J. and Slonimski, P. P. (1989) Birth of the D-E-A-D box. *Nature.* **337**: 121-122.

62. Nielsen, P. J. and Trachsel, H. (1988) The mouse protein synthesis initiation factor 4A gene family includes two related functional genes which are differentially expressed. *EMBO J.* **7**: 2097-2105.

63. Weinstein, D. C., Honore, E. and Hemmati-Brivanlou, A. (1997) Epidermal induction and inhibition of neural fate by translation initiation factor 4AIII. *Development.* **124**: 4235-4242.

64. Yoder-Hill, J., Pause, A., Sonenberg, N. and Merrick, W. C. (1993) The p46 subunit of eukaryotic initiation factor (eIF)-4F exchanges with eIF-4A. *J. Biol. Chem.* **268**: 5566-5573.

65. Altmann, M., Muller, P. P., Wittmer, B., Ruchti, F., Lanker, S. and Trachsel, H. (1993) A Saccharomyces cerevisiae homologue of mammalian translation initiation factor 4B contributes to RNA helicase activity. *EMBO J.* **12**: 3997-4003.

66. Coppolecchia, R., Buser, P., Stotz, A. and Linder, P. (1993) A new yeast translation initiation factor suppresses a mutation in the eIF-4A RNA helicase. *EMBO J.* **12**: 4005-4011.

67. Pause, A., Methot, N., Svitkin, Y., Merrick, W. C. and Sonenberg, N. (1994) Dominant negative mutants of mammalian translation initiation factor eIF-4A define a critical role for eIF-4F in cap-dependent and cap-independent initiation of translation. *EMBO J.* **13**: 1205-1215.

68. Duncan, R. and Hershey, J. W. (1983) Identification and quantitation of levels of protein synthesis initiation factors in crude HeLa cell lysates by two-dimensional polyacrylamide gel electrophoresis. *J. Biol. Chem.* **258**: 7228-7235.

69. Parkin, N. T., Cohen, E. A., Darveau, A., Rosen, C., Haseltine, W. and Sonenberg, N. (1988) Mutational analysis of the 5' non-coding region of human immunodeficiency virus type 1: effects of secondary structure on translation. *EMBO J.* **7**: 2831-2837.

70. Morgan, M. and Shatkin, A. (1980) Initiation of reovirus transcription by inosine 5'-triphosphate and properties of 7-methylinosine-capped, inosine-substituted messenger ribonucleic acids. *Biochemistry.* **19**: 5960-5966.

71. Kozak, M. (1980) Influence of mRNA secondary structure on binding and migration of 40S ribosomal subunits. *Cell.* **19**: 79-90.

72. Lee, K. A., Guertin, D. and Sonenberg, N. (1983) mRNA secondary structure as a determinant in cap recognition and initiation complex formation. ATP-Mg2+ independent cross-linking of cap binding proteins to m7I-capped inosine-substituted reovirus mRNA. *J. Biol. Chem.* **258**: 707-710.

73. Milburn, S., Hershey, J., Davies, M., Kelleher, K. and Kaufman, R. (1990) Cloning and expression of eukaryotic initiation factor 4B cDNA: sequence determination identifies a common RNA recognition motif. *EMBO J.* **9**: 2783-2790.

74. Benne, R. and Hershey, J. W. (1976) Purification and characterization of initiation factor IF-E3 from rabbit reticulocytes. *Proc. Natl. Acad. Sci. U S A.* **73**: 3005-3009.

75. Pestova, T. V., Maslova, S. V., Potapov, V. K. and Agol, V. I. (1989) Distinct modes of poliovirus polyprotein initiation in vitro. *Virus Res.* **14**: 107-118.

76. Pestova, T. V., Shatsky, I. N. and Hellen, C. U. T. (1996) Functional dissection of eukaryotic initiation factor 4F: the 4A subunit and the central domain of the 4G subunit are sufficient to mediate internal entry of 43S preinitiation complexes. *Mol. Cell. Biol.* **16**: 6870-6878.

77. Methot, N., Song, M. and Sonenberg, N. (1996) A region rich in aspartic acid, arginine, tyrosine, and glycine (DRYG) mediates eukaryotic initiation factor 4B (eIF4B) self-association and interaction with eIF3. *Mol. Cell. Biol.* **16**: 5328-5334.

78. Methot, N., Rom, E., Olsen, H. and Sonenberg, N. (1997) The human homologue of the yeast Prt1 protein is an integral part of the eukaryotic initiation factor 3 complex and interacts with p170. *J. Biol. Chem.* **272**: 1110-1116.

79. Methot, N., Pickett, G., Keene, J. and Sonenberg, N. (1996) In vitro RNA selection identifies RNA ligands that specifically bind to eukaryotic translation initiation factor 4B: the role of the RNA remotif. *RNA.* **2**: 38-50.

80. Naranda, T., Strong, W. B., Menaya, J., Fabbri, B. J. and Hershey, J. W. (1994) Two structural domains of initiation factor eIF-4B are involved in binding to RNA. *J. Biol. Chem.* **269**: 14465-14472.

81. Methot, N., Pause, A., Hershey, J. and Sonenberg, N. (1994) The translation initiation factor eIF-4B contains an RNA-binding region that is distinct and independent from its ribonucleoprotein consensus sequence. *Mol. Cell. Biol.* **14**: 2307-2316.

82. Richter-Cook, N. J., Dever, T. E., Hensold, J. O. and Merrick, W. C. (1998) Purification and characterization of a new eukaryotic protein translation factor. Eukaryotic initiation factor 4H. *J. Biol. Chem.* **273**: 7579-7587.

83. Mueller, P. P. and Hinnebusch, A. G. (1986) Multiple upstream AUG codons mediate translational control of GCN4. *Cell.* **45**: 201-207.

84. Kozak, M. and Shatkin, A. J. (1977) Sequences of two 5'-terminal ribosome-protected fragments from reovirus messenger RNAs. *J. Mol. Biol.* **112**: 75-96.

85. Futterer, J., Kiss-Laszlo, Z. and Hohn, T. (1993) Nonlinear ribosome migration on cauliflower mosaic virus 35S RNA. *Cell.* **73**: 789-802.

86. Yueh, A. and Schneider, R. J. (1996) Selective translation initiation by ribosome jumping in adenovirus-infected and heat-shocked cells. *Genes Dev.* **10**: 1557-1567.

87. Latorre, P., Kolakofsky, D. and Curran, J. (1998) Sendai virus Y proteins are initiated by a ribosomal shunt. *Mol. Cell. Biol.* **18**: 5021-5031.

88. Pelletier, J. and Sonenberg, N. (1988) Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature.* **334**: 320-325.

89. Jang, S. K., Kräusslich, H.-G., Nicklin, M. J. H., Duke, G. M., Palmenberg, A. C. and Wimmer, E. (1988) A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. *J. Virol.* **62**: 2636-2643.

90. Jackson, R. J. and Kaminski, A. (1995) Internal initiation of translation in eukaryotes: the picornavirus paradigm and beyond. *RNA.* **1**: 985-1000.

91. Ehrenfeld, E. (1996) Initiation of translation by picornavirus RNAs. *In Translational control.* J. W. B. Hershey, M. Mathews and N. Sonenberg, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 549-573.

92. Pestova, T. V., Shatsky, I. N., Fletcher, S. P., Jackson, R. J. and Hellen, C. U. (1998) A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon during internal translation initiation of hepatitis C and classical swine fever virus RNAs. *Genes Dev.* **12**: 67-83.

93. Pestova, T. V. and Hellen, C. U. (1999) Internal initiation of translation of bovine viral diarrhea virus RNA. *Virology.* **258**: 249-256.

94. Kleijn, M., Scheper, G. C., Voorma, H. O. and Thomas, A. A. M. (1998) Regulation of translation initiation factors by signal transduction. *Eur. J. Biochem.* **253**: 531-544.

95. Schneider, R. J. (2000) Translational control during heat shock. *In Translational control of gene expression.* N. Sonenberg, J. W. B. Hershey and M. B. Mathews, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 581-593.

96. Lodish, H. F. (1976) Translational control of protein synthesis. *Annu. Rev. Biochem.* **45**: 39-72.

97. Koromilas, A. E., Lazaris-Karatzas, A. and Sonenberg, N. (1992) mRNAs containing extensive secondary structure in their 5' non-coding region translate efficiently in cells overexpressing initiation factor eIF-4E. *EMBO J.* **11**: 4153-4158.

98. Svitkin, Y. V., Pause, A., Haghighat, A., Pyronnet, S., Witherell, G., Belsham, G. J. and Sonenberg, N. (2001) The requirement for eukaryotic initiation factor 4A (eIF4A) in translation is in direct proportion to the degree of mRNA 5' secondary structure. *RNA.* **7**: 382-394.

99. Altmann, M., Wittmer, B., Méthot, N., Sonenberg, N. and Trachsel, H. (1995) The Saccharomyces cerevisiae translation initiation factor Tif3 and its mammalian homologue, eIF-4B, have RNA annealing activity. *EMBO J.* **14**: 3820-3827.

100. Manzella, J. M., Rychlik, W., Rhoads, R. E., Hershey, J. W. and Blackshear, P. J. (1991) Insulin induction of ornithine decarboxylase. Importance of mRNA secondary structure and phosphorylation of eucaryotic initiation factors eIF-4B and eIF-4E. *J. Biol. Chem.* **266**: 2383-2389.

101. Shantz, L. M., Hu, R. H. and Pegg, A. E. (1996) Regulation of ornithine decarboxylase in a transformed cell line that overexpresses translation initiation factor eIF-4E. *Cancer Res.* **56**: 3265-3269.

102. Kozak, M. (1991) An analysis of vertebrate mRNA sequences: intimations of translational control. *J. Cell Biol.* **115**: 887-903.

103. Hershey, J. W. B. and Miyamoto, S. (2000) Translational control and cancer. *In Translational control of gene expression.* N. Sonenberg, J. W. B. Hershey and M. B. Mathews, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 637-654.

104. De Benedetti, A. and Harris, A. (1999) eIF4E expression in tumours. *Int. J. Biochem. Cell Biol.* **31**: 59-72.

105. Sonenberg, N., Hershey, J. W. B. and Mathews, M. B. (2000) Translational control of gene expression. Cold Spring Harbor Laboratory Press, Plainview, N.Y.

106. Fumagalli, S. and Thomas, G. (2000) S6 phosphorylation and signal transduction. *In Translational control of gene expression.* N. Sonenberg, J. W. B. Hershey and M. B. Mathews, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 695-718.

107. Zong, Q., Schummer, M., Hood, L. and Morris, D. R. (1999) Messenger RNA translation state: the second dimension of high-throughput expression screening. *Proc. Natl. Acad. Sci. U S A.* **96**: 10632-10636.

108. Mikulits, W., Pradet-Balade, B., Habermann, B., Beug, H., Garcia-Sanz, J. A. and Mullner, E. W. (2000) Isolation of translationally controlled mRNAs by differential screening. *FASEB J.* **14**: 1641-1652.

109. Johannes, G., Carter, M. S., Eisen, M. B., Brown, P. O. and Sarnow, P. (1999) Identification of eukaryotic mRNAs that are translated at reduced cap binding complex eIF4F concentrations using a cDNA microarray. *Proc. Natl. Acad. Sci. U S A.* **96**: 13118-13123.

110. Bu, X., Haas, D. W. and Hagedorn, C. H. (1993) Novel phosphorylation sites of eukaryotic initiation factor-4F and evidence that phosphorylation stabilizes interactions of the p25 and p220 subunits. *J. Biol. Chem.* **268**: 4975-4978.

111. Whalen, S. G., Gingras, A.-C., Amankwa, L., Mader, S., Branton, P. E., Aebersold, R. and Sonenberg, N. (1996) Phosphorylation of eIF-4E on serine 209 by protein kinase C is inhibited by the translational repressors, 4E-binding proteins. *J. Biol. Chem.* **271**: 11831-11837.

112. Joshi, B., Cai, A. L., Keiper, B. D., Minich, W. B., Mendez, R., Beach, C. M., Stepinski, J., Stolarski, R., Darzynkiewicz, E. and Rhoads, R. E. (1995) Phosphorylation of eukaryotic protein synthesis initiation factor 4E at Ser-209. *J. Biol. Chem.* **270**: 14597-14603.

113. Sonenberg, N. (1996) mRNA 5' cap-binding protein eIF4E and control of cell growth. *In Translational control.* J. W. B. Hershey, M. Mathews and N. Sonenberg, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 249-269.

114. Bonneau, A. M. and Sonenberg, N. (1987) Involvement of the 24-kDa cap-binding protein in regulation of protein synthesis in mitosis. *J. Biol. Chem.* **262**: 11134-11139.

115. Huang, J. T. and Schneider, R. J. (1991) Adenovirus inhibition of cellular protein synthesis involves inactivation of cap-binding protein. *Cell.* **65**: 271-280.

116. Fan, H. and Penman, S. (1970) Regulation of protein synthesis in mammalian cells. II. Inhibition of protein synthesis at the level of initiation during mitosis. *J. Mol. Biol.* **50**: 655-670.

117. Pyronnet, S., Pradayrol, L. and Sonenberg, N. (2000) A cell cycle-dependent internal ribosome entry site. *Mol. Cell.* **5**: 607-616.

118. Cornelis, S., Bruynooghe, Y., Denecker, G., Van Huffel, S., Tinton, S. and Beyaert, R. (2000) Identification and characterization of a novel cell cycle-regulated internal ribosome entry site. *Mol. Cell Biol.* **5**: 597-605.

119. Duncan, R. F. and Hershey, J. W. (1989) Protein synthesis and protein phosphorylation during heat stress, recovery, and adaptation. *J. Cell Biol.* **109**: 1467-1481.

120. Feigenblum, D. and Schneider, R. J. (1993) Modification of eukaryotic initiation factor 4F during infection by influenza virus. *J. Virol.* **67**: 3027-3035.

121. Kleijn, M., Vrins, C. L., Voorma, H. O. and Thomas, A. A. (1996) Phosphorylation state of the cap-binding protein eIF4E during viral infection. *Virology.* **217**: 486-494.

122. Loreni, F., Thomas, G. and Amaldi, F. (2000) Transcription inhibitors stimulate translation of 5' TOP mRNAs through activation of S6 kinase and the mTOR/FRAP signalling pathway. *Eur. J. Biochem.* **267**: 6594-6601.

123. Feigenblum, D. and Schneider, R. J. (1996) Cap-binding protein (eukaryotic initiation factor 4E) and 4E-inactivating protein BP-1 independently regulate cap-dependent translation. *Mol. Cell. Biol.* **16**: 5450-5457.

124. Gingras, A.-C., Kennedy, S. G., O'Leary, M. A., Sonenberg, N. and Hay, N. (1998) 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. *Genes Dev.* **12**: 502-513.

125. Kimball, S. R. and Jefferson, L. S. (2000) Regulation of translation initiation in mammalian cells by amino acids. *In Translational control of gene expression.* N. Sonenberg, J. W. B. Hershey and M. B. Mathews, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 561-580.

126. Rao, G. N., Griendling, K. K., Frederickson, R. M., Sonenberg, N. and Alexander, R. W. (1994) Angiotensin II induces phosphorylation of eukaryotic protein synthesis initiation factor 4E in vascular smooth muscle cells. *J. Biol. Chem.* **269**: 7180-7184.

127. Fleurent, M., Gingras, A.-C., Sonenberg, N. and Meloche, S. (1997) Angiotensin II stimulates phosphorylation of the translational repressor 4E-binding protein 1 by a mitogen-activated protein kinase-independent mechanism. *J. Biol. Chem.* **272**: 4006-4012.

128. von Manteuffel, S. R., Gingras, A.-C., Ming, X. F., Sonenberg, N. and Thomas, G. (1996) 4E-BP1 phosphorylation is mediated by the FRAP-p70s6k pathway and is independent of mitogen-activated protein kinase. *Proc. Natl. Acad. Sci. U S A.* **93**: 4076-4080.

129. Beretta, L., Singer, N. G., Hinderer, R., Gingras, A.-C., Richardson, B., Hanash, S. M. and Sonenberg, N. (1998) Differential regulation of translation and eIF4E phosphorylation during human thymocyte maturation. *J. Immunol.* **160**: 3269-3273.

130. Wang, X., Flynn, A., Waskiewicz, A. J., Webb, B. L., Vries, R. G., Baines, I. A., Cooper, J. A. and Proud, C. G. (1998) The phosphorylation of eukaryotic initiation factor eIF4E in response to phorbol esters, cell stresses, and cytokines is mediated by distinct MAP kinase pathways. *J. Biol. Chem.* **273**: 9373-9377.

131. Bragado, M. J., Tashiro, M. and Williams, J. A. (2000) Regulation of the initiation of pancreatic digestive enzyme protein synthesis by cholecystokinin in rat pancreas in vivo. *Gastroenterology.* **119**: 1731-1739.

132. Bragado, M. J., Groblewski, G. E. and Williams, J. A. (1998) Regulation of protein synthesis by cholecystokinin in rat pancreatic acini involves PHAS-I and the p70 S6 kinase pathway. *Gastroenterology.* **115**: 733-742.

133. Morley, S. J., Rau, M., Kay, J. E. and Pain, V. M. (1993) Increased phosphorylation of eukaryotic initiation factor 4 alpha during early activation of T

lymphocytes correlates with increased initiation factor 4F complex formation. *Eur. J. Biochem.* **218**: 39-48.

134. Polakiewicz, R. D., Schieferl, S. M., Gingras, A. C., Sonenberg, N. and Comb, M. J. (1998) mu-Opioid receptor activates signaling pathways implicated in cell survival and translational control. *J. Biol. Chem.* **273**: 23534-23541.

135. Donaldson, R. W., Hagedorn, C. H. and Cohen, S. (1991) Epidermal growth factor or okadaic acid stimulates phosphorylation of eukaryotic initiation factor 4F. *J. Biol. Chem.* **266**: 3162-3166.

136. Kleijn, M., Voorma, H. O. and Thomas, A. A. (1995) Phosphorylation of eIF-4E and initiation of protein synthesis in P19 embryonal carcinoma cells. *J. Cell Biochem.* **59**: 443-452.

137. Blackshear, P. J., Nemenoff, R. A. and Avruch, J. (1983) Insulin and growth factors stimulate the phosphorylation of a Mr-22000 protein in 3T3-L1 adipocytes. *Biochem. J.* **214**: 11-19.

138. Lin, T. A., Kong, X., Saltiel, A. R., Blackshear, P. J. and Lawrence, J. C., Jr (1995) Control of PHAS-I by insulin in 3T3-L1 adipocytes. Synthesis, degradation, and phosphorylation by a rapamycin-sensitive and mitogen-activated protein kinase-independent pathway. *J. Biol. Chem.* **270**: 18531-18538.

139. Kleijn, M., Korthout, M. M., Voorma, H. O. and Thomas, A. A. (1996) Phosphorylation of the eIF4E-binding protein PHAS-I after exposure of PC12 cells to EGF and NGF. *FEBS Lett.* **396**: 165-171.

140. Pyronnet, S., Gingras, A.-C., Bouisson, M., Kowalski-Chauvel, A., Seva, C., Vaysse, N., Sonenberg, N. and Pradayrol, L. (1998) Gastrin induces phosphorylation of eIF4E binding protein 1 and translation initiation of ornithine decarboxylase mRNA. *Oncogene.* **16**: 2219-2227.

141. Aronica, S. M., Gingras, A.-C., Sonenberg, N., Cooper, S., Hague, N. and Broxmeyer, H. E. (1997) Macrophage inflammatory protein-1alpha and interferon-inducible protein 10 inhibit synergistically induced growth factor stimulation of MAP kinase activity and suppress phosphorylation of eukaryotic initiation factor 4E and 4E binding protein 1. *Blood.* **89**: 3582-3595.

142. Scheper, G. C., Mulder, J., Kleijn, M., Voorma, H. O., Thomas, A. A. and van Wijk, R. (1997) Inactivation of eIF2B and phosphorylation of PHAS-I in heat-shocked rat hepatoma cells. *J. Biol. Chem.* **272**: 26850-26856.

143. Takeuchi, K., Shibamoto, S., Nagamine, K., Shigemori, I., Omura, S., Kitamura, N. and Ito, F. (2001) Signaling pathways leading to transcription and translation cooperatively regulate the transient increase in expression of c-Fos protein. *J. Biol. Chem.* **276**: 26077-26083.

144. Xu, G., Marshall, C. A., Lin, T. A., Kwon, G., Munivenkatappa, R. B., Hill, J. R., Lawrence, J. C., Jr and McDaniel, M. L. (1998) Insulin mediates glucose-stimulated phosphorylation of PHAS-I by pancreatic beta cells. An insulin-receptor mechanism for autoregulation of protein synthesis by translation. *J. Biol. Chem.* **273**: 4485-4491.

145. Morley, S. J. and McKendrick, L. (1997) Involvement of stress-activated protein kinase and p38/RK mitogen-activated protein kinase signaling pathways in the enhanced phosphorylation of initiation factor 4E in NIH 3T3 cells. *J. Biol. Chem.* **272**: 17887-17893.

146. Frederickson, R. M., Montine, K. S. and Sonenberg, N. (1991) Phosphorylation of eukaryotic translation initiation factor 4E is increased in Src-transformed cell lines. *Mol. Cell. Biol.* **11**: 2896-2900.

147. Morley, S. J. and Traugh, J. A. (1990) Differential stimulation of phosphorylation of initiation factors eIF-4F, eIF-4B, eIF-3, and ribosomal protein S6 by insulin and phorbol esters. *J. Biol. Chem.* **265**: 10611-10616.

148. Arnott, C. H., Atkinson, P. G., Sale, E. M. and Sale, G. J. (1997) PHAS-I phosphorylation in response to foetal bovine serum (FBS) is regulated by an ERK1/ERK2-independent and rapamycin-sensitive pathway in 3T3-L1 adipocytes. *FEBS Lett.* **406**: 179-183.

149. Flynn, A. and Proud, C. G. (1996) Insulin-stimulated phosphorylation of initiation factor 4E is mediated by the MAP kinase pathway. *FEBS Lett.* **389**: 162-166.

150. Flynn, A. and Proud, C. G. (1996) Insulin and phorbol ester stimulate initiation factor eIF-4E phosphorylation by distinct pathways in Chinese hamster ovary cells overexpressing the insulin receptor. *Eur. J. Biochem.* **236**: 40-47.

151. Kimball, S. R., Jefferson, L. S., Fadden, P., Haystead, T. A. and Lawrence, J. C., Jr (1996) Insulin and diabetes cause reciprocal changes in the association of eIF-4E and PHAS-I in rat skeletal muscle. *Am. J. Physiol.* **270**: C705-709.

152. Azpiazu, I., Saltiel, A. R., DePaoli-Roach, A. A. and Lawrence, J. C., Jr (1996) Regulation of both glycogen synthase and PHAS-I by insulin in rat skeletal muscle involves mitogen-activated protein kinase-independent and rapamycin-sensitive pathways. *J. Biol. Chem.* **271**: 5033-5039.

153. Mendez, R., Myers MG, J., White, M. and Rhoads, R. (1996) Stimulation of protein synthesis, eukaryotic translation initiation factor 4E phosphorylation, and PHAS-I phosphorylation by insulin requires insulin receptor substrate 1 and phosphatidylinositol 3-kinase. *Mol. Cell. Biol.* **16**: 2857-2864.

154. Hara, K., Yonezawa, K., Weng, Q. P., Kozlowski, M. T., Belham, C. and Avruch, J. (1998) Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. *J. Biol. Chem.* **273**: 14484-14494.

155. Bhandari, B. K., Feliers, D., Duraisamy, S., Stewart, J. L., Gingras, A.-C., Abboud, H. E., Choudhury, G. G., Sonenberg, N. and Kasinath, B. S. (2001) Insulin regulation of protein translation repressor 4E-BP1, an eIF4E-binding protein, in renal epithelial cells. *Kidney Int.* **59**: 866-875.

156. Wang, L., Wang, X. and Proud, C. G. (2000) Activation of mRNA translation in rat cardiac myocytes by insulin involves multiple rapamycin-sensitive steps. *Am. J. Physiol. Heart Circ. Physiol.* **278**: H1056-1068.

157. Graves, L. M., Bornfeldt, K. E., Argast, G. M., Krebs, E. G., Kong, X., Lin, T. A. and Lawrence, J. C., Jr (1995) cAMP- and rapamycin-sensitive regulation of the association of eukaryotic initiation factor 4E and the translational regulator PHAS-I in aortic smooth muscle cells. *Proc. Natl. Acad. Sci. U S A.* **92**: 7222-7226.

158. Rychlik, W., Rush, J. S., Rhoads, R. E. and Waechter, C. J. (1990) Increased rate of phosphorylation-dephosphorylation of the translational initiation factor eIF-4E

correlates with the induction of protein and glycoprotein biosynthesis in activated B lymphocytes. *J. Biol. Chem.* **265**: 19467-19471.

159. Potter, M. W., Shah, S. A., Elbirt, K. K. and Callery, M. P. (2001) Endotoxin (LPS) stimulates 4E-BP1/PHAS-I phosphorylation in macrophages. *J Surg Res.* **97**: 54-59.

160. Mick, S., Abramson, R., Ray, B., Merrick, W., Thach, R. and Hagedorn, C. (1988) Induction of eIF-4E phosphorylation by the addition of L-pyrroline-5-carboxylic acid to rabbit reticulocyte lysate. *FEBS Lett.* **236**: 484-488.

161. Frederickson, R. M., Mushynski, W. E. and Sonenberg, N. (1992) Phosphorylation of translation initiation factor eIF-4E is induced in a ras-dependent manner during nerve growth factor-mediated PC12 cell differentiation. *Mol. Cell. Biol.* **12**: 1239-1247.

162. Bu, X. and Hagedorn, C. H. (1991) Platelet-derived growth factor stimulates phosphorylation of the 25 kDa mRNA cap binding protein (eIF-4E) in human lung fibroblasts. *FEBS Lett.* **283**: 219-222.

163. Boal, T. R., Chiorini, J. A., Cohen, R. B., Miyamoto, S., Frederickson, R. M., Sonenberg, N. and Safer, B. (1993) Regulation of eukaryotic translation initiation factor expression during T-cell activation. *Biochim. Biophys. Acta.* **1176**: 257-264.

164. Rybkin, I. I., Cross, M. E., McReynolds, E. M., Lin, R. Z. and Ballou, L. M. (2000) α1A Adrenergic receptor induces eukaryotic initiation factor 4E-binding protein 1 phosphorylation via a Ca^{2+}-dependent phatway independent of phosphatidylinositol 3-kinase/Akt. *J. Biol. Chem.* **275**: 5460-5465.

165. Rao, G. N., Madamanchi, N. R., Lele, M., Gadiparthi, L., Gingras, A.-C., Eling, T. E. and Sonenberg, N. (1999) A potential role for extracellular signal-regulated kinases in prostaglandin F2alpha-induced protein synthesis in smooth muscle cells. *J. Biol. Chem.* **274**: 12925-12932.

166. Beretta, L., Gingras, A.-C., Svitkin, Y. V., Hall, M. N. and Sonenberg, N. (1996) Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. *EMBO J.* **15**: 658-664.

167. Kaspar, R. L., Rychlik, W., White, M. W., Rhoads, R. E. and Morris, D. R. (1990) Simultaneous cytoplasmic redistribution of ribosomal protein L32 mRNA and phosphorylation of eukaryotic initiation factor 4E after mitogenic stimulation of Swiss 3T3 cells. *J. Biol. Chem.* **265**: 3619-3622.

168. Marino, M. W., Feld, L. J., Jaffe, E. A., Pfeffer, L. M., Han, H. M. and Donner, D. B. (1991) Phosphorylation of the proto-oncogene product eukaryotic initiation factor 4E is a common cellular response to tumor necrosis factor. *J. Biol. Chem.* **266**: 2685-2688.

169. Robinson, M. J. and Cobb, M. H. (1997) Mitogen-activated protein kinase pathways. *Curr. Opin. Cell Biol.* **9**: 180-186.

170. Waskiewicz, A. J. and Cooper, J. A. (1995) Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeast. *Curr. Opin. Cell Biol.* **7**: 798-805.

171. Rinker-Schaeffer, C. W., Austin, V., Zimmer, S. and Rhoads, R. E. (1992) Ras transformation of cloned rat embryo fibroblasts results in increased rates of protein synthesis and phosphorylation of eukaryotic initiation factor 4E. *J. Biol. Chem.* **267**: 10659-10664.

172. Cobb, M. H. and Goldsmith, E. J. (1995) How MAP kinases are regulated. *J. Biol. Chem.* **270**: 14843-14846.

173. Young, P. R., McLaughlin, M. M., Kumar, S., Kassis, S., Doyle, M. L., McNulty, D., Gallagher, T. F., Fisher, S., McDonnell, P. C., Carr, S. A., Huddleston, M. J., Seibel, G., Porter, T. G., Livi, G. P., Adams, J. L. and Lee, J. C. (1997) Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site. *J. Biol. Chem.* **272**: 12116-12121.

174. Fukunaga, R. and Hunter, T. (1997) MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. *EMBO J.* **16**: 1921-1933.

175. Waskiewicz, A. J., Flynn, A., Proud, C. G. and Cooper, J. A. (1997) Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. *EMBO J.* **16**: 1909-1920.

176. Waskiewicz, A. J., Johnson, J. C., Penn, B., Mahalingam, M., Kimball, S. R. and Cooper, J. A. (1999) Phosphorylation of the cap-binding protein eukaryotic translation initiation factor 4E by protein kinase Mnk1 in vivo. *Mol. Cell. Biol.* **19**: 1871-1880.

177. Edery, I., Altmann, M. and Sonenberg, N. (1988) High-level synthesis in Escherichia coli of functional cap-binding eukaryotic initiation factor eIF-4E and affinity purification using a simplified cap-analog resin. *Gene.* **74**: 517-525.

178. Carberry, S. E., Rhoads, R. E. and Goss, D. J. (1989) A spectroscopic study of the binding of m7GTP and m7GpppG to human protein synthesis initiation factor 4E. *Biochemistry.* **28**: 8078-8083.

179. Minich, W., Balasta, M., Goss, D. and Rhoads, R. (1994) Chromatographic resolution of in vivo phosphorylated and nonphosphorylated eukaryotic translation initiation factor eIF-4E: increased cap affinity of the phosphorylated form. *Proc. Natl. Acad. Sci. U S A.* **91**: 7668-7672.

180. Morley, S. J. and Traugh, J. A. (1993) Stimulation of translation in 3T3-L1 cells in response to insulin and phorbol ester is directly correlated with increased phosphate labelling of initiation factor (eIF-) 4F and ribosomal protein S6. *Biochimie.* **75**: 985-989.

181. Morley, S. J. and Pain, V. M. (1995) Hormone-induced meiotic maturation in Xenopus oocytes occurs independently of p70s6k activation and is associated with enhanced initiation factor (eIF)-4F phosphorylation and complex formation. *J. Cell Sci.* **108 (Pt 4)**: 1751-1760.

182. Morley, S. J. and Pain, V. M. (1995) Translational regulation during activation of porcine peripheral blood lymphocytes: association and phosphorylation of the alpha and gamma subunits of the initiation factor complex eIF-4F. *Biochem. J.* **312 (Pt 2)**: 627-635.

183. Tuazon, P. T., Merrick, W. C. and Traugh, J. A. (1989) Comparative analysis of phosphorylation of translational initiation and elongation factors by seven protein kinases. *J. Biol. Chem.* **264**: 2773-2777.

184. Raught, B., Gingras, A.-C., Gygi, S. P., Imataka, H., Morino, S., Gradi, A., Aebersold, R. and Sonenberg, N. (2000) Serum-stimulated, rapamycin-sensitive

phosphorylation sites in the eukaryotic translation initiation factor 4GI. *EMBO J.* **19**: 434-444.

185. Mahalingam, M. and Templeton, D. J. (1996) Constitutive activation of S6 kinase by deletion of amino-terminal autoinhibitory and rapamycin sensitivity domains. *Mol. Cell. Biol.* **16**: 405-413.

186. Dennis, P. B., Pullen, N., Kozma, S. C. and Thomas, G. (1996) The principal rapamycin-sensitive p70(s6k) phosphorylation sites, T-229 and T-389, are differentially regulated by rapamycin-insensitive kinase kinases. *Mol. Cell. Biol.* **16**: 6242-6251.

187. Bushell, M., Poncet, D., Marissen, W. E., Flotow, H., Lloyd, R. E., Clemens, M. J. and Morley, S. J. (2000) Cleavage of polypeptide chain initiation factor eIF4GI during apoptosis in lymphoma cells: characterisation of an internal fragment generated by caspase-3-mediated cleavage. **7**: 628-636.

188. Benne, R., Edman, J., Traut, R. R. and Hershey, J. W. B. (1978) Phosphorylation of eukaryotic protein synthesis initiation factors. *Proc. Natl. Acad. Sci. U S A.* **75**: 108-112.

189. Benne, R., Luedi, M. and Hershey, J. W. B. (1977) Purification and characterization of initiation factors IF-E4 and IF-E6 from rabbit reticulocytes. *J. Biol. Chem.* **252**: 5798-5803.

190. Schreier, M. H., Erni, B. and Staehelin, T. (1977) Initiation of mammalian protein synthesis. I. Purification and characterization of seven initiation factors. *J. Mol. Biol.* **116**: 727-753.

191. Duncan, R. and Hershey, J. W. (1985) Regulation of initiation factors during translational repression caused by serum depletion. Covalent modification. *J. Biol. Chem.* **260**: 5493-5497.

192. Morley, S. J. and Traugh, J. A. (1989) Phorbol esters stimulate phosphorylation of eukaryotic initiation factors 3, 4B, and 4F. *J. Biol. Chem.* **264**: 2401-2404.

193. Peiretti, F., Raught, B., Gingras, A.-C., Sonenberg, N. and Hershey, J. W. B. (2001) The eukaryotic translation initiation factor eIF4B is a substrate of the p70 ribosomal S6 kinase (S6K) 1. *in preparation.*

194. Lawrence, J. C., Jr and Abraham, R. T. (1997) PHAS/4E-BPs as regulators of mRNA translation and cell proliferation. *Trends Biochem. Sci.* **22**: 345-349.

195. Pause, A., Belsham, G. J., Gingras, A.-C., Donzé, O., Lin, T. A., Lawrence, J. C., Jr and Sonenberg, N. (1994) Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. *Nature.* **371**: 762-767.

196. Poulin, F., Gingras, A.-C., Olsen, H., Chevalier, S. and Sonenberg, N. (1998) 4E-BP3, a new member of the eukaryotic initiation factor 4E-binding protein family. *J. Biol. Chem.* **273**: 14002-14007.

197. Altmann, M., Schmitz, N., Berset, C. and Trachsel, H. (1997) A novel inhibitor of cap-dependent translation initiation in yeast: p20 competes with eIF4G for binding to eIF4E. *EMBO J.* **16**: 1114-1121.

198. Haghighat, A., Mader, S., Pause, A. and Sonenberg, N. (1995) Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E. *EMBO J.* **14**: 5701-5709.

199. Fletcher, C. M., McGuire, A. M., Gingras, A.-C., Li, H., Matsuo, H., Sonenberg, N. and Wagner, G. (1998) 4E binding proteins inhibit the translation factor eIF4E without folded structure. *Biochemistry.* **37**: 9-15.

200. Fletcher, C. M. and Wagner, G. (1998) The interaction of eIF4E with 4E-BP1 is an induced fit to a completely disordered protein. *Protein Sci.* **7**: 1639-1642.

201. Hershey, P. E., McWhirter, S. M., Gross, J. D., Wagner, G., Alber, T. and Sachs, A. B. (1999) The Cap-binding protein eIF4E promotes folding of a functional domain of yeast translation initiation factor eIF4G1. *J. Biol. Chem.* **274**: 21297-21304.

202. Ohlmann, T., Pain, V. M., Wood, W., Rau, M. and Morley, S. J. (1997) The proteolytic cleavage of eukaryotic initiation factor (eIF) 4G is prevented by eIF4E binding protein (PHAS-I; 4E-BP1) in the reticulocyte lysate. *EMBO J.* **16**: 844-855.

203. Haghighat, A., Svitkin, Y., Novoa, I., Kuechler, E., Skern, T. and Sonenberg, N. (1996) The eIF4G-eIF4E complex is the target for direct cleavage by the rhinovirus 2A proteinase. *J. Virol.* **70**: 8444-8450.

204. Lin, T. A., Kong, X., Haystead, T. A., Pause, A., Belsham, G., Sonenberg, N. and Lawrence, J. C., Jr (1994) PHAS-I as a link between mitogen-activated protein kinase and translation initiation. *Science.* **266**: 653-656.

205. Fadden, P., Haystead, T. A. and Lawrence, J. C., Jr (1997) Identification of phosphorylation sites in the translational regulator, PHAS-I, that are controlled by insulin and rapamycin in rat adipocytes. *J. Biol. Chem.* **272**: 10240-10247.

206. Belsham, G. J. and Denton, R. M. (1980) The effect of insulin and adrenaline on the phosphorylation of a 22 000-molecular weight protein within isolated fat cells; possible identification as the inhibitor-1 of the 'general phosphatase'. *Biochem. Soc. Trans.* **8**: 382-383.

207. Belsham, G. J., Brownsey, R. W. and Denton, R. M. (1982) Reversibility of the insulin-stimulated phosphorylation of ATP citrate lyase and a cytoplasmic protein of subunit Mr 22000 in adipose tissue. *Biochem. J.* **204**: 345-352.

208. Blackshear, P. J., Nemenoff, R. A. and Avruch, J. (1982) Preliminary characterization of a heat-stable protein from rat adipose tissue whose phosphorylation is stimulated by insulin. *Biochem. J.* **204**: 817-824.

209. Hu, C., Pang, S., Kong, X., Velleca, M. and Lawrence, J. C., Jr (1994) Molecular cloning and tissue distribution of PHAS-I, an intracellular target for insulin and growth factors. *Proc. Natl. Acad. Sci. U S A.* **91**: 3730-3734.

210. Schneider, R. J. (2000) Adenovirus inhibition of cellular protein synthesis and preferential translation of viral mRNAs. *In Translational control of gene expression.* N. Sonenberg, J. W. B. Hershey and M. B. Mathews, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 901-914.

211. Gingras, A.-C., Svitkin, Y., Belsham, G. J., Pause, A. and Sonenberg, N. (1996) Activation of the translational suppressor 4E-BP1 following infection with encephalomyocarditis virus and poliovirus. *Proc. Natl. Acad. Sci. U S A.* **93**: 5578-5583.

212. Vries, R. G., Flynn, A., Patel, J. C., Wang, X., Denton, R. M. and Proud, C. G. (1997) Heat shock increases the association of binding protein-1 with initiation factor 4E. *J. Biol. Chem.* **272**: 32779-32784.

213. Parrott, L. A. and Templeton, D. J. (1999) Osmotic stress inhibits p70/85 S6 kinase through activation of a protein phosphatase. *J. Biol. Chem.* **274**: 24731-24736.

214. Heesom, K. J., Avison, M. B., Diggle, T. A. and Denton, R. M. (1998) Insulin-stimulated kinase from rat fat cells that phosphorylates initiation factor-4E binding protein 1 on the rapamycin-insensitive site (serine-111). *Biochem. J.* **336**: 39-48.

215. Gingras, A.-C., Gygi, S. P., Raught, B., Polakiewicz, R. D., Abraham, R. T., Hoekstra, M. F., Aebersold, R. and Sonenberg, N. (1999) Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes Dev.* **13**: 1422-1437.

216. Gingras, A.-C., Raught, B., Gygi, S. P., Niedzwiecka, A., Miron, M., Burley, S. K., Polakiewicz, R. D., Wyslouch-Cieszynska, A., Aebersold, R. and Sonenberg, N. (2001) Hierarchical phosphorylation of the translational inhibitor 4E-BP1. *submitted.*

217. Fadden, P., Haystead, T. A. and Lawrence, J. C., Jr (1998) Phosphorylation of the translational regulator, PHAS-I, by protein kinase CK2. *FEBS Lett.* **435**: 105-109.

218. Raught, B., Gingras, A.-C. and Sonenberg, N. (2001) Differential phosphorylation of 4E-BP1 and 4E-BP2 influence the kinetics of release from eIF4E. *in preparation.*

219. Gingras, A.-C. and Sonenberg, N. (1997) Adenovirus infection inactivates the translational inhibitors 4E-BP1 and 4E-BP2. *Virology.* **237**: 182-186.

220. Lin, T. A. and Lawrence, J. C., Jr (1996) Control of the translational regulators PHAS-I and PHAS-II by insulin and cAMP in 3T3-L1 adipocytes. *J. Biol. Chem.* **271**: 30199-30204.

221. Haystead, T. A., Haystead, C. M., Hu, C., Lin, T. A. and Lawrence J C, J. (1994) Phosphorylation of PHAS-I by mitogen-activated protein (MAP) kinase. Identification of a site phosphorylated by MAP kinase in vitro and in response to insulin in rat adipocytes. *J. Biol. Chem.* **269**: 23185-23191.

222. Karim, M. M., Hughes, J. M. X., Warwicker, J., Scheper, G. C., Proud, C. G. and McCarthy, J. E. G. (2001) A quantitative molecular model for modulation of mammalian translation by the eIF4E-binding protein 1. *J. Biol. Chem.* **276**: 20750-20757.

223. von Manteuffel, S. R., Dennis, P. B., Pullen, N., Gingras, A.-C., Sonenberg, N. and Thomas, G. (1997) The insulin-induced signalling pathway leading to S6 and initiation factor 4E binding protein 1 phosphorylation bifurcates at a rapamycin-sensitive point immediately upstream of p70s6k. *Mol. Cell. Biol.* **17**: 5426-5436.

224. Fruman, D. A., Meyers, R. E. and Cantley, L. C. (1998) Phosphoinositide kinases. *Annu. Rev. Biochem.* **67**: 481-507.

225. Di Cristofano, A. and Pandolfi, P. P. (2000) The multiple roles of PTEN in tumor suppression. *Cell.* **100**: 387-390.

226. Cantley, L. C. and Neel, B. G. (1999) New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc. Natl. Acad. Sci. U S A.* **96**: 4240-4245.

227. Wymann, M. P., Bulgarelli-Leva, G., Zvelebil, M. J., Pirola, L., Vanhaesebroeck, B., Waterfield, M. D. and Panayotou, G. (1996) Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol. Cell. Biol.* **16**: 1722-1733.

228. Vlahos, C. J., Matter, W. F., Hui, K. Y. and Brown, R. F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.* **2169**: 5241-5248.

229. Ui, M., Okada, T., Hazeki, K. and Hazeki, O. (1995) Wortmannin as a unique probe for an intracellular signalling protein, phosphoinositide 3-kinase. *Trends Biochem. Sci.* **20**: 303-307.

230. Brunn, G. J., Williams, J., Sabers, C., Wiederrecht, G., Lawrence, J. C., Jr and Abraham, R. T. (1996) Direct inhibition of the signaling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002. *EMBO J.* **15**: 5256-5267.

231. Sarkaria, J. N., Tibbetts, R. S., Busby, E. C., Kennedy, A. P., Hill, D. E. and Abraham, R. T. (1998) Inhibition of phosphoinositide 3-kinase related kinases by the radiosensitizing agent wortmannin. *Cancer Res.* **58**: 4375-4382.

232. Sarkaria, J. N., Busby, E. C., Tibbetts, R. S., Roos, P., Taya, Y., Karnitz, L. M. and Abraham, R. T. (1999) Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. *Cancer Res.* **59**: 4375-4382.

233. Nakanishi, S., Catt, K. J. and Balla, T. (1995) A wortmannin-sensitive phosphatidylinositol 4-kinase that regulates hormone-sensitive pools of inositolphospholipids. *Proc. Natl. Acad. Sci. U S A.* **92**: 5317-5321.

234. Downing, G. J., Kim, S., Nakanishi, S., Catt, K. J. and Balla, T. (1996) Characterization of a soluble adrenal phosphatidylinositol 4-kinase reveals wortmannin sensitivity of type III phosphatidylinositol kinases. *Biochemistry.* **35**: 3587-3594.

235. Franke, T. F., Kaplan, D. R. and Cantley, L. C. (1997) PI3K: downstream AKTion blocks apoptosis. *Cell.* **88**: 435-437.

236. Bellacosa, A., Testa, J. R., Staal, S. P. and Tsichlis, P. N. (1991) A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science.* **254**: 274-277.

237. Chan, T. O., Rittenhouse, S. E. and Tsichlis, P. N. (1999) AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu. Rev. Biochem.* **68**: 965-1014.

238. Datta, S. R., Brunet, A. and Greenberg, M. E. (1999) Cellular survival: a play in three Akts. *Genes Dev.* **13**: 2905-2927.

239. Franke, T. F., Yang, S. I., Chan, T. O., Datta, K., Kazlauskas, A., Morrison, D. K., Kaplan, D. R. and Tsichlis, P. N. (1995) The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell.* **81**: 727-736.

240. Burgering, B. M. and Coffer, P. J. (1995) Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature.* **376**: 599-602.

241. Kohn, A. D., Summers, S. A., Birnbaum, M. J. and Roth, R. A. (1996) Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J. Biol. Chem.* **271**: 31372-31378.

242. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P. and Hemmings, B. A. (1996) Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J.* **15**: 6541-6551.

243. Stambolic, V., Suzuki, A., de la Pompa, J. L., Brothers, G. M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J. M., Siderovski, D. P. and Mak, T. W. (1998) Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell.* **95**: 29-39.

244. Rohn, J. L., Hueber, A. O., McCarthy, N. J., Lyon, D., Navarro, P., Burgering, B. M. and Evan, G. I. (1998) The opposing roles of the Akt and c-Myc signalling pathways in survival from CD95-mediated apoptosis. *Oncogene.* **17**: 2811-2818.

245. Vezina, C., Kudelski, A. and Sehgal, S. N. (1975) Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. *J. Antibiot. (Tokyo).* **28**: 721-726.

246. Schmelzle, T. and Hall, M. N. (2000) TOR, a central controller of cell growth. *Cell.* **103**: 253-262.

247. Abraham, R. T. and Wiederrecht, G. J. (1996) Immunopharmacology of rapamycin. *Annu. Rev. Immunol.* **14**: 483-510.

248. Kahan, B. D. (2000) Efficacy of sirolimus compared with azathioprine for reduction of acute renal allograft rejection: a randomised multicentre study. The Rapamune US Study Group. *Lancet.* **356**: 194-202.

249. Barbet, N. C., Schneider, U., Helliwell, S. B., Stansfield, I., Tuite, M. F. and Hall, M. N. (1996) TOR controls translation initiation and early G1 progression in yeast. *Mol. Biol. Cell.* **7**: 25-42.

250. Polymenis, M. and Schmidt, E. V. (1997) Coupling of cell division to cell growth by translational control of the G1 cyclin CLN3 in yeast. *Genes Dev.* **11**: 2522-2531.

251. Gallego, C., Gari, E., Colomina, N., Herrero, E. and Aldea, M. (1997) The Cln3 cyclin is down-regulated by translational repression and degradation during the G1 arrest caused by nitrogen deprivation in budding yeast. *EMBO J.* **16**: 7196-7206.

252. Danaie, P., Altmann, M., Hall, M. N., Trachsel, H. and Helliwell, S. B. (1999) CLN3 expression is sufficient to restore G1-to-S-phase progression in Saccharomyces cerevisiae mutants defective in translation initiation factor eIF4E. *Biochem. J.* **340 (Pt 1)**: 135-141.

253. Pedersen, S., Celis, J. E., Nielsen, J., Christiansen, J. and Nielsen, F. C. (1997) Distinct repression of translation by wortmannin and rapamycin. *Eur. J. Biochem.* **247**: 449-456.

254. Terada, N., Patel, H. R., Takase, K., Kohno, K., Nairn, A. C. and Gelfand, E. W. (1994) Rapamycin selectively inhibits translation of mRNAs encoding elongation factors and ribosomal proteins. *Proc. Natl. Acad. Sci. U S A.* **91**: 11477-11481.

255. Jefferies, H. B., Reinhard, C., Kozma, S. C. and Thomas, G. (1994) Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family. *Proc. Natl. Acad. Sci. U S A.* **91**: 4441-4445.

256. Jefferies, H. B. and Thomas, G. (1996) Ribosomal protein S6 phosphorylation and signal transduction. *In Translational control.* J. W. B. Hershey, M. Mathews and N. Sonenberg, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 389-409.

257. Meyuhas, O., Avni, D. and Shama, S. (1996) Translational control of ribosomal protein mRNAs in eukaryotes. *In Translational control.* J. W. B. Hershey, M. Mathews and N. Sonenberg, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 363-388.

258. Jefferies, H. B., Fumagalli, S., Dennis, P. B., Reinhard, C., Pearson, R. B. and Thomas, G. (1997) Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k. *EMBO J.* **16**: 3693-3704.

259. Schwab, M. S., Kim, S. H., Terada, N., Edfjäll, C., Kozma, S. C., Thomas, G. and Maller, J. L. (1999) p70(S6K) controls selective mRNA translation during oocyte maturation and early embryogenesis in Xenopus laevis. *Mol. Cell. Biol.* **19**: 2485-2494.

260. Nielsen, F. C., Ostergaard, L., Nielsen, J. and Christiansen, J. (1995) Growth-dependent translation of IGF-II mRNA by a rapamycin-sensitive pathway. *Nature.* **377**: 358-362.

261. Nielsen, J., Christiansen, J., Lykke-Andersen, J., Johnsen, A. H., Wewer, U. M. and Nielsen, F. C. (1999) A family of insulin-like growth factor II mRNA-binding proteins represses translation in late development. *Mol. Cell. Biol.* **19**: 1262-1270.

262. Seidel, E. R. and Ragan, V. L. (1997) Inhibition by rapamycin of ornithine decarboxylase and epithelial cell proliferation in intestinal IEC-6 cells in culture. *Br. J. Pharmacol.* **120**: 571-574.

263. West, M. J., Stoneley, M. and Willis, A. E. (1998) Translational induction of the c-myc oncogene via activation of the FRAP/mTOR signalling pathway. *Oncogene.* **17**: 769-780.

264. Taha, C., Liu, Z., Jin, J., Al-Hasani, H., Sonenberg, N. and Klip, A. (1999) Opposite translational control of GLUT1 and GLUT4 glucose transporter mRNAs in response to insulin. Role of mammalian target of rapamycin, protein kinase B, and phosphatidylinositol 3-kinase in GLUT1 mRNA translation. *J. Biol. Chem.* **274**: 33085-33091.

265. Muise-Helmericks, R. C., Grimes, H. L., Bellacosa, A., Malstrom, S. E., Tsichlis, P. N. and Rosen, N. (1998) Cyclin D expression is controlled post-transcriptionally via a phosphatidylinositol 3-kinase/Akt-dependent pathway. *J. Biol. Chem.* **273**: 29864-29872.

266. Harding, M. W., Galat, A., Uehling, D. E. and Schreiber, S. L. (1989) A receptor for the immunosuppressant FK506 is a cis-trans peptidyl-prolyl isomerase. *Nature.* **341**: 758-760.

267. Siekierka, J. J., Hung, S. H., Poe, M., Lin, C. S. and Sigal, N. H. (1989) A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. *Nature.* **341**: 755-757.

268. Siekierka, J. J., Wiederrecht, G., Greulich, H., Boulton, D., Hung, S. H., Cryan, J., Hodges, P. J. and Sigal, N. H. (1990) The cytosolic-binding protein for the immunosuppressant FK-506 is both a ubiquitous and highly conserved peptidyl-prolyl cis-trans isomerase. *J. Biol. Chem.* **265**: 21011-21015.

269. Gothel, S. F. and Marahiel, M. A. (1999) Peptidyl-prolyl cis-trans isomerases, a superfamily of ubiquitous folding catalysts. *Cell Mol Life Sci.* **55**: 423-436.

270. Heitman, J., Movva, N. R., Hiestand, P. C. and Hall, M. N. (1991) FK 506-binding protein proline rotamase is a target for the immunosuppressive agent FK 506 in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U S A.* **88**: 1948-1952.

271. Koltin, Y., Faucette, L., Bergsma, D. J., Levy, M. A., Cafferkey, R., Koser, P. L., Johnson, R. K. and Livi, G. P. (1991) Rapamycin sensitivity in Saccharomyces cerevisiae is mediated by a peptidyl-prolyl cis-trans isomerase related to human FK506-binding protein. *Mol. Cell. Biol.* **11**: 1718-1723.

272. Wiederrecht, G., Brizuela, L., Elliston, K., Sigal, N. H. and Siekierka, J. J. (1991) FKB1 encodes a nonessential FK 506-binding protein in Saccharomyces cerevisiae and contains regions suggesting homology to the cyclophilins. *Proc. Natl. Acad. Sci. U S A.* **88**: 1029-1033.

273. Dolinski, K., Muir, S., Cardenas, M. and Heitman, J. (1997) All cyclophilins and FK506 binding proteins are, individually and collectively, dispensable for viability in Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U S A.* **94**: 13093-13098.

274. Cafferkey, R., Young, P. R., McLaughlin, M. M., Bergsma, D. J., Koltin, Y., Sathe, G. M., Faucette, L., Eng, W. K., Johnson, R. K. and Livi, G. P. (1993) Dominant missense mutations in a novel yeast protein related to mammalian phosphatidylinositol 3-kinase and VPS34 abrogate rapamycin cytotoxicity. *Mol. Cell. Biol.* **13**: 6012-6023.

275. Heitman, J., Movva, N. R. and Hall, M. N. (1991) Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science.* **253**: 905-909.

276. Helliwell, S. B., Wagner, P., Kunz, J., Deuter-Reinhard, M., Henriquez, R. and Hall, M. N. (1994) TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologues in yeast. *Mol. Biol. Cell.* **5**: 105-118.

277. Kunz, J., Henriquez, R., Schneider, U., Deuter-Reinhard, M., Movva, N. R. and Hall, M. N. (1993) Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. *Cell.* **73**: 585-596.

278. Lorenz, M. C. and Heitman, J. (1995) TOR mutations confer rapamycin resistance by preventing interaction with FKBP12-rapamycin. *J. Biol. Chem.* **270**: 27531-27537.

279. Zheng, X. F., Florentino, D., Chen, J., Crabtree, G. R. and Schreiber, S. L. (1995) TOR kinase domains are required for two distinct functions, only one of which is inhibited by rapamycin. *Cell.* **82**: 121-130.

280. Schmidt, A., Kunz, J. and Hall, M. N. (1996) TOR2 is required for organization of the actin cytoskeleton in yeast. *Proc. Natl. Acad. Sci. U S A.* **93**: 13780-13785.

281. Schmidt, A., Bickle, M., Beck, T. and Hall, M. N. (1997) The yeast phosphatidylinositol kinase homolog TOR2 activates RHO1 and RHO2 via the exchange factor ROM2. *Cell.* **88**: 531-542.

282. Brown, E. J., Albers, M. W., Shin, T. B., Ichikawa, K., Keith, C. T., Lane, W. S. and Schreiber, S. L. (1994) A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature.* **369**: 756-758.

283. Chen, Y., Chen, H., Rhoad, A. E., Warner, L., Caggiano, T. J., Failli, A., Zhang, H., Hsiao, C. L., Nakanishi, K. and Molnar-Kimber, K. L. (1994) A putative sirolimus (rapamycin) effector protein. *Biochem. Biophys. Res. Commun.* **203**: 1-7.

284. Chiu, M. I., Katz, H. and Berlin, V. (1994) RAPT1, a mammalian homolog of yeast Tor, interacts with the FKBP12/rapamycin complex. *Proc. Natl. Acad. Sci. U S A.* **91**: 12574-12578.

285. Sabatini, D. M., Erdjument-Bromage, H., Lui, M., Tempst, P. and Snyder, S. H. (1994) RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell.* **78**: 35-43.

286. Sabers, C. J., Martin, M. M., Brunn, G. J., Williams, J. M., Dumont, F. J., Wiederrecht, G. and Abraham, R. T. (1995) Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells. *J. Biol. Chem.* **270**: 815-822.

287. Alarcon, C. M., Heitman, J. and Cardenas, M. E. (1999) Protein kinase activity and identification of a toxic effector domain of the target of rapamycin TOR proteins in yeast. *Mol. Biol. Cell.* **10**: 2531-2546.

288. Bosotti, R., Isacchi, A. and Sonnhammer, E. L. (2000) FAT: a novel domain in PIK-related kinases. *Trends Biochem. Sci.* **25**: 225-227.

289. Cafferkey, R., McLaughlin, M. M., Young, P. R., Johnson, R. K. and Livi, G. P. (1994) Yeast TOR (DRR) proteins: amino-acid sequence alignment and identification of structural motifs. *Gene.* **141**: 133-136.

290. Choi, J., Chen, J., Schreiber, S. L. and Clardy, J. (1996) Structure of the FKBP12-rapamycin complex interacting with the binding domain of human FRAP. *Science.* **273**: 239-242.

291. Andrade, M. A. and Bork, P. (1995) HEAT repeats in the Huntington's disease protein. *Nat. Genet.* **11**: 115-116.

292. Groves, M. R. and Barford, D. (1999) Topological characteristics of helical repeat proteins. *Curr. Opin. Struct. Biol.* **9**: 383-389.

293. Groves, M. R., Hanlon, N., Turowski, P., Hemmings, B. A. and Barford, D. (1999) The structure of the protein phosphatase 2A PR65/A subunit reveals the conformation of its 15 tandemly repeated HEAT motifs. *Cell.* **96**: 99-110.

294. Marcotrigiano, J., Lomakin, I. B., Sonenberg, N., Pestova, T. V., Hellen, C. U. T. and Burley, S. K. (2001) A conserved HEAT domain within eIF4G directs assembly of the translation initiation machinery. *Mol. Cell.* **7**: 193-203.

295. Cingolani, G., Petosa, C., Weis, K. and Muller, C. W. (1999) Structure of importin-beta bound to the IBB domain of importin-alpha. *Nature.* **399**: 221-229.

296. Chook, Y. M. and Blobel, G. (1999) Structure of the nuclear transport complex karyopherin-beta2-Ran x GppNHp. *Nature.* **399**: 230-237.

297. Vetter, I. R., Arndt, A., Kutay, U., Gorlich, D. and Wittinghofer, A. (1999) Structural view of the Ran-Importin beta interaction at 2.3 A resolution. *Cell.* **97**: 635-646.

298. Sabatini, D. M., Barrow, R. K., Blackshaw, S., Burnett, P. E., Lai, M. M., Field, M. E., Bahr, B. A., Kirsch, J., Betz, H. and Snyder, S. H. (1999) Interaction of RAFT1 with gephyrin required for rapamycin-sensitive signaling. *Science.* **284**: 1161-1164.

299. Kunz, J., Schneider, U., Howald, I., Schmidt, A. and Hall, M. N. (2000) HEAT repeats mediate plasma membrane localization of Tor2p in yeast. *J. Biol. Chem.* **275**: 37011-37020.

300. Leibowitz, R. and Penman, S. (1971) Regulation of protein synthesis in HeLa cells. 3. Inhibition during poliovirus infection. *J. Virol.* **8**: 661-668.

301. Kaufmann, Y., Goldstein, E. and Penman, S. (1976) Poliovirus-induced inhibition of polypeptide initiation in vitro on native polyribosomes. *Proc. Natl. Acad. Sci. U S A.* **73**: 1834-1838.

302. Abreu, S. L. and Lucas-Lenard, J. (1976) Cellular protein synthesis shutoff by mengovirus: translation of nonviral and viral mRNA's in extracts from uninfected and infected Ehrlich ascites tumor cells. *J. Virol.* **18**: 182-194.

303. Svitkin, Y. V., Ginevskaya, V. A., Ugarova, T. Y. and Agol, V. I. (1978) A cell-free model of the encephalomyocarditis virus-induced inhibition of host cell protein synthesis. *Virology.* **87**: 199-203.

304. Helentjaris, T. and Ehrenfeld, E. (1978) Control of protein synthesis in extracts from poliovirus-infected cells. I. mRNA discrimination by crude initiation factors. *J. Virol.* **26**: 510-521.

305. Etchison, D., Milburn, S. C., Edery, I., Sonenberg, N. and Hershey, J. W. (1982) Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eucaryotic initiation factor 3 and a cap binding protein complex. *J. Biol. Chem.* **257**: 14806-14810.

306. Bonneau, A. M. and Sonenberg, N. (1987) Proteolysis of the p220 component of the cap-binding protein complex is not sufficient for complete inhibition of host cell protein synthesis after poliovirus infection. *J. Virol.* **61**: 986-991.

307. Irurzun, A., Sanchez-Palomino, S., Novoa, I. and Carrasco, L. (1995) Monensin and nigericin prevent the inhibition of host translation by poliovirus, without affecting p220 cleavage. *J. Virol.* **69**: 7453-7460.

308. Perez, L. and Carrasco, L. (1992) Lack of direct correlation between p220 cleavage and the shut-off of host translation after poliovirus infection. *Virology.* **189**: 178-186.

309. Jen, G. and Thach, R. E. (1982) Inhibition of host translation in encephalomyocarditis virus-infected L cells: a novel mechanism. *J. Virol.* **43**: 250-261.

310. Mosenkis, J., Daniels-McQueen, S., Janovec, S., Duncan, R., Hershey, J. W., Grifo, J. A., Merrick, W. C. and Thach, R. E. (1985) Shutoff of host translation by encephalomyocarditis virus infection does not involve cleavage of the eucaryotic initiation factor 4F polypeptide that accompanies poliovirus infection. *J. Virol.* **54**: 643-645.

311. Rose, J. K., Trachsel, H., Leong, K. and Baltimore, D. (1978) Inhibition of translation by poliovirus: inactivation of a specific initiation factor. *Proc. Natl. Acad. Sci. U S A.* **75**: 2732-2736.

312. Lawrence, C. and Thach, R. E. (1974) Encephalomyocarditis virus infection of mouse plasmacytoma cells. I. Inhibition of cellular protein synthesis. *J. Virol.* **14**: 598-610.

313. Svitkin, Y. V., Ugarova, T. Y., Ginevskaya, V. A., Kalinina, N. O., Scarlat, I. V. and Agol, V. I. (1974) Efficiency of translation of viral and cellular mRNA's in extracts from cells infected with encephalomyocarditis virus. *Intervirology.* **4**: 214-220.

314. Dolph, P. J., Racaniello, V., Villamarin, A., Palladino, F. and Schneider, R. J. (1988) The adenovirus tripartite leader may eliminate the requirement for cap-binding protein complex during translation initiation. *J. Virol.* **62**: 2059-2066.

315. Dolph, P. J., Huang, J. T. and Schneider, R. J. (1990) Translation by the adenovirus tripartite leader: elements which determine independence from cap-binding protein complex. *J. Virol.* **64**: 2669-2677.

316. Huang, J. T. and Schneider, R. J. (1990) Adenovirus inhibition of cellular protein synthesis is prevented by the drug 2-aminopurine. *Proc. Natl. Acad. Sci. U S A.* **87**: 7115-7119.

317. Feigenblum, D., Walker, R. and Schneider, R. J. (1998) Adenovirus induction of an interferon-regulatory factor during entry into the late phase of infection. *J. Virol.* **72**: 9257-9266.

318. Bablanian, R. (1972) Depression of macromolecular synthesis in cells infected with guanidine-dependent poliovirus under restrictive conditions. *Virology.* **47**: 255-259.

319. Black, T. L., Safer, B., Hovanessian, A. and Katze, M. G. (1989) The cellular 68,000-Mr protein kinase is highly autophosphorylated and activated yet significantly degraded during poliovirus infection: implications for translational regulation. *J. Virol.* **63**: 2244-2251.

320. Baglioni, C., Simili, M. and Shafritz, D. A. (1978) Initiation activity of EMC virus RNA, binding to initiation factor eIF-4B and shut-off of host cell protein synthesis. *Nature.* **275**: 240-243.

321. Duke, G. M., Hoffman, M. A. and Palmenberg, A. C. (1992) Sequence and structural elements that contribute to efficient encephalomyocarditis virus RNA translation. *J. Virol.* **66**: 1602-1609.

322. Carrasco, L. and Smith, A. E. (1976) Sodium ions and the shut-off of host cell protein synthesis by picornaviruses. *Nature.* **264**: 807-809.

323. Svitkin, Y. V., Gradi, A., Imataka, H., Morino, S. and Sonenberg, N. (1999) Eukaryotic initiation factor 4GII (eIF4GII), but not eIF4GI, cleavage correlates with inhibition of host cell protein synthesis after human rhinovirus infection. *J. Virol.* **73**: 3467-3472.

324. Gradi, A., Svitkin, Y. V., Imataka, H. and Sonenberg, N. (1998) Proteolysis of human eukaryotic translation initiation factor eIF4GII, but not eIF4GI, coincides with the shutoff of host protein synthesis after poliovirus infection. *Proc. Natl. Acad. Sci. U S A.* **95**: 11089-11094.

325. Beretta, L., Svitkin, Y. V. and Sonenberg, N. (1996) Rapamycin stimulates viral protein synthesis and augments the shutoff of host protein synthesis upon picornavirus infection. *J. Virol.* **70**: 8993-8996.

326. Svitkin, Y. V., Hahn, H., Gingras, A.-C., Palmenberg, A. C. and Sonenberg, N. (1998) Rapamycin and wortmannin enhance replication of a defective encephalomyocarditis virus. *J. Virol.* **72**: 5811-5819.

327. Rueckert, R. R. and Pallansch, M. A. (1981) Preparation and characterization of encephalomyocarditis (EMC) virus. *Methods Enzymol.* **78**: 315-325.

328. Dalgarno, L., Martin, E. M., Liu, S. L. and Work, T. S. (1966) Characterization of the products formed by the RNA polymerases of cells infected with encephalomyocarditis virus. *J. Mol. Biol.* **15**: 77-91.

329. Blanar, M. A. and Rutter, W. J. (1992) Interaction cloning: identification of a helix-loop-helix zipper protein that interacts with c-Fos. *Science.* **256**: 1014-1018.

330. Svitkin, Y. V. and Agol, V. I. (1978) Complete translation of encephalomyocarditis virus RNA and faithful cleavage of virus-specific proteins in a cell-free system from Krebs-2 cells. *FEBS Lett.* **87**: 7-11.

331. Svitkin, Y. V., Lyapustin, V. N., Lashkevich, V. A. and Agol, V. I. (1984) Differences between translation products of tick-borne encephalitis virus RNA in cell-free systems from Krebs-2 cells and rabbit reticulocytes: involvement of membranes in the processing of nascent precursors of flavivirus structural proteins. *Virology.* **135**: 536-541.

332. Barbeau, D., Charbonneau, R., Whalen, S. G., Bayley, S. T. and Branton, P. E. (1994) Functional interactions within adenovirus E1A protein complexes. *Oncogene.* **9**: 359-373.

333. Gingras, A. C., Raught, B. and Sonenberg, N. (2001) Regulation of translation initiation by FRAP/mTOR. *Genes Dev.* **15**: 807-826.

334. de Groot, R. P., Schouten, G. J., de Wit, L., Ballou, L. M., van der Eb, A. J. and Zantema, A. (1995) Induction of the mitogen-activated p70 S6 kinase by adenovirus E1A. *Oncogene.* **10**: 543-548.

335. Khwaja, A., Rodriguez-Viciana, P., Wennstrom, S., Warne, P. H. and Downward, J. (1997) Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *EMBO J.* **16**: 2783-2793.

336. Kennedy, S. G., Wagner, A. J., Conzen, S. D., Jordan, J., Bellacosa, A., Tsichlis, P. N. and Hay, N. (1997) The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. *Genes Dev.* **11**: 701-713.

337. Ahmed, N. N., Grimes, H. L., Bellacosa, A., Chan, T. O. and Tsichlis, P. N. (1997) Transduction of interleukin-2 antiapoptotic and proliferative signals via Akt protein kinase. *Proc. Natl. Acad. Sci. U S A.* **94**: 3627-3632.

338. Brown, E. J., Beal, P. A., Keith, C. T., Chen, J., Shin, T. B. and Schreiber, S. L. (1995) Control of p70 s6 kinase by kinase activity of FRAP in vivo. *Nature.* **377**: 441-446.

339. Brunn, G. J., Hudson, C. C., Sekulic, A., Williams, J. M., Hosoi, H., Houghton, P. J., Lawrence, J. C., Jr and Abraham, R. T. (1997) Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. *Science.* **277**: 99-101.

340. Takata, M., Ogawa, W., Kitamura, T., Hino, Y., Kuroda, S., Kotani, K., Klip, A., Gingras, A.-C., Sonenberg, N. and Kasuga, M. (1999) Requirement for Akt (protein kinase B) in insulin-induced activation of glycogen synthase and phosphorylation of 4E-BP1 (PHAS-1). *J. Biol. Chem.* **274**: 20611-20618.

341. Dufner, A., Andjelkovic, M., Burgering, B. M., Hemmings, B. A. and Thomas, G. (1999) Protein kinase B localization and activation differentially affect S6 kinase 1 activity and eukaryotic translation initiation factor 4E-binding protein 1 phosphorylation. *Mol. Cell. Biol.* **19**: 4525-4534.

342. Kohn, A. D., Barthel, A., Kovacina, K. S., Boge, A., Wallach, B., Summers, S. A., Birnbaum, M. J., Scott, P. H., Lawrence, J. C., Jr and Roth, R. A. (1998) Construction and characterization of a conditionally active version of the serine/threonine kinase Akt. *J. Biol. Chem.* **273**: 11937-11943.

343. Wu, X., Senechal, K., Neshat, M. S., Whang, Y. E. and Sawyers, C. L. (1998) The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway. *Proc. Natl. Acad. Sci. U S A.* **95**: 15587-15591.

344. Ueki, K., Yamamoto-Honda, R., Kaburagi, Y., Yamauchi, T., Tobe, K., Burgering, B. M., Coffer, P. J., Komuro, I., Akanuma, Y., Yazaki, Y. and Kadowaki, T. (1998) Potential role of protein kinase B in insulin-induced glucose transport, glycogen synthesis, and protein synthesis. *J. Biol. Chem.* **273**: 5315-5322.

345. Pullen, N., Dennis, P. B., Andjelkovic, M., Dufner, A., Kozma, S. C., Hemmings, B. A. and Thomas, G. (1998) Phosphorylation and activation of p70s6k by PDK1. *Science.* **279**: 707-710.

346. Polunovsky, V. A., Rosenwald, I. B., Tan, A., White, J., Chiang, L., Sonenberg, N. and Bitterman, P. B. (1996) Translational control of programmed cell death: eukaryotic translation initiation factor 4E blocks apoptosis in growth-factor-restricted fibroblasts with physiologically expressed or deregulated Myc. *Mol. Cell. Biol.* **16**: 6573-6581.

347. Polunovsky, V. A., Gingras, A. C., Sonenberg, N., Peterson, M., Tan, A., Rubins, J. B., Manivel, J. C. and Bitterman, P. B. (2000) Translational control of the antiapoptotic function of Ras. *J. Biol. Chem.* **275**: 24776-24780.

348. Rousseau, D., Gingras, A.-C., Pause, A. and Sonenberg, N. (1996) The eIF4E-binding proteins 1 and 2 are negative regulators of cell growth. *Oncogene.* **13**: 2415-2420.

349. Wagner, A. J., Kokontis, J. M. and Hay, N. (1994) Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21waf1/cip1. *Genes Dev.* **8**: 2817-2830.

350. Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

351. van der Geer, P., Luo, K., Sefton, B. M. and Hunter, T. (1994) Phosphopeptide mapping and phosphoamino acid analysis on cellulose thin-layer plates. *In Cell biology: a laboratory handbook.* J. E. Celis, eds. Academic Press, San Diego. 422-448.

352. Brunn, G. J., Fadden, P., Haystead, T. A. J. and Lawrence, J. C., Jr (1997) The mammalian target of rapamycin phosphorylates sites having a (Ser/Thr)-Pro motif and is activated by antibodies to a region near its COOH terminus. *J. Biol. Chem.* **272**: 32547-32550.

353. Burnett, P. E., Barrow, R. K., Cohen, N. A., Snyder, S. H. and Sabatini, D. M. (1998) RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc. Natl. Acad. Sci. U S A.* **95**: 1432-1437.

354. Scott, P. H., Brunn, G. J., Kohn, A. D., Roth, R. A. and Lawrence, J. C., J.r (1998) Evidence of insulin-stimulated phosphorylation and activation of the mammalian target of rapamycin mediated by a protein kinase B signaling pathway. *Proc. Natl. Acad. Sci. U S A.* **95**: 7772-7777.

355. Fiol, C. J., Wang, A., Roeske, R. W. and Roach, P. J. (1990) Ordered multisite protein phosphorylation. Analysis of glycogen synthase kinase 3 action using model peptide substrates. *J. Biol. Chem.* **265**: 6061-6065.

356. Flotow, H. and Roach, P. J. (1991) Role of acidic residues as substrate determinants for casein kinase I. *J. Biol. Chem.* **266**: 3724-3727.

357. Muslin, A. J., Tanner, J. W., Allen, P. M. and Shaw, A. S. (1996) Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell.* **84**: 889-897.

358. Yaffe, M. B., Rittinger, K., Volinia, S., Caron, P. R., Aitken, A., Leffers, H., Gamblin, S. J., Smerdon, S. J. and Cantley, L. C. (1997) The structural basis for 14-3-3:phosphopeptide binding specificity. *Cell.* **91**: 961-971.

359. Lawrence, J. C., Jr, Fadden, P., Haystead, T. A. and Lin, T. A. (1997) PHAS proteins as mediators of the actions of insulin, growth factors and cAMP on protein synthesis and cell proliferation. *Adv. Enzyme Regul.* **37**: 239-267.

360. Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y. and Ziv, Y. (1998) Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science.* **281**: 1674-1677.

361. Yang, D.-Q. and Kastan, M. B. (2000) Participation of ATM in insulin signaling through phosphorylation of eIF-4E-binding protein 1. *Nature Cell Biol.* **2**: 893-898.

362. Scott, P. H. and Lawrence, J. C., Jr (1998) Attenuation of mammalian Target of Rapamycin activity by increased cAMP in 3T3-L1 cells. *J. Biol. Chem.* **272**: 34496-34501.

363. Chen, J., Peterson, R. T. and Schreiber, S. L. (1998) Alpha 4 associates with protein phosphatases 2A, 4, and 6. *Biochem. Biophys. Res. Commun.* **247**: 827-832.

364. Nanahoshi, M., Nishiuma, T., Tsujishita, Y., Hara, K., Inui, S., Sakaguchi, N. and Yonezawa, K. (1998) Regulation of protein phosphatase 2A catalytic activity by alpha4 protein and its yeast homolog TAP42. *Biochem. Biophys. Res. Commun.* **251**: 52-526.

365. Kim, J. E. and Chen, J. (2000) Cytoplasmic-nuclear shuttling of FKBP12-rapamycin-associated protein is involved in rapamycin-sensitive signaling and translation initiation. *Proc. Natl. Acad. Sci. U S A.* **97**: 14340-14345.

366. Chen, C. A. and Okayama, H. (1988) Calcium phosphate-mediated gene transfer: a highly efficient transfection system for stably transforming cells with plasmid DNA. *Biotechniques.* **6**: 632-638.

367. Watts, J. D., Affolter, M., Krebs, D. L., Wange, R. L., Samelson, L. E. and Aebersold, R. (1994) Identification by electrospray ionization mass spectrometry of the sites of tyrosine phosphorylation induced in activated Jurkat T cells on the protein tyrosine kinase ZAP-70. *J. Biol. Chem.* **269**: 29520-29529.

368. Gygi, S. P., Rochon, Y., Franza, B. R. and Aebersold, R. (1999) Correlation between protein and mRNA abundance in yeast. *Mol. Cell. Biol.* **19**: 1720-1730.

369. Eng, J., McCormack, A. L. and Yates, J. R. (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J. Am. Soc. Mass Spectrom.* **5**: 976-989.

370. Yates, J. R., III, Eng, J. K., McCormack, A. L. and Schieltz, D. (1995) Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. *Anal. Chem.* **67**: 1426-1436.

371. Mothe-Satney, I., Yang, D., Fadden, P., Haystead, T. A. and Lawrence, J. C., Jr (2000) Multiple mechanisms control phosphorylation of PHAS-I in five (S/T)P sites that govern translational repression. *Mol. Cell. Biol.* **20**: 3558-3567.

372. Mothe-Satney, I., Brunn, G. J., McMahon, L. P., Capaldo, C. T., Abraham, R. T. and Lawrence, J. C., Jr. (2000) Mammalian Target of Rapamycin-dependent phosphorylation of PHAS-I in Four (S/T)P Sites detected by phospho-specific antibodies. *J. Biol. Chem.* **275**: 33836-33843.

373. Heesom, K. J. and Denton, R. M. (1999) Dissociation of the eukaryotic initiation factor-4E/4E-BP1 complex involves phosphorylation of 4E-BP1 by an mTOR-associated kinase. *FEBS Lett.* **457**: 489-493.

374. Yang, D., Brunn, G. J. and Lawrence, J. C. J. (1999) Mutational analysis of sites in the translational regulator, PHAS-I, that are selectively phosphorylated by mTOR. *FEBS Lett.* **453**: 387-390.

375. Peterson, R. T., Desai, B. N., Hardwick, J. S. and Schreiber, S. L. (1999) Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycin associated protein. *Proc. Natl. Acad. Sci. U S A.* **96**: 4438-4442.

376. Gygi, S. P., Han, D. K., Gingras, A. C., Sonenberg, N. and Aebersold, R. (1999) Protein analysis by mass spectrometry and sequence database searching: tools for cancer research in the post-genomic era. *Electrophoresis.* **20**: 310-319.

377. Schnolzer, M., Alewood, P., Jones, A., Alewood, D. and Kent, S. B. (1992) In situ neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. *Int. J. Pept. Protein Res.* **40**: 180-193.

378. Wakamiya, T., Saruto, K., Yasuoka, J. and Kusumoto, S. (1994) An efficient procedure for solid-phase synthesis of phosphopeptides by Fmoc strategy. *Chemistry Letters.* 1099-1102.

379. Blachut-Okrasinska, E., Bojarska, E., Niedzwiecka, A., Chlebicka, L., Darzynkiewicz, E., Stolarski, R., Stepinski, J. and Antosiewicz, J. M. (2000) Stopped-flow and Brownian dynamics studies of electrostatic effects in the kinetics of binding of 7-methyl-GpppG to the protein eIF4E. *Eur. Biophys. J.* **29**: 487-498.

380. Peterson, R. T., Beal, P. A., Comb, M. J. and Schreiber, S. L. (2000) FKBP12-rapamycin-associated protein (FRAP) autophosphorylates at serine 2481 under translationally repressive conditions. *J. Biol. Chem.* **275**: 7416-7423.

381. Raught, B., Gingras, A.-C. and Sonenberg, N. (2000) Regulation of ribosomal recruitment in eukaryotes. *In Translational control of gene expression.* N. Sonenberg, J. W. B. Hershey and M. B. Mathews, eds. Cold Spring Harbor Laboratory Press, Plainview, N.Y. 245-294.

382. Gingras, A.-C., Raught, B. and Sonenberg, N. (2001) Control of translation by the target of rapamycin proteins. *In Prog. Mol. Subcell. Biol.* R. E. Rhoads, eds. Springer-Verlag, Berlin. 143.

383. Thomas, G. and Hall, M. N. (1997) TOR signalling and control of cell growth. *Curr. Opin. Cell. Biol.* **9**: 782-787.

384. Mahalingam, M. and Templeton, D. (1996) Constitutive activation of S6 kinase by deletion of amino-terminal autoinhibitory and rapamycin sensitivity domains. *Mol. Cell. Biol.* **16**: 405-413.

385. Sekulic, A., Hudson, C. C., Homme, J. L., Yin, P., Otterness, D. M., Karnitz, L. M. and Abraham, R. T. (2000) A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. *Cancer Res.* **60**: 3504-3513.

386. Navé, B. T., Ouwens, M., Withers, D. J., Alessi, D. R. and Shepherd, P. R. (1999) Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. *Biochem. J.* **344 Pt 2**: 427-431.

387. Zhang, H., Stallock, J. P., Ng, J. C., Reinhard, C. and Neufeld, T. P. (2000) Regulation of cellular growth by the *Drosophila* target of rapamycin *dTOR*. *Genes Dev.* **14**: 2712-2724.

388. Dennis, P. B., Fumagalli, S. and Thomas, G. (1999) Target of rapamycin (TOR): balancing the opposing forces of protein synthesis and degradation. *Curr. Op. Genet. Dev.* **9**: 49-54.

389. Xu, G., Kwon, G., Marshall, C. A., Lin, T. A., Lawrence, J. C., Jr and McDaniel, M. L. (1998) Branched-chain amino acids are essential in the regulation of PHAS-I and

p70 S6 kinase by pancreatic beta-cells. A possible role in protein translation and mitogenic signaling. *J. Biol. Chem.* **273**: 28178-28184.

390. Iiboshi, Y., Papst, P. J., Kawasome, H., Hosoi, H., Abraham, R. T., Houghton, P. J. and Terada, N. (1999) Amino acid-dependent control of p70(s6k). Involvement of tRNA aminoacylation in the regulation. *J. Biol. Chem.* **274**: 1092-1099.

391. Campbell, L. E., Wang, X. and Proud, C. G. (1999) Nutrients differentially regulate multiple translation factors and their control by insulin. *Biochem. J.* **344**: 433-441.

392. Kimball, S. R., Shantz, L. M., Horetsky, R. L. and Jefferson, L. S. (1999) Leucine regulates translation of specific mRNAs in L6 myoblasts through mTOR-mediated changes in availability of eIF4E and phosphorylation of ribosomal protein S6. *J. Biol. Chem.* **274**: 11647-11652.

393. Patti, M. E., Brambilla, E., Luzi, L., Landaker, E. J. and Kahn, C. R. (1998) Bidirectional modulation of insulin action by amino acids. *J. Clin. Invest.* **101**: 1519-1529.

394. Shigemitsu, K., Tsujishita, Y., Hara, K., Nanahoshi, M., Avruch, J. and Yonezawa, K. (1999) Regulation of translational effectors by amino acid and mammalian target of rapamycin signaling pathways. Possible involvement of autophagy in cultured hepatoma cells. *J. Biol. Chem.* **274**: 1058-1065.

395. Wang, X., Campbell, L. E., Miller, C. M. and Proud, C. G. (1998) Amino acid availability regulates p70 S6 kinase and multiple translation factors. *Biochem. J.* **334**: 261-267.

396. Cardenas, M. E., Cutler, N. S., Lorenz, M. C., Di Como, C. J. and Heitman, J. (1999) The TOR signaling cascade regulates gene expression in response to nutrients. *Genes Dev.* **13**: 3271-3279.

397. Hardwick, J. S., Kuruvilla, F. G., Tong, J. K., Shamji, A. F. and Schreiber, S. L. (1999) Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins. *Proc. Natl. Acad. Sci. U S A.* **96**: 14866-14870.

398. Beck, T. and Hall, M. N. (1999) The TOR signalling pathway controls nuclear localization of nutrient-regulated transcription factors. *Nature.* **402**: 689-692.

399. Shamji, A. F., Kuruvilla, F. G. and Schreiber, S. L. (2000) Partitioning the transcriptional program induced by rapamycin among the effectors of the Tor proteins. *Curr. Biol.* **10**: 1574-1581.

400. Brown, E. J. and Schreiber, S. L. (1996) A signaling pathway to translational control. *Cell.* **86**: 517-520.

401. Lazaris-Karatzas, A., Montine, K. S. and Sonenberg, N. (1990) Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap. *Nature.* **345**: 544-547.

402. Rosenwald, I. B., Chen, J. J., Wang, S., Savas, L., London, I. M. and Pullman, J. (1999) Upregulation of protein synthesis initiation factor eIF-4E is an early event during colon carcinogenesis. *Oncogene.* **18**: 2507-2517.

403. Khaleghpour, K., Pyronnet, S., Gingras, A.-C. and Sonenberg, N. (1999) Translational homeostasis: eukaryotic translation initiation factor 4E control of 4E-binding protein 1 and p70 S6 kinase activities. *Mol. Cell. Biol.* **19**: 4302-4310.

404. Raught, B., Gingras, A.-C., James, A., Medina, D., Sonenberg, N. and Rosen, J. M. (1996) Expression of a translationally regulated, dominant-negative CCAAT/enhancer-binding protein beta isoform and up-regulation of the eukaryotic translation initiation factor 2alpha are correlated with neoplastic transformation of mammary epithelial cells. *Cancer Res.* **56**: 4382-4386.

405. Di Como, C. J. and Arndt, K. T. (1996) Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases. *Genes Dev.* **10**: 1904-1916.

406. Schmidt, A., Beck, T., Koller, A., Kunz, J. and Hall, M. N. (1998) The TOR nutrient signalling pathway phosphorylates NPR1 and inhibits turnover of the tryptophan permease. *EMBO J.* **17**: 6924-6931.

407. Jiang, Y. and Broach, J. R. (1999) Tor proteins and protein phosphatase 2A reciprocally regulate Tap42 in controlling cell growth in yeast. *EMBO J.* **18**: 2782-2792.

408. Harris, D. M., Myrick, T. L. and Rundle, S. J. (1999) The Arabidopsis homolog of yeast TAP42 and mammalian alpha4 binds to the catalytic subunit of protein phosphatase 2A and is induced by chilling. *Plant. Physiol.* **121**: 609-617.

409. Inui, S., Kuwahara, K., Mizutani, J., Maeda, K., Kawai, T., Nakayasu, H. and Sakaguchi, N. (1995) Molecular cloning of a cDNA clone encoding a phosphoprotein component related to the Ig receptor-mediated signal transduction. *J. Immunol.* **154**: 2714-2723.

410. Onda, M., Inui, S., Maeda, K., Suzuki, M., Takahashi, E. and Sakaguchi, N. (1997) Expression and chromosomal localization of the human alpha 4/IGBP1 gene, the structure of which is closely related to the yeast TAP42 protein of the rapamycin-sensitive signal transduction pathway. *Genomics.* **46**: 373-378.

411. Inui, S., Sanjo, H., Maeda, K., Yamamoto, H., Miyamoto, E. and Sakaguchi, N. (1998) Ig receptor binding protein 1 (alpha4) is associated with a rapamycin-sensitive signal transduction in lymphocytes through direct binding to the catalytic subunit of protein phosphatase 2A. *Blood.* **92**: 539-546.

412. Murata, K., Wu, J. and Brautigan, D. L. (1997) B cell receptor-associated protein alpha4 displays rapamycin-sensitive binding directly to the catalytic subunit of protein phosphatase 2A. *Proc. Natl. Acad. Sci. U S A.* **94**: 10624-10629.

413. Nanahoshi, M., Tsujishita, Y., Tokunaga, C., Inui, S., Sakaguchi, N., Hara, K. and Yonezawa, K. (1999) Alpha4 protein as a common regulator of type 2A-related serine/threonine protein phosphatases. *FEBS Lett.* **446**: 108-112.

414. Kloeker, S. and Wadzinski, B. E. (1999) Purification and Identification of a novel subunit of protein serine/threonine phosphatase 4. *J. Biol. Chem.* **274**: 5339-5347.

415. Lu, K. P. (2000) Phosphorylation-dependent prolyl isomerization: a novel cell cycle regulatory mechanism. *Prog. Cell Cycle Res.* **4**: 83-96.

416. Raught, B., Gingras, A.-C. and Sonenberg, N. (2001) The target of rapamycin (TOR) proteins. *Proc. Natl. Acad. Sci. U S A.* **98**: 7037-7044.

417. Warner, J. R. (1999) The economics of ribosome biosynthesis in yeast. *Trends Biochem. Sci.* **24**: 437-440.

418. Powers, T. and Walter, P. (1999) Regulation of ribosome biogenesis by the rapamycin-sensitive TOR-signaling pathway in Saccharomyces cerevisiae. *Mol. Biol. Cell.* **10**: 987-1000.

419. Zaragoza, D., Ghavidel, A., Heitman, J. and Schultz, M. C. (1998) Rapamycin induces the G(0) program of transcriptional repression in yeast by interfering with the TOR signaling pathway. *Mol. Cell. Biol.* **18**: 4463-4470.

420. Leicht, M., Simm, A., Bertsch, G. and Hoppe, J. (1996) Okadaic acid induces cellular hypertrophy in AKR-2B fibroblasts: involvement of the p70S6 kinase in the onset of protein and rRNA synthesis. *Cell Growth Differ.* **7**: 1199-1209.

421. Mahajan, P. B. (1994) Modulation of transcription of rRNA genes by rapamycin. *Int J Immunopharmacol.* **16**: 711-721.

422. Chan, T.-F., Carvalho, J., Riles, L. and Zheng, X. F. S. (2000) A chemical genomics approach toward understanding the global functions of the target of rapamycin protein (TOR). *Proc. Natl. Acad. Sci. U S A.* **97**: 13227-13232.

423. Beck, T., Schmidt, A. and Hall, M. N. (1999) Starvation induces vacuolar targeting and degradation of the tryptophan permease in yeast. *J. Cell Biol.* **146**: 1227-1238.

424. Klionsky, D. J. and Emr, S. D. (2000) Autophagy as a Regulated Pathway of Cellular Degradation. *Science.* **290**: 1717-1721.

425. Kim, J. and Klionsky, D. J. (2000) Autophagy, cytoplasm-to-vacuole targeting pathway, and pexophagy in yeast and mammalian cells. *Annu. Rev. Biochem.* **69**: 303-342.

426. Klionsky, D. J. and Ohsumi, Y. (1999) Vacuolar import of proteins and organelles from the cytoplasm. *Annu Rev Cell Dev Biol.* **15**: 1-32.

427. Noda, T. and Ohsumi, Y. (1998) Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *J. Biol. Chem.* **273**: 3963-3966.

428. Blommaart, E. F., Luiken, J. J., Blommaart, P. J., van Woerkom, G. M. and Meijer, A. J. (1995) Phosphorylation of ribosomal protein S6 is inhibitory for autophagy in isolated rat hepatocytes. *J. Biol. Chem.* **270**: 2320-2326.

429. Leevers, S. J., Weinkove, D., MacDougall, L. K., Hafen, E. and Waterfield, M. D. (1996) The Drosophila phosphoinositide 3-kinase Dp110 promotes cell growth. *EMBO J.* **15**: 6584-6594.

430. Weinkove, D., Neufeld, T. P., Twardzik, T., Waterfield, M. D. and Leevers, S. J. (1999) Regulation of imaginal disc cell size, cell number and organ size by Drosophila class I(A) phosphoinositide 3-kinase and its adaptor. *Curr. Biol.* **9**: 1019-1029.

431. Verdu, J., Buratovich, M. A., Wilder, E. L. and Birnbaum, M. J. (1999) Cell-autonomous regulation of cell and organ growth in Drosophila by Akt/PKB. *Nat Cell Biol.* **1**: 500-506.

432. Gao, X., Neufeld, T. P. and Pan, D. (2000) Drosophila PTEN regulates cell growth and proliferation through PI3K-dependent and -independent pathways. *Developmental Biol.* **221**: 404-418.

433. Goberdhan, D. C., Paricio, N., Goodman, E. C., Mlodzik, M. and Wilson, C. (1999) Drosophila tumor suppressor PTEN controls cell size and number by antagonizing the Chico/PI3-kinase signaling pathway. *Genes Dev.* **13**: 3244-3258.

434. Huang, H., Potter, C. J., Tao, W., Li, D. M., Brogiolo, W., Hafen, E., Sun, H. and Xu, T. (1999) PTEN affects cell size, cell proliferation and apoptosis during Drosophila eye development. *Development.* **126**: 5365-5372.

435. Oldham, S., Montagne, J., Radimerski, T., Thomas, G. and Hafen, E. (2000) Genetic and biochemical characterization of dTOR, the Drosophila homolog of the target of rapamycin. *Genes Dev.* **14**: 2689-2694.

436. Britton, J. S. and Edgar, B. A. (1998) Environmental control of the cell cycle in Drosophila: nutrition activates mitotic and endoreplicative cells by distinct mechanisms. *Development.* **125**: 2149-2158.

437. Bernal, A. and Kimbrell, D. A. (2000) Drosophila Thor participates in host immune defense and connects a translational regulator with innate immunity. *Proc. Natl. Acad. Sci. U S A.* **97**: 6019-6024.

438. Stewart, M. J., Berry, C. O., Zilberman, F., Thomas, G. and Kozma, S. C. (1996) The Drosophila p70s6k homolog exhibits conserved regulatory elements and rapamycin sensitivity. *Proc. Natl. Acad. Sci. U S A.* **93**: 10791-10796.

439. Miron, M., Verdú, J., Lachance, P. E. D., Birnbaum, M. J., Lasko, P. F. and Sonenberg, N. (2001) The translational inhibitor 4E-BP is an effector of PI3K/Akt signaling in *Drosophila*, and regulates cell growth. *Nature Cell Biol.* **3**: 596-601.

440. Montagne, J., Stewart, M. J., Stocker, H., Hafen, E., Kozma, S. C. and Thomas, G. (1999) Drosophila S6 kinase: a regulator of cell size. *Science.* **285**: 2126-2129.

441. Shioi, T., Kang, P. M., Douglas, P. S., Hampe, J., Yballe, C. M., Lawitts, J., Cantley, L. C. and Izumo, S. (2000) The conserved phosphoinositide 3-kinase pathway determines heart size in mice. *EMBO J.* **19**: 2537-2548.

442. Hentges, K., Thompson, K. and Peterson, A. (1999) The flat-top gene is required for the expansion and regionalization of the telencephalic primordium. *Development.* **126**: 1601-1609.

443. Shima, H., Pende, M., Chen, Y., Fumagalli, S., Thomas, G. and Kozma, S. C. (1998) Disruption of the p70S6k/p85S6k gene reveals a small mouse phenotype and a new functional S6 kinase. *EMBO J.* **17**: 6649-6659.

444. Pende, M., Kozma, S. C., Jaquet, M., Oorschot, V., Burcelin, R., Le Marchand-Brustel, Y., Klumperman, J., Thorens, B. and Thomas, G. (2000) Hypoinsulinaemia, glucose intolerance and diminished β-cell size in S6K1-deficient mice. *Nature.* **408**: 994-997.

445. Blackshear, P. J., Stumpo, D. J., Carballo, E. and Lawrence J C, J. (1997) Disruption of the gene encoding the mitogen-regulated translational modulator PHAS-I in mice. *J. Biol. Chem.* **272**: 31510-31514.

446. Tsukiyama-Kohara, K., Poulin, F., Kohara, M., Demaria, C. T., Cheng, A., Wu, Z., Gingras, A.-C., Katsume, A., Elcheby, M., Spiegelman, B. M., Harper, M.-E.,

Tremblay, M. L. and Sonenberg, N. (2001) Adipose tissue reduction in mice lacking the translational inibitor 4E-BP1. *submitted.*

447. Sugimoto, Y., Whitman, M., Cantley, L. C. and Erikson, R. L. (1984) Evidence that the Rous sarcoma virus transforming gene product phosphorylates phosphatidylinositol and diacylglycerol. *Proc. Natl. Acad. Sci. U S A.* **81**: 2117-2121.

448. Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. and Soltoff, S. (1991) Oncogenes and signal transduction. *Cell.* **64**: 281-302.

449. Chang, H. W., Aoki, M., Fruman, D., Auger, K. R., Bellacosa, A., Tsichlis, P. N., Cantley, L. C., Roberts, T. M. and Vogt, P. K. (1997) Transformation of chicken cells by the gene encoding the catalytic subunit of PI 3-kinase. *Science.* **276**: 1848-1850.

450. Jimenez, C., Jones, D. R., Rodriguez-Viciana, P., Gonzalez-Garcia, A., Leonardo, E., Wennstrom, S., von Kobbe, C., Toran, J. L., R-Borlado, L., Calvo, V., Copin, S. G., Albar, J. P., Gaspar, M. L., Diez, E., Marcos, M. A., Downward, J., Martinez-A, C., Merida, I. and Carrera, A. C. (1998) Identification and characterization of a new oncogene derived from the regulatory subunit of phosphoinositide 3-kinase. *EMBO J.* **17**: 743-753.

451. Borlado, L. R., Redondo, C., Alvarez, B., Jimenez, C., Criado, L. M., Flores, J., Marcos, M. A., Martinez-A, C., Balomenos, D. and Carrera, A. C. (2000) Increased phosphoinositide 3-kinase activity induces a lymphoproliferative disorder and contributes to tumor generation in vivo. *FASEB J.* **14**: 895-903.

452. Shayesteh, L., Lu, Y., Kuo, W. L., Baldocchi, R., Godfrey, T., Collins, C., Pinkel, D., Powell, B., Mills, G. B. and Gray, J. W. (1999) PIK3CA is implicated as an oncogene in ovarian cancer. *Nat. Genet.* **21**: 99-102.

453. Hu, L., Zaloudek, C., Mills, G. B., Gray, J. and Jaffe, R. B. (2000) In vivo and in vitro ovarian carcinoma growth inhibition by a phosphatidylinositol 3-kinase inhibitor (LY294002). *Clin Cancer Res.* **6**: 880-886.

454. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S. H., Giovanella, B. C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M. H. and Parsons, R. (1997) PTEN, a putative protein

tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science.* **275**: 1943-1947.

455. Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K., Lin, H., Ligon, A. H., Langford, L. A., Baumgard, M. L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D. H. and Tavtigian, S. V. (1997) Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat. Genet.* **15**: 356-362.

456. Liaw, D., Marsh, D. J., Li, J., Dahia, P. L., Wang, S. I., Zheng, Z., Bose, S., Call, K. M., Tsou, H. C., Peacocke, M., Eng, C. and Parsons, R. (1997) Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nat. Genet.* **16**: 64-67.

457. Marsh, D. J., Dahia, P. L., Zheng, Z., Liaw, D., Parsons, R., Gorlin, R. J. and Eng, C. (1997) Germline mutations in PTEN are present in Bannayan-Zonana syndrome. *Nat. Genet.* **16**: 333-334.

458. Di Cristofano, A., Pesce, B., Cordon-Cardo, C. and Pandolfi, P. P. (1998) Pten is essential for embryonic development and tumour suppression. *Nat. Genet.* **19**: 348-355.

459. Suzuki, A., de la Pompa, J. L., Stambolic, V., Elia, A. J., Sasaki, T., del Barco Barrantes, I., Ho, A., Wakeham, A., Itie, A., Khoo, W., Fukumoto, M. and Mak, T. W. (1998) High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. *Curr. Biol.* **8**: 1169-1178.

460. Podsypanina, K., Ellenson, L. H., Nemes, A., Gu, J., Tamura, M., Yamada, K. M., Cordon-Cardo, C., Catoretti, G., Fisher, P. E. and Parsons, R. (1999) Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. *Proc. Natl. Acad. Sci. U S A.* **96**: 1563-1568.

461. Cheng, J. Q., Altomare, D. A., Klein, M. A., Lee, W. C., Kruh, G. D., Lissy, N. A. and Testa, J. R. (1997) Transforming activity and mitosis-related expression of the AKT2 oncogene: evidence suggesting a link between cell cycle regulation and oncogenesis. *Oncogene.* **14**: 2793-2801.

462. Mirza, A. M., Kohn, A. D., Roth, R. A. and McMahon, M. (2000) Oncogenic transformation of cells by a conditionally active form of the protein kinase Akt/PKB. *Cell Growth Differ.* **11**: 279-292.

463. Nakatani, K., Thompson, D. A., Barthel, A., Sakaue, H., Liu, W., Weigel, R. J. and Roth, R. A. (1999) Up-regulation of Akt3 in estrogen receptor-deficient breast cancers and androgen-independent prostate cancer lines. *J. Biol. Chem.* **274**: 21528-21532.

464. Ruggeri, B. A., Huang, L., Wood, M., Cheng, J. Q. and Testa, J. R. (1998) Amplification and overexpression of the AKT2 oncogene in a subset of human pancreatic ductal adenocarcinomas. *Mol Carcinog.* **21**: 81-86.

465. Cheng, J. Q., Godwin, A. K., Bellacosa, A., Taguchi, T., Franke, T. F., Hamilton, T. C., Tsichlis, P. N. and Testa, J. R. (1992) AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. *Proc. Natl. Acad. Sci. U S A.* **89**: 9267-9271.

466. Cheng, J. Q., Ruggeri, B., Klein, W. M., Sonoda, G., Altomare, D. A., Watson, D. K. and Testa, J. R. (1996) Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA. *Proc. Natl. Acad. Sci. U S A.* **93**: 3636-3641.

467. Bellacosa, A., de Feo, D., Godwin, A. K., Bell, D. W., Cheng, J. Q., Altomare, D. A., Wan, M., Dubeau, L., Scambia, G., Masciullo, V. and al, e. (1995) Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. *Int. J. Cancer.* **64**: 280-285.

468. Aoki, M., Blazek, E. and Vogt, P. K. (2001) A role of the kinase mTOR in cellular transformation induced by the oncoproteins P3k and Akt. *Proc. Natl. Acad. Sci. U S A.* **98**: 136-141.

469. Gibbons, J. J., Discafani, C., Peterson, R., Hernandez, R., Skotnicki, J. and Frost, P. (1999) The effect of CCI-779, a novel macrolide anti-tumor agent, on the growth of human tumor cells *in vitro* and in nude mouse xenografts *in vivo*. *Proc Amer Ass Cancer Res.* **40**: 301.

470. Majewski, M., Korecka, M., Kossev, P., Li, S., Goldman, J., Moore, J., Silberstein, L. E., Nowell, P. C., Schuler, W., Shaw, L. M. and Wasik, M. A. (2000) The immunosuppressive macrolide RAD inhibits growth of human Epstein-Barr virus-transformed B lymphocytes in vitro and in vivo: A potential approach to prevention and treatment of posttransplant lymphoproliferative disorders. *Proc. Natl. Acad. Sci. U S A.* **97**: 4285-4290.

471. Duncan, R., Milburn, S. C. and Hershey, J. W. (1987) Regulated phosphorylation and low abundance of HeLa cell initiation factor eIF-4F suggest a role in translational control. Heat shock effects on eIF-4F. *J. Biol. Chem.* **262**: 380-388.

472. Hiremath, L. S., Webb, N. R. and Rhoads, R. E. (1985) Immunological detection of the messenger RNA cap-binding protein. *J. Biol. Chem.* **260**: 7843-7849.

473. Rousseau, D., Kaspar, R., Rosenwald, I., Gehrke, L. and Sonenberg, N. (1996) Translation initiation of ornithine decarboxylase and nucleocytoplasmic transport of cyclin D1 mRNA are increased in cells overexpressing eukaryotic initiation factor 4E. *Proc. Natl. Acad. Sci. U S A.* **93**: 1065-1070.

474. De Benedetti, A., Joshi, B., Graff, J. R. and Zimmer, S. G. (1994) CHO cells transformed by the translation factor eIF-4E display increased c-*myc* expression, but require overexpression of Max for tumorigenicity. *Mol. Cell. Diff.* **2**: 347-371.

475. Fukuchi-Shimogori, T., Ishii, I., Kashiwagi, K., Mashiba, H., Ekimoto, H. and Igarashi, K. (1997) Malignant transformation by overproduction of translation initiation factor eIF4G. *Cancer Res.* **57**: 5041-5044.

476. Brass, N., Heckel, D., Sahin, U., Pfreundschuh, M., Sybrecht, G. W. and Meese, E. (1997) Translation initiation factor eIF-4gamma is encoded by an amplified gene and induces an immune response in squamous cell lung carcinoma. *Hum. Mol. Genet.* **6**: 33-39.

477. Eberle, J., Krasagakis, K. and Orfanos, C. E. (1997) Translation initiation factor eIF-4A1 mRNA is consistently overexpressed in human melanoma cells in vitro. *Int. J. Cancer.* **71**: 396-401.

ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

1) Picornaviruses (encephalomyocarditis virus, EMCV, and poliovirus) dephosphorylate and activate 4E-BP1. In the case of EMCV, this dephosphorylation coincides with the shutoff of host cell protein synthesis. In the case of poliovirus, the dephosphorylation depends on viral replication, and lags behind the shutoff. Infection with adenovirus induces hyperphosphorylation of 4E-BP1 early in infection, during which translation of the viral RNA proceeds via a cap-dependent mechanism. 4E-BP1 remains in the hyperphosphorylated state even at later times after infection, during which the shutoff of host protein synthesis occurs.

2) PI3 kinase, Akt/PKB and FRAP/mTOR relay signals to 4E-BP1 phosphorylation. PI3-kinase-induced phosphorylation of 4E-BP1 is sensitive to wortmannin, indicating that PI3K is the wortmannin-sensitive step in this pathway. Activated Akt/PKB induces phosphorylation of 4E-BP1 and 4E-BP2 in a wortmannin-resistant, but rapamycin-sensitive manner. Akt/PKB induces phosphorylation of 4E-BP1 on the same sites that are phosphorylated following insulin or growth factor stimulation. The effects of the Akt/PKB-induced 4E-BP1 phosphorylation are the inactivation of 4E-BP1, and the release of eIF4E. Rapamycin-sensitivity of Akt/PKB-induced 4E-BP1 phosphorylation can be overcome by overexpression of a rapamycin-resistant FRAP/mTOR. Thus, the activities of these kinases in mediating 4E-BP1 phosphorylation can be ordered: PI3K>Akt/PKB>FRAP/mTOR.

3) 4E-BP1 phosphorylation on the multiple phosphorylation sites occurs via a step-wise mechanism. A combination of phosphopeptide-mapping and mass spectrometry allowed the identification of two *in vivo* sites, Thr37 and Thr46. Phosphorylation of Thr37 and Thr46 is obtained *in vitro* with immunoprecipitates of FRAP/mTOR. Phosphorylation of these two sites is insufficient to mediate release from eIF4E. *In vivo*, Thr37 and Thr46 are phosphorylated under basal (i.e. serum-starved) conditions, and the phosphorylation of these sites is only slightly affected by incubation with PI3K or FRAP/mTOR inhibitors. The phosphorylation of two sites, Thr37 and Thr46, is required for the phosphorylation of the other 4E-BP1 phosphorylation sites.

4) The 4E-BP1 phosphorylation sites which exhibit the strongest serum-dependence, Ser65 and Thr70, were identified by a combination of phosphopeptide mapping and mass spectrometry. These sites are also extremely sensitive to inhibition of PI3 kinase and FRAP/mTOR signaling. Phosphate addition onto 4E-BP1 was further ordered, via the use of a combination of two-dimensional isoelectric focusing/SDS-PAGE and phosphospecific antibodies. Phosphorylation of Thr37 and Thr46 precedes that of Thr70, which itself occurs prior to Ser65 phosphorylation. Thus, the phosphorylation of 4E-BP1 culminates at Ser65. Phosphorylation of Ser65 alone, or Ser65 and Thr70 in combination, is however insufficient to mediate release from eIF4E, indicating that phosphorylation of a combination of sites (probably Thr37, Thr46, Ser65 and Thr70) is necessary to mediate release.