A role for the Nck Adapter in Protein Translation

Sem Kebache

Department of Medicine

Division of Experimental Medicine

McGill University

Montreal, Quebec, Canada

A thesis submitted to the Faculty of Graduate and Post-doctoral Studies in partial fulfillment of the requirements of the degree of Doctor of Philosophy.

Your file Votre référence
ISBN: 0-612-88495-3
Our file Notre référence
ISBN: 0-612-88495-3

"The only real valuable thing is intuition."

"If we knew what we were doing, it wouldn't be called research, would it? "

Albert Einstein (1879-1955)

ABSTRACT

In mammals, Nck, represented by two genes, is a 47kDa protein lacking intrinsic enzymatic function. It is composed solely of three N-terminal Src-homology 3 (SH3) domains and a single C-terminal Src-homology 2 (SH2) domain. Nck is classified as an adapter molecule that links cell surface receptors, via its SH2 domain, to downstream effectors, through its SH3 domains. Two cDNAs coding for the carboxy-terminal region of the β subunit of the eukaryotic initiation factor 2 (eIF2β) were isolated from a two-hybrid screen to identify new effector molecules interacting with the SH3 domains of Nck. eIF2β is a component of the molecular complex eIF2 responsible for one of the early steps in the initiation of protein synthesis. In this thesis, I determined *in vivo* that the first and the third SH3 domains of Nck were both required for its interaction with eIF2β. Furthermore, Nck and eIF2β colocalized in an enriched ribosomal fraction. The localization of Nck in this compartment is enhanced upon insulin stimulation. I also established that Nck-1 overexpression is concomitant with an increase in protein translation. Interestingly, the effect of Nck on translation was dependent on its first and third SH3 domains originally found to mediate its interaction with eIF2β. To elucidate how Nck modulates protein translation, I examined whether Nck maintains its positive effects under ER stress conditions where protein synthesis is normally inhibited. Interestingly, my data revealed that Nck-1 overexpression antagonized ER stress-induced inhibition of translation. Furthermore, I demonstrated that overexpression of Nck-1 prevented PERK activation, eIF2α phosphorylation, BiP induction and cell survival in response to ER stress treatments. This integrates

Nck in the regulation of the ER unfolded protein response (UPR). I also provided evidence that the effects of Nck on the UPR involve its second SH3 domain and a phosphatase-dependent mechanism. Finally, using Nck deficient cells, I established that Nck is required for an optimal protein synthesis. All together these data uncovered for the first time a role for an SH2/SH3 adapter in the regulation of protein synthesis through its direct interaction with a component of the translation machinery as well as in modulating ER stress-induced UPR. These observations open up a new field of investigation on how major signaling molecules regulate cellular responses such as protein translation. Furthermore, this new information may allow the design of new therapeutic strategies in common diseases such as cancer, neurodegenerative disorders and diabetes.

RÉSUMÉ

Chez les mammifères, Nck représenté par deux gènes, est une protéine de 47kDa dépourvue de fonction enzymatique intrinsèque. Nck possède dans sa région N-terminale, trois domaines SH3 (homologue 3 de Src) et dans sa région C-terminale, un seul domaine SH2 (homologue 2 de Src). Nck est classé comme une molécule adaptatrice du fait qu'elle lie par l'entremise de son domaine SH2, les récepteurs à la surface de la cellule, aux molécules effectrices intracellulaires associées à ses domaines SH3. Un criblage dans un systeme 2-hybrides chez la levure nous a permis d'isoler deux clones représentant la région carboxy-termnale de la sous-unité β du facteur d'initiation eukaryotique eIF2 (eIF2β), qui interagissaient avec les domaines SH3 de Nck. eIF2β une composante du complexe moléculaire eIF2 qui est responsable d'une des étapes précoce de l'initiation de la synthèse protéique. Dans cette thèse, j'ai montré que le premier et le troisième domaines SH3 de Nck sont tout deux nécessaires pour son interaction avec eIF2β. De plus, j'ai démontré que Nck et eIF2β colocalisent dans une fraction enrichie en ribosomes. Cette localisation est accentuée suite à une stimulation par l'insuline. J'ai aussi établi que la surexpression de Nck-1 coincide avec une augmentation de la synthèse protéique. Cet effet de Nck-1 sur la traduction implique les mêmes domaines SH3 qui sont responsables de son interaction avec eIF2β. Pour élucider les mécanismes utilisés par Nck pour influencer la traduction des protéines, j'ai examiné si ces effets étaient préservés dans des conditions où la traduction est inhibée comme par example en réponse à des situations de stress du réticulum endoplasmique (RE). Mes données

révèlent que dans ces conditions, la surexpréssion de Nck-1 prévient l'inhibition de la traduction. J'ai démontré que la surexpréssion de Nck-1 empèche l'activation de PERK, la phosphorylation de eIF2α, l'induction de la molécule chaperone BiP et ne protége pas les cellules de la mort programmée, intégrant ainsi Nck dans la régulation de la signalisation du RE en réponse au repliement inapproprié des protéines (UPR). De plus, j'ai observé que les effets de Nck sur la translation dans les conditions de stress, sont médiés par son deuxième domaine SH3 et un mécanisme qui implique l'activation d'une phosphatase de type sérine/thréonine. Finalement, en utilisant des cellules dépourvues en Nck, j'ai établi que Nck est requis pour qu'un niveau optimal de synthèse protéique soit conservé. Dans leur ensemble, mes données me permettent pour la première fois d'impliquer un adaptateur SH2/SH3 à la fois dans la régulation de la synthèse protéique par son interaction directe avec une composante de la machinerie de traduction des protéines, mais aussi, par la même occasion dans la modulation de la réponse du RE aux conditions de stress. Ces observations ouvrent une nouvelle avenue de recherche qui nous permettra de mieux comprendre comment les molécules de signalisation régulent les réponses cellulaires comme la traduction protéique. Ces nouvelles informations pouront conduire à l'élaboration de nouvelles modalités thérapeutiques de maladies courantes comme le cancer, les maladies neurodégénératives et le diabète.

ACKNOWLEDGMENTS

I dedicate this thesis to my family, especially to my mother and my deceased father whose love and unconditional support have played a major role in helping me to attain this achievement and to whom I will be eternally indebted.

I would like also to express my sincere and deepest thanks to my supervisor, Dr. Louise Larose, for all her help, guidance, encouragement and support throughout the duration of my Ph.D. studies and beyond. She has been an endless source of energy, enthusiasm and inspiration and her lab has provided me with an ideal place to complete my work for which I am grateful.

My thanks go also to the members of my thesis committee, namely: Dr. Katherine Cianflone, Dr. Simon Wing and Dr. Jean-Jacques Lebrun for helpful discussions, suggestions, guidance and support.

Thanks to all members of the Polypeptide Hormone Laboratory, past and present, especially Dr. Barry Posner, Dr. Michael Greenwood and Simon Wing for their valuable help and scientific discussions; Gerald Baquiran, Nathalie Bedard, Victor Dumas, Sheryl Jackson, Mary Lapenna, Tabitha Tosti, Jocelyn Reid, Alejandro Balbis, Mei Kong, my group: Dongmei Zuo, Eric Cardin and Jenny Ping Yang, for their precious technical supports and friendship, the members of Dr. John, J. M. Bergeron, especially Pamela Cameron and Ali Fazel

for their experimental help and my friends: Dr. Eric Chevet and Dr. Duc Thang Nguyen for their encouragement, support, discussions and collaborative work.

I am also extremely grateful to Dr. Gerald Price: the chairman of the Division of Experimental Medicine for his precious advice and guidance and also many thanks to his secretary, Dominique Besso for her kindness, serenity and reliability.

Last but not least, I extend my most sincere thanks to the Canadian Diabetes Association in memory of Aurele Labelle for its financial support.

PREFACE

This thesis has been written according to the guidelines for a manuscript-based thesis issued by the Faculty of Graduate and Post-doctoral Studies of McGill University. These guidelines read in part:

1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.)

2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceeding and following each manuscript are mandatory.

3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts.

The thesis must include the following:

1. a table of contents;
2. a brief abstract in both English and French;
3. an introduction which clearly states the rational and objectives of the research;
4. a comprehensive review of the literature (in addition to that covered in the introduction to each paper);
5. a final conclusion and summary;
6. a thorough bibliography;
7. Appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material.

4. As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

5. In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers.

This thesis consists of a General Introduction, One published paper, a second manuscript submitted for publication and a general Discussion. A section concerning the 'Contribution of the Authors' is included.

CONTRIBUTIONS OF AUTHORS

Two first-author publications arose from the work in this thesis:

1) **Sem Kebache**, Dongmei Zuo, Eric Chevet and Louise Larose, "Modulation of Protein Translation by Nck-1." *Proc. Nat. Acad. Sci,* USA, 2002, **99:** 5406-5411.

2) **Sem Kebache**, Duc Thang Nguyen, Eric Chevet and Louise Larose, "Nck-1 antagonizes Endoplasmic Reticulum stress-induced Inhibition of Translation." *J. Biol. Chem*, submitted.

The specific contributions of other authors to these manuscripts are as follows.

All other experimental work reported in these papers were performed by myself

(see 'Contribution to Original Research'):

1) D.Z. provided the data for figure.1C and figure.1D. E.C. provided experimental expertise for the sucrose cushion and subcellular fractionation methods. L.L. provided data for Table.1.

2) D.T.N. and E.C. provided data for figure.2D.

Other publications

1) Jiang-Feng Liu, Eric Chevet, **Sem Kebache**, Gilles Lemaitre, Denis Barritault, Louise Larose and Michel Crepin, "Functional Rac-1 and Nck signaling networks are required for FGF-2-induced DNA synthesis in MCF-7 cells."*Oncogene,* 1999, 18: 6425-6433.

I provided the RT-PCR and the Southern blot analysis for LAT protein.

2) Duc Thang Nguyen, Sarah Jenna, Ali Fazel, **Sem Kebache**, Anouk Emadali, Hetty N. Wong, Louise Larose, John. J. M. Bergeron, Randall J. Kaufman and Eric Chevet, " Mechanism of Regulation of Endoplasmic Reticulum Stress Response by STAT-5 and Nck Downstream of IREα." *In preparation*.

I constructed the GSTNck-1 fusion proteins.

ABREVIATIONS

4EBP1 : eIF4E binding protein 1

Arp2/3 : actin related protein 2/3

ATF6 : activating transcription factor 6

ATP : adenosine triphosphate

BiP : immunoglobulin heavy-chain-binding protein

cDNA : complemantary Deoxyribonucleic acid

CEBP : CAAT enhancer binding protein

CHOP : CEBP homologous protein

CKI : casein kinase I

CrPV : cricket paralysis virus

DCC : deleted colorectal cancer

DNA : deoxyribonucleic acid

dsRNA : double stranded ribonucleic acid

DTT : dithiothreitol

eIF : eukaryotic initiation factor

EGF : epidermal growth factor

EGFR : epidermal growth factor receptor

ERK : extracellular signal-regulated kinase

FAK : focal adhesion kinase

FGF : fibroblast growth factor

FLT-1 : fms-like tyrosine kinase

GADD153 : growth arrest and DNA damage inducible 153

GADD34 : growth arrest and DNA damage inducible 34

GAP : GTPase-activating protein

GBD : GTPase binding domain

GCN2 : general control non-repressed 2

GDP : guanosine 5'-diphosphate

GEF : guanine nucleotide exchange factor

GFP : green fluorescent protein

GPCR : G protein-coupled receptor

GRP : glucose-regulated protein family

GSK-3 : glycogen synthase kinase-3

GTP : guanosine 5'-triphosphate

HIV : human immunodeficiency virus

HSV : herpes simplex virus

HRI : heme-regulated inhibitor

IGF : insulin growth factor

IR : insulin receptor

IRE1 : inositol requiring 1

IRES : internal ribosomal entry site

IRS : insulin receptor substrate

JNK : Jun N-terminal kinase

MAPK : mitogen-activated protein kinase

MEK : mitogen or extracellular regulated kinase

MEKK : mitogen or extracellular regulated kinase kinase

Met : methionine

Mnk : MAPK-interacting kinase

mRNA : messenger ribonucleic acid

mTOR : mammalian target of rapamycin

NAK : Nck-associated kinase

NF-κB : nuclear factor κB

NIDDM : non-insulin dependent diabetes mellitus

NIK : Nck-interacting kinase

ORF : open reading frame

Pak : p21-activated serine/threonine kinase

PDGF : platelet-derived growth factor

PDGFR : platelet-derived growth factor receptor

PDK : phosphatidylinositol-dependent kinase

PERK : PKR–like endoplasmic reticulum kinase

PI3-K : phosphatidylinositol 3-kinase

PKA : protein kinase A

PKB : protein kinase B

PKC : protein kinase C

PKN : lipid-regulated protein kinase N

PKR : interferon inducible double-stranded RNA-activated-kinase

PLCγ : phospholipase Cγ

PS1 : presenilin-1

PTB : phosphotyrosine binding domain

PtdIns : phosphatidylinositols

PTK : protein tyrosine kinase

RKS : receptor kinase substrate

RNA : ribonucleic acid

RNAse : endoribonuclease

RTK : receptors tyrosine kinase

SAPK : stress activated protein kinase

SDS-PAGE: sodium deodecyl-sulfate polyacrylamide gel electrophoresis

Ser : serine

SH2 : Src-homology domain 2

SH3 : Src-homology domain 3

SH-PTP2 : SH2-containing phosphotyrosine phosphatase 2

Sos : son of sevenless

SP1/SP2 : site-1 protease and site-2 protease

S/TPK : serine/threonine protein kinase

STAT-5 : signal transducer and activator of transcription

Thr : threonine

Tir : translocated intimin receptor

TNF : tumor necrosis factor

$tRNA_i$: transfer ribonucleic acid initiator

Tyr : tyrosine

UPR : unfolded protein response

UTR : untranslated region

WAS : Wiskott-Aldrich syndrom

WASP : Wiskott-Aldrich syndrom protein

WIP : WASP interacting protein

XBP1 : bZIP transcription factor X-box binding protein 1

TABLE OF CONTENTS

LIST OF FIGURES

Chapter I

Chapter II

Chapter III

LIST OF TABLES

Chapter II

CHAPTER I

Introduction and Literature Review

A. Signal Transduction: *General Concepts*

Transduction of extracellular signals from the plasma membrane to intracellular targets has been an intensive field of study for the understanding of cell functions such as proliferation, differentiation, gene transcription, development, cell motility, survival and protein translation. Intracellular signaling triggered by external stimuli (i.e. growth factors) is initiated by the activation of transmembrane receptors tyrosine kinases (RTKs), which play an important role in these processes. Ligand-induced activation of RTKs is characterized by receptor dimerization and transphosphorylation of tyrosine residues (Tyr) on the cytoplasmic domain of the receptors. This enhances the intrinsic tyrosine kinase activity of the RTKs and creates high affinity docking sites for proteins possessing Src-homology domain 2 (SH2) and/or phosphotyrosine-binding (PTB) domains (Fig.1) (1). The PTB domain was first identified in the Shc signaling protein, as a region enabling binding to phosphorylated Tyr sites on activated receptor tyrosine kinases (2, 3). However, recent studies showed that PTB domain-like protein modules can also bind to proteins independently of tyrosine phosphorylation (4, 5). Unlike SH2 domains, PTB domains share low sequence homology amongst themselves and exhibit extremely high ligand binding selectivity (reviewed in 6). Shc and the insulin receptor substrate 1-4 (IRS-1-4) are among the PTB-containing proteins commonly called docking proteins (6).

SH2 domain-containing proteins, on the other hand, can be grouped into two classes: Enzymes, such as Src family tyrosine kinases, PLCγ, and rasGAP, and

adapter proteins, a group of SH2- and SH3 domain-containing proteins, devoid of any enzymatic activity (reviewed in 7-9). This group mainly includes Crk (Crk-II and Crk-L) (reviewed in 10), Grb2 (Sem-5/Ash) (11), Nck (Nckα/Nck/Nck1 and Nckβ/Grb4/Nck-2) (reviews by 12-14), Grb10 (15) and the p85 regulatory subunit of the phosphatidylinositol 3-kinase (PI3-K) (16, 17). These adapters are believed to link cell surface receptors, via their SH2 domain, to downstream effectors via their SH3 domains (Fig.1). Such proteins are thought to be responsible for the formation of multi molecular complexes in specific subcellular compartments, like plasma membranes, endosomes and cytoskeleton (9, 18-21).

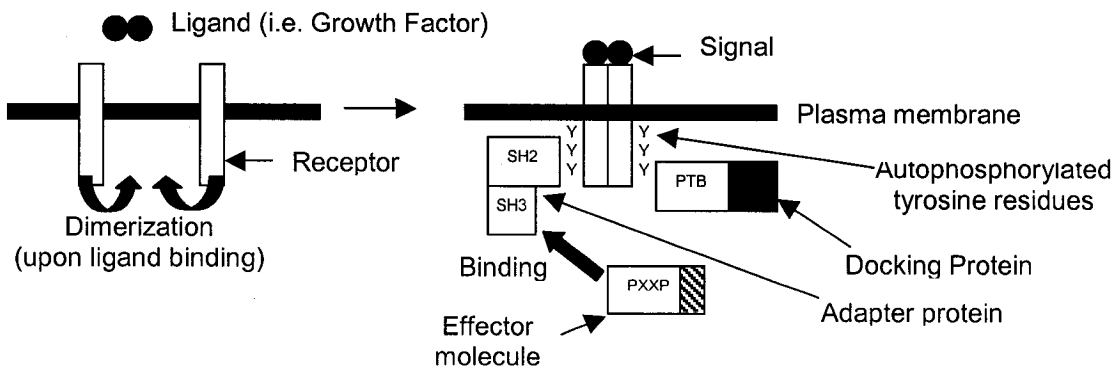


Fig.1. Ligand-Induced Receptor Tyrosine Kinase Activation.

B. The Nck adapter protein

Nck was identified during the screening of a human melanoma complemantary deoxyribonucleic acid (cDNA) expression library, using a monoclonal antibody specific to the melanoma MUC18 antigen (22). Later, a murine homologue of Nck, termed Grb4 and also referred as Nck-2 (Nckβ) was isolated when identifying novel SH2 domain containing proteins bound to the carboxy-terminal of the EGF receptor (23). Both human and mouse Nck isoform cDNAs encode a 47 kDa protein consisting entirely of three N-terminal SH3 domains and a single C-terminal SH2 domain (Fig. 2). Implying a function in diverse cellular events, the Nck mRNA transcript and protein are expressed in all tissues and cell types tested (24, 25). Upon growth factor stimulation, Nck undergoes intensive phosphorylation on threonine and serine residues and in some occasions also on tyrosine residues (24-26).

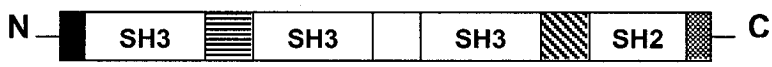


Fig.2. Structure of the adapter protein Nck (See text for details).

Nck-1 (Nckα) and Nck-2 (Nckβ) are encoded by two distinctive genes. The human Nck-1 gene is localized to the q21 locus of the chromosome 3. This chromosomal locus has been associated with various human hematopoietic malignancies, suggesting a potential role for Nck in oncogenesis. The human Nck-2 gene resides at the q12 on chromosome 2 (27-29). The amino acid variations between Nck-1 and Nck-2 are observed largely in the interval sequences between the SH domains, but overall they share 67% homology. Additional comparisons within the SH2 and SH3 domains show an amino acid identity of 80% between the two isoforms (14).

Single Nck-like genes have been reported in lower organisms such as Xenopus (30), Drosophila (31) and *C. elegans* (28). Comparisons of overall sequences and individual domains of the human Nck-1 show 87% and 44% amino acid identity with Xenopus Nck and Drosophila Nck (dreadlocks, dock), respectively. The human Nck-2 shares also 68% and 43% amino acid identity with the latter (14). These high levels of conservation among species suggest that Nck functions must be preserved through evolution.

I. Tyrosine-phosphorylated proteins that associate the SH2 domain of Nck

Several RTKs and substrates of receptor tyrosine kinases have been shown to interact with the SH2 domain of Nck (Fig.3). Via its SH2 domain, Nck associates with activated epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptor tyrosine kinases (24-26, 32). A phosphotyrosine site (pY751) in the PDGF receptor cytoplasmic domain that is known to bind one of the two SH2 domains of the p85 subunit of PI3-K has been shown also to associate directly with the SH2 of Nck (33). This suggests that Nck may compete with PI3-K for the binding to PDGFR and antagonize the activation of PI3-K (33). However, this hypothesis needs to be validated. In contrast, association between Nck and the EGFR has been shown to be indirect via the cooperation of a 62kDa tyrosine phosphorylated docking protein, the GAP- associated $p62^{dok}$ protein (34). Upon insulin stimulation, association of Nck through its SH2 domain, with the phosphotyrosine residue Y361 on $p62^{dok}$ has also been reported (35). In addition, $p62^{dok}$ has been involved in the recruitment of Nck into a molecular complex containing the EphB2 receptor, a member of the Eph RTKs that regulate axonal guidance and neuronal bundling (36). Moreover, in another system, stimulation of EphB1 and EphB2 results in a complex formation between Nck, Nck-interacting kinase (NIK), Dok-1, RasGAP and an unidentified 145kDa tyrosine phosphoprotein (37). It is hypothesized that tyrosine-phosphorylated Dok directly interacts with the SH2 domain of Nck, while NIK is bound through the SH3 domains of Nck. In this model, it is believed that the recruitment of NIK to phosphotyrosine docking molecules by Nck is an important step in EphB receptor

signaling. EphB1 receptor has been shown to bind not only Nck but also its Drosophila homologue Dock, which has been implicated in photoreceptor axonal guidance and targeting (31, 38). Moreover, activation of EphB1 by its ligand, ephrin-B1/Fc, is shown to recruit Nck to native receptor complexes and activate c-Jun kinase (JNK/SAPK) (38).

In addition to $p62^{dok}$, several other docking proteins have been reported to interact with Nck. For instance, Nck forms a stable complex *in vivo* with IRS-1 and IRS-3 in insulin-stimulated cells (39, 40). These interactions result from the binding of the tyrosine-phosphorylated IRS proteins to the SH2 domain of Nck. Interestingly, these interactions of Nck with IRS proteins and the insulin-induced binding of Nck with Dok point out a role for Nck in signaling pathways downstream of the insulin receptor. However, to date no additional information has contributed to clearly establish a role of Nck in mediating insulin actions.

The SH2 domain of Nck has been reported *in vitro* to associate with a tyrosine kinase activity attributed to c-Src (32). However, this is not believed to result from a direct association between Nck and c-Src but rather through the docking protein $p130^{Cas-1}$ which would link Nck and c-Src (41). In addition, a recent study showed that both Nck isoforms could interact with the focal adhesion kinase (FAK), a cytoplasmic protein tyrosine kinase critically involved in the control of cell motility (42). This interaction is mediated by the SH2 domain of Nck and depends on the phosphorylation of Tyr397 on FAK, a site involved in the regulation of cell motility (42). Knowing that FAK and $p130^{Cas-1}$ are recruited

downstream to integrin activation, these findings suggest a model where fibronectin-mediated integrin engagement induces FAK activation, tyrosine phophorylation of $p130^{Cas-1}$ and results in the formation of a multimeric signaling complex containing Nck. Overall these information raise the importance to address the role of Nck in the modulation of cell motility.

Nck has been implicated in bacterial virulence (43, 44). In fact, the enteropathogenic *Escherichia coli* (EPEC) a bacterial pathogen that cause infantile diarrhea worldwide, injects a bacterial protein namely, the translocated intimin receptor (Tir), into the host-cell plasma membrane where it acts as a receptor for the bacterial outer membrane protein, intimin (45). The interaction of Tir and intimin triggers a marked rearrangement of the host actin cytoskeleton into pedestrals beneath adherent bacteria, thus conferring to this pathogen its potential virulence. Recent studies in EPEC have shown direct binding of Nck with Tir, in an SH2-dependent manner. Furthermore, Nck-Tir interaction is required for the recruitment of both N-WASP and the actin related protein (Arp2/3) complex. The assembly of this multi molecular complex, clearly dependent on Nck, leads to the formation of the EPEC pedestal.

II. Effector molecules that associate the SH3 domains of Nck.

A number of proteins interacting with the SH3 domains of Nck have been identified. These include guanine exchange factor (GEF), Serine/Threonine protein kinases (S/TPK), protein tyrosine kinases (PTK) and various molecules involved in the rearrangement of the cytoskeleton (summarized in Fig.3).

One of the first SH3 binding partners for Nck initially identified was the guanine exchange factor son of sevenless (Sos), suggesting that Nck, similarly to the adapter protein Grb2, is involved in Ras activation (46, 47). This was demonstrated by the fact that overexpression of Nck in NIH-3T3 cells strongly activates a gene reporter regulated by the *fos* promoter and this can be blocked by dominant negative Ras. In addition, an inhibitory effect of dominant negative Nck (with mutations that prevent the binding of its SH domains) was observed on fibroblast growth factor (FGF)-induced extracellular signal-regulated kinase 1 (ERK1) activation in *Xenopus* animal caps. Targeting the first two SH3 domains of Nck to the plasma membrane activated ERK1 in the absence of any growth factor. This suggests that the recruitment of Sos at the membrane through Nck is sufficient for Ras and ERK1 activation (48) and that, in addition to Grb2, Nck could contribute to Ras activation. However, no Nck-dependent recruitment of Sos by activated receptor tyrosine kinases, such as the PDGF or EGF receptors, have been demonstrated so far. In contrast, using Nck constructs containing mutations in the SH2 or SH3 domains, it was demonstrated that Nck had no effect on ERK activation in response to EGF stimulation (49). In addition, the lack

of effect of neutralizing anti-Nck antibody, microinjected into fibroblasts on c-Fos expression, strongly suggests that Nck does not contribute to PDGF-dependent Ras activation (50). On the other hand, overexpression of Nck in PC12 cells blocked NGF- and bFGF-induced neurite outgrowth, indicating an inhibitory effect of Nck on Ras-dependent differentiation (51). In the light of these divergent results, it remains to establish clearly whether the interaction of Nck with Sos is mediating Ras activation.

Members of the Pak family of Ser/Thr kinases are believed to be the targets of the small G-proteins, Rac1 and Cdc42. Paks play an important role in a wide range of cellular events, such as organization of the actin cytoskeleton and stress response (52). Mammals express at least four Pak isoforms: Pak1 (Pakα), Pak2 (Pakβ), Pak3 (Pakχ) and Pak4 (52). Two members of the Pak family, Pak1 and Pak3, have been shown to be constitutively associated in quiescent and growth factor-stimulated cells with the second SH3 domain of Nck (53-56) and it is well established that Nck links RTKs, Rho family GTPases and Pak S/TPKs (53-55). Furthermore, membrane localization of Pak1, either by direct fusion of a myristoylation signal to Pak1 or by translocation mediated by the binding to the second SH3 domain of Nck, is sufficient to activate Pak1 kinase activity as well as the MAP kinase cascade (53). Therefore, it is believed that the recruitment of Nck by the RTKs serves to translocate Pak1 at the membrane (53), thus bringing Pak1 in close proximity of the GTP-bound of Rac1 and Cdc42. However, even though EGF enhanced Pak1 activity under a

mechanism dependent on GTP bound form Rac1 and Cdc42, the mechanism by which Pak is activated at the membrane level remains to be defined (54, 57).

Another Ser/Thr kinase shown to associate with the second SH3 domain of Nck is PRK2 (58), a homologue of PKN. In PRK2, the proline-rich consensus sequence, which mediates its interaction with Nck, is similar to the one found in Pak1, Pak3 and Sos. This suggests that Sos, PAK and PRK2 may compete for the same binding site on Nck. PRK2 is known, as PKN, to associate with the GTP-bound form of the RhoA GTPase and leads to the transcriptional activation of a RhoA dependent reporter gene. Together, these data strongly suggest a functional role for Nck in the activation of the small GTPases through the involvement of exchange factors Sos or S/TPKs (Pak1, Pak3, and PRK2). The Nck-Sos complex may activate the p21ras/ERK signaling cascade, while the Nck-Pak1, -Pak3, or -PRK2 complexes may link the various Rho GTPases to the regulation of the actin dynamics and the activation of the SAPK/JNK and p38 signaling pathways (59). Since the members of the Ras superfamily are known to elicit distinct cytoskeletal rearrangements and gene regulatory events, Nck by interacting with upstream activators or downstream effectors of the small GTPases could contribute to diverse cell responses involving differentiation, proliferation, and/or migration. Indeed, using a peptide harboring the proline-rich region of Pak fused to the HIV tat protein to facilitate its entry into the cells, it was observed that localization of Pak was disrupted in endothelial cells, and that cell migration and contractility was inhibited (60). Moreover, the Pak peptide

specifically blocked angiogenesis in a chick chorioallantoic membrane assay (60).

NIK or MAP kinase kinase kinase kinase (MAP4K4), which includes significant sequence homology with Pak, has been isolated in a yeast two-hybrid screen when trying to identify new interacting proteins that could associate with the second SH3 domains of Nck (61). Two proline-rich sequences within the central region of NIK interact with the SH3 domains of Nck. Transient overexpression of NIK activates the JNK/SAPK pathway (61). However, NIK activation is not dependent on activated Rho GTPases, as seen with Pak1. NIK activation presumably occurs upon a direct interaction with MEKK, via a novel C-terminal NIK regulatory region. MEKK association leading to NIK activation likely accounts for the ability of NIK overexpression to activate the JNK/SAPK pathway. Nck through its SH3 domain recruits NIK to receptor or nonreceptor tyrosine kinases leading to activation of JNK signaling pathways and regulation of actin cytoskeleton. In fact, this could be the mechanism by which the EphB1 receptor through Nck activates JNK and leads to actin remodeling for axonal guidance (38).

KETTE, a member of the HEM family in Drosophila, has been recently implicated in cytoskeletal organization during axon pathfinding (62). KETTE protein is a homologue of the protein HEM-2/Nap1 whose interaction with Nck has been well characterized (63, 64). Nck/Nap1 interaction is mediated through the first SH3 domain of Nck (63, 64). Recently, isolation of human Nap1

(NCKAP1), a novel apoptosis-related gene whose expression appeared to be strongly down regulated in Alzheimer's disease, has been shown to bind Nck (65). From a yeast two-hybrid screen, a protein designated hNap1BP (for human Nap1 binding protein) has been identified (66). hNap1BP contains an SH3 domain, a PEST domain and a proline-rich region responsible for its interaction with the SH3 domains of c-Abl and Nck (66). However, to date, no specific cellular functions have been attributed to the Nap1 and Nap1BP proteins.

More recently, Li et al. have shown in commissural neurons that Nck-1 binds the deleted colorectal cancer (DCC) receptor for netrin-1. Netrins are known to guide the migration of cells and axonal growth cones during development. This association between Nck and DCC is dependent on the first and third SH3 domains of Nck and appears to be constitutive. In DCC expressing cells, Nck-1 relocalized to the cytoskeleton and this appears to be dependent on DCC (67). These studies strengthen the role for Nck in signaling events that lead to actin-based cytoskeleton rearrangement responsible for axonal guidance.

Nck-2, but not Nck-1 binds through a non-conventional SH3 domain interaction with PINCH, a LIM-containing protein implicated in integrin signaling (68). This was demonstrated by functional mutations of the conserved tryptophan residues in the third SH3 domain of Nck-2, which usually abolishes the binding of the SH3 domain to proline-rich motifs but did not prevent PINCH binding (69). Nevertheless, in this study it is suggested that PINCH may function

as a linker protein, recruiting ILK to Nck-2 (69). They further proposed an ILK-PINCH-Nck2 signaling mechanism in which ILK by interacting with integrins may recruit PINCH and Nck-2 to focal adhesion complexes (69). In these complexes, Nck-2 may also recruit ligand activated RTKs (e.g. PDGFR) establishing a functional link between integrin and growth factor signaling.

WASP is also identified as an Nck interacting protein (70). WASP was originally isolated as the gene mutated in the Wiskott–Aldrich Syndrome, a severe X-linked immunodeficiency disease associated with thrombocytopenia, eczema and recurrent infections (71). Mutation in WASP leads to reduced mobility of lymphoid immune cells, suggesting that WASP and its relatives play a critical role in organization of the actin cytoskeleton (72-74). The WASP-family of proteins consists of WASP, N-WASP and WAVE (72). Neural WASP (N-WASP) promotes actin polymerization by stimulating the actin-nucleating activity of the Arp2/3-complex, in a Cdc42 dependent manner (75). This leads to the formation of branched actin filaments, the main structures involved in membrane ruffling and lamellipodia (75). Nck association is likely to occur with the C-terminal proline-rich region of WASP (70). Several lines of evidence suggest that Nck/WASP interaction represents another bridge between RTKs and regulation of the actin dynamics. Molecular complexes containing WASP and Nck have been demonstrated in various systems and conditions. For instance, N-WASP is required for the actin tail-based motility of vaccinia virus in infected cells and its recruitment to the site of actin polymerization on vaccinia requires also Nck and WASP interacting protein (WIP) (76, 77). Recently, Grb2 was described as an

additional component of the vaccinia actin tail-forming complex (78). Grb2 alone is unable to nucleate actin tails, but it cooperates with Nck to stabilize and/or activate the vaccinia actin-nucleating complex containing WASP (78). In addition, Nck, similar to GTP-Rac1 activates actin nucleation through the release of WAVE1, a WASP homologue, from a large molecular complex where it is kept inactivated (79).

Other Nck interacting proteins suggested a role for Nck in regulating actin dynamics. In fact, Nck2, via its second and third SH3 domains, binds to DOCK180 (80). DOCK180 participates in integrin signaling by activating Rac and inducing membrane ruffling and cell migration (81-83). In addition, reported Nck-dynamin and Nck-synaptojanin complexes may couple cell surface receptors to endocytosis and vesicle trafficking (84).

As mentioned previously, a potential role for Nck in insulin signaling is suggested by the observation that through its SH2 domain, Nck is capable of an insulin-dependent association with IRS-1 and IRS-3 (39, 40). In contrast, an SH3 mediated interaction of Nck-2 with IRS-1 has also been reported (85). Other than its association with IRS, Nck function in insulin signaling has still to be firmly established.

Our laboratory has identified an Nck associated Ser/Thr kinase activity mediated by the γ2 isoform of casein kinase I (CKIγ2) (86). The Nck/CKI-γ2 interaction is constitutive and primarily dependent on the third SH3 domain of Nck. Although the physiological significance of this interaction is still unknown, CKIγ2 has been shown to phosphorylate and antagonize the activity of the ligand-bound PDGF receptor (87). From this study, it was suggested that Nck could physically link the activated PDGF receptor to CKIγ2, however this still remains to be demonstrated.

Nck through its SH3 domains also associates Sam68, a c-Src substrate during mitosis (88) and an RNA binding protein (89). Since Sam68 shuttles between the cytoplasm and the nucleus (88), its interaction with Nck could mediate nuclear localization of Nck (89). Interestingly, Sam68 is phosphorylated on tyrosine in insulin-stimulated cells where it translocates from the nucleus to the cytoplasm. Tyrosine phosphorylated Sam68 associates with the SH2 domains of p85 of PI3-K and GAP, both *in vivo* and *in vitro* (90). Its association with p85 allows Sam68 to be in close proximity of the IR in order to be more phosphorylated (90). In addition, Sam68 co-precipitates with IRS-1 and IR (91). Nevertheless, the physiological conditions and significance for the interaction of Nck with Sam68, remains to be established (89). On the other hand, the SH3 domains of Nck also associate with Abl, a nuclear resident tyrosine kinase (92), strengthening a potential role for Nck in the nucleus. The Nck-Abl complex appears to activate the Abl kinase activity, which plays a critical role in gene

expression and development (93, 94). The c-Cbl protooncogene product also interacts with Nck (95). $p120^{Cbl}$ (Cbl) is a 120kDa cytoplasmic protein possessing a putative N-terminal nuclear localization signal, a RING DNA binding motif, and a proline-rich region (96). Its association with Nck seems constitutive and occurs via more than one SH3 domain of Nck. Cbl becomes tyrosine-phosphorylated upon various growth factor stimulation (96) and under this condition, Nck via its SH2 domain can also binds Cbl (97). Interestingly, Cbl is associated with the ubiquitination and degradation of RTKs, but also with insulin-induced increased glucose transport in adipocytes, suggesting that Nck may participate in these RTK driven crucial cellular mechanisms. Here again, this remains to be further investigated.

III. Biological significance of Nck-mediated protein interactions

Various studies strongly support the ability of the different domains of Nck to participate in the regulation of diverse signal transduction events. Depending on the extracellular stimulus, Nck may be capable of selectively regulating different responses within a cell.

1. Cell proliferation.

A dominant role for Nck in cell proliferation was provided by the fact that overexpression of Nck in PC12 cells, while inhibiting NGF-dependent cell differentiation, favors proliferation (51). Nck, through its SH3 domains, is proposed to translocate into the activated RTK complex, various regulators of mitogenesis. A role for Nck in mitogenesis was also suggested when it was shown that Nck was required for DNA synthesis in response to PDGF stimulation (50). In addition, the role of Nck in mitogenesis was further strengthened by Roche et al., which demonstrated that microinjected anti-Nck antibodies and dominant negative forms of Nck, inhibit the PDGF-induced fibroblast entry into S phase (50). PDGF, by binding to the PDGFR, leads to the receptor autophosphorylation/activation and the recruitment of Nck through direct binding of its SH2 domain to pY751 of the PDGFR cytoplasmic region (33). Interestingly, Ming et al. have identified signals emanating from PDGF RTK pY751 as essential for the Ras-independent activation of the 70kDa and 85kDa S6 kinases (98). The S6 kinases, bound to and activated by Ras and Cdc42, have a regulatory control

of cellular proliferation (99, 100). It remains unknown whether PI3-K or Nck or both mediate PDGF-induced S6 kinase activation. On the other hand, an alternative way by which Nck can link PDGFR to DNA synthesis is similar to the function of Grb2, which translocates Sos at the membrane, a mechanism known to activate the Ras/ERK signaling cascade. This mechanism is suggested from the fact that Nck binds the guanine exchange factor Sos and activates a gene reporter regulated by the c-fos gene promoter, in a Ras-dependent manner (46). Thus, dominant negative Ras blocked Nck-induced c-fos gene transcription (46). Interestingly, overexpression of Nck-2 but not Nck-1, inhibited PDGF-stimulated DNA synthesis in NIH-3T3 cells (28), suggesting that both Nck isoforms may differentially regulate mitogenesis.

Few studies have shown that Nck also behaves as an oncogne. The oncogenic potential of Nck was revealed when its overexpression in NIH-3T3 and chick fibroblasts was shown to lead to anchorage-independent growth and abnormal morphology (25, 32). Moreover, its oncogenic property was confirmed when subcutaneous injection of the Nck overexpressing chick cells into nude mice was shown to result in tumor formation (32).

2. Actin cytoskeletal dynamics.

The fact that several Nck-associated proteins are involved in functional interactions with members of the Rho subfamily of GTPases suggests that Nck participates in regulating dynamics of the actin cytoskeleton. A role for Nck in this process is supported by genetic analysis of Dock, the Drosophila homologue of the human Nck. During a genetic screen to identify effectors involved in axonal pathfinding, dock was identified as an essential gene for proper photoreceptor axon targeting (31). In this study, Garrity and colleagues proposed that Nck plays a similar role in forming precise patterns of neuronal connections in vertebrates. To test this hypothesis, Rao and colleagues studied various mutant forms of Dock for rescuing the *dock* null phenotype (101). They found that the wild-type Nck-1 was able to rescue the R cell projection defects in *dock* mutants, supporting the hypothesis that Dock and Nck are functionally conserved during evolution. A recent study showed that Drosophila Pak is required in the Dock pathway regulating R cell axon guidance and targeting (102). It was found that Pak and Dock colocalize to R cell axons and growth cones, and physically interact. Furthermore, another essential component in photoreceptor axon guidance has been identified. Indeed, mutations in the gene encoding Trio, a guanine nucleotide exchange factor for Rho GTPase, result in projection defects similar to those observed in both Pak and Dock mutants (103).

3. Lymphocyte activation

Following T cell receptor activation, the SLP-76 scaffolding molecule undergoes intensive phosphorylation on tyrosine residues and recruits Vav1, a GEF for small Rho GTPases and the adapter Nck, both through their SH2 domains (104-106). This molecular complex allows the activated Rho family GTPases to regulate target effectors interacting with Nck. In agreement with this hypothesis, T cell receptor-dependent activation of Pak1 and inducible association of Pak1 with Nck have been observed (104). In addition, dominant negative Pak1 and Nck specifically inhibited T cell receptor-mediated activation of the nuclear factor of activated T cells (NFAT) transcription factor (104). In contrast to these findings, Ku et al. (107) reported that Pak1 activation required neither SLP-76 nor direct binding to Nck. Instead, efficient Pak1 activation required active GTPases and Pix, a guanine nucleotide exchange factor for Rho GTPases. Furthermore, a stable trimolecular complex was detected, consisting of Pak1, Pix and p95PKL (107).

Involvement of Nck downstream of T lymphocyte receptors signaling was suggested from an experiment using the yeast two-hybrid where it was shown that Nck interacted with a proline-rich sequence of the T-cell receptor CD3ε cytoplasmic tail (108). This study also revealed that antigen binding to CD3ε induces a conformational change that allows the recruitment of Nck to the receptor.

Nck was also reported as a signaling molecule involved in B cell receptor signaling. Mizumo et al. demonstrated that Nck associates, in an SH2 dependent manner, with the B cell linker protein (BLNK), a substrate of the tyrosine phosphatase SHP-1 (109). B cell receptor (BCR) induces activation of JNK and this is significantly enhanced in cells expressing a form of SHP-1 lacking phosphatase activity (SHP-1-C/S). Expression of dominant-negative forms of Nck inhibited BCR-induced JNK activation and Nck SH2 mutants suppressed BCR-induced apoptosis in SHP-1-C/S -expressing cells (109).

4. Glucose homeostasis

In addition to its binding to key molecules downstream of the insulin receptor, suggesting an involvement of Nck in mediating insulin action (39, 40, 68, 85), its expression levels vary in various diabetic conditions. Indeed, in streptozotocin-treated rats (Type-I diabetes), the levels of Nck in liver are reduced whereas they remain unchanged in adipose tissue (110). On the other hand, in obese insulin resistant diabetic KKA^{y} mice, an animal model of NIDDM or Type-II diabetes, Nck levels remained unchanged in liver and dramatically increased in adipose tissue (111). Interestingly, Nck expression is increased in skeletal muscle of exercise-trained rats, which are more sensitive to insulin (112). Taken together, these data suggest that modulation of Nck expression, with other key signaling molecules, in insulin target tissues might be associated with altered

glucose metabolism as seen in pathological conditions such as Type-I and Type-II diabetes mellitus.

IV. Conclusion

Significant advances have been made in identifying the physiological role of the Nck adapter in various signal transduction pathways. However, evidence strongly suggests that the Nck proteins are implicated in the organization of actin cytoskeleton underlying cell movement and axon guidance in flies. Several signaling molecules have emerged as proteins associating with Nck. The physiological importance behind these protein–protein interactions is still poorly understood. Even though individual Nck isoforms can play specific roles in various cell types (28, 68, 113, 114), mice deficient in either Nck-1 or Nck-2 are normal, whereas a double knock out of both Nck isoforms resulted in early embryonic lethality (T. Pawson, personal communication). This suggests that Nck-1 and Nck-2 are functionally redundant and that at least the presence of one isoform is required during development.

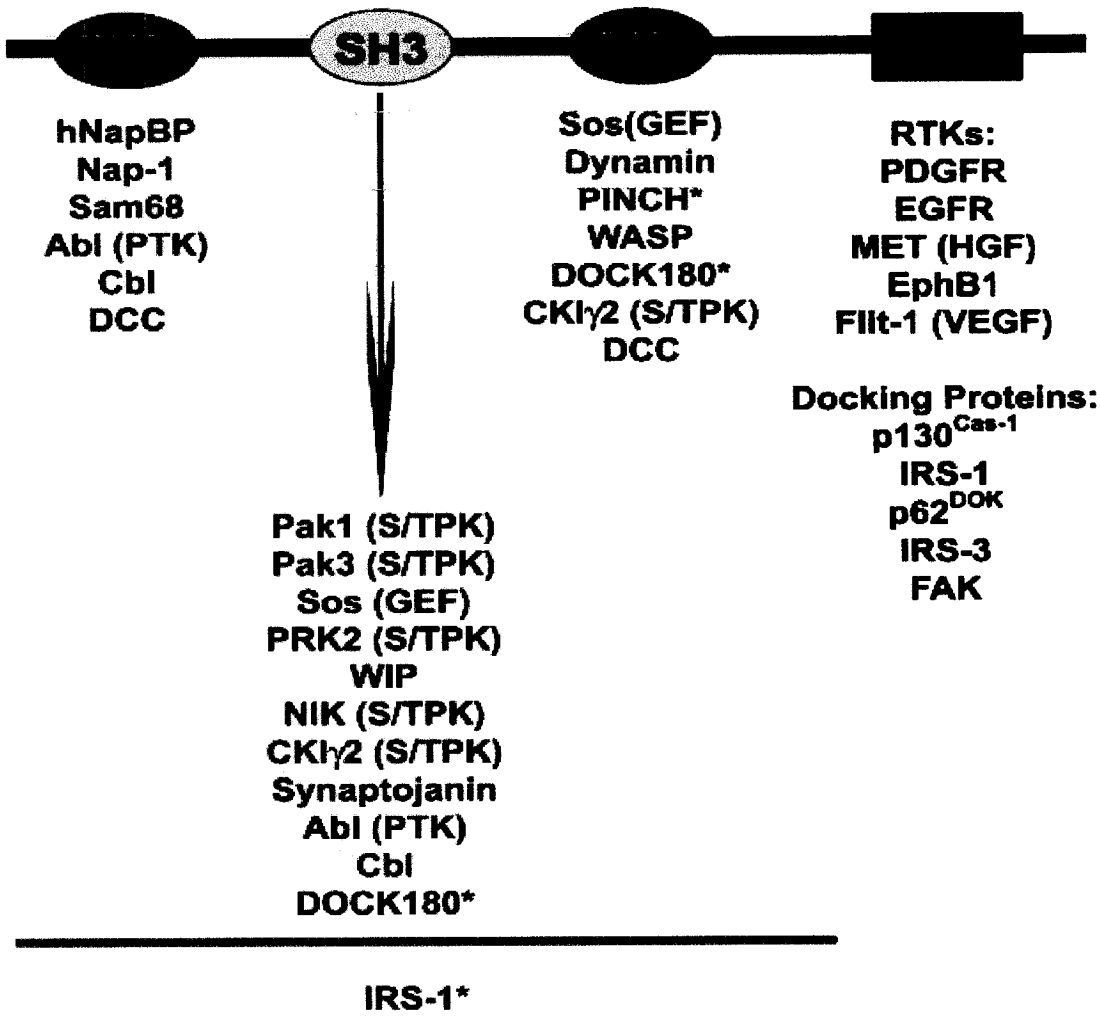


Fig.3. Current list of Nck1/Nck2 binding proteins and their association with Nck domains. The effector proteins with an asterisk have been characterized only for their binding to Nck-2.

C. Protein Translation.

Protein translation is conventionally divided into three stages, initiation, elongation and termination, and each is mediated by protein factors, termed initiation, elongation and release factors. Initiation seems to be the major site of regulation and modulation of eukaryotic initiation factors (eIFs), especially by phosphorylation, has been the center of attention. In addition to ribosomes, at least 25 protein factors organized in multiprotein complexes are involved in mechanisms responsible for the initiation of translation of cellular mRNAs. The most well characterized include the Cap-dependent and –independent initiation mechanisms.

I. Cap-dependent initiation of translation

Cap-dependent initiation of protein translation has been well described elsewhere (reviewed in 115-118). Briefly, following ribosomal subunit dissociation, which is assisted by eIF3, the small (40S) ribosomal subunit (carrying eIF3) associates with a ternary complex to generate a 43S pre-initiation complex. This ternary complex contains the methionine (Met)-charged initiator tRNA and the initiation factor eIF2 bound to GTP. The 43S complex is then recruited, by the eIF-4 group of initiation factors, to the 5' end of the mRNA (Fig.4). Mammalian eIF4F is composed of three subunits: eIF4E (which binds to the m7GpppG cap), eIF4A (an ATPase-dependent RNA helicase) and eIF4G (which through its interaction with eIF3 aids the binding of the 43S pre-initiation

complex). The helicase activity contained in eIF4F unwinds secondary structures of the 5' UTR mRNAs. This helicase activity is stimulated by eIF4B, which binds to eIF4F. The interaction of eIF4B with eIF3 is believed to stabilize the 43S complex. Once bound, the 43S complex along with eIF4 complex and mRNAs form the 48S mRNA complex. This complex migrates along the 5' UTR in an ATP-dependent manner described as scanning, until it encounters an initiator AUG codon, normally the first AUG. Following AUG recognition, eIF5 triggers hydrolysis of the GTP on eIF2 in the ternary complex, initiation factors are released and the large (60S) ribosomal subunit joins the remaining 40S ribosomal subunit bound to mRNA, to form the 80S initiation complex (119). At this step, elongation now begins (Fig.4).

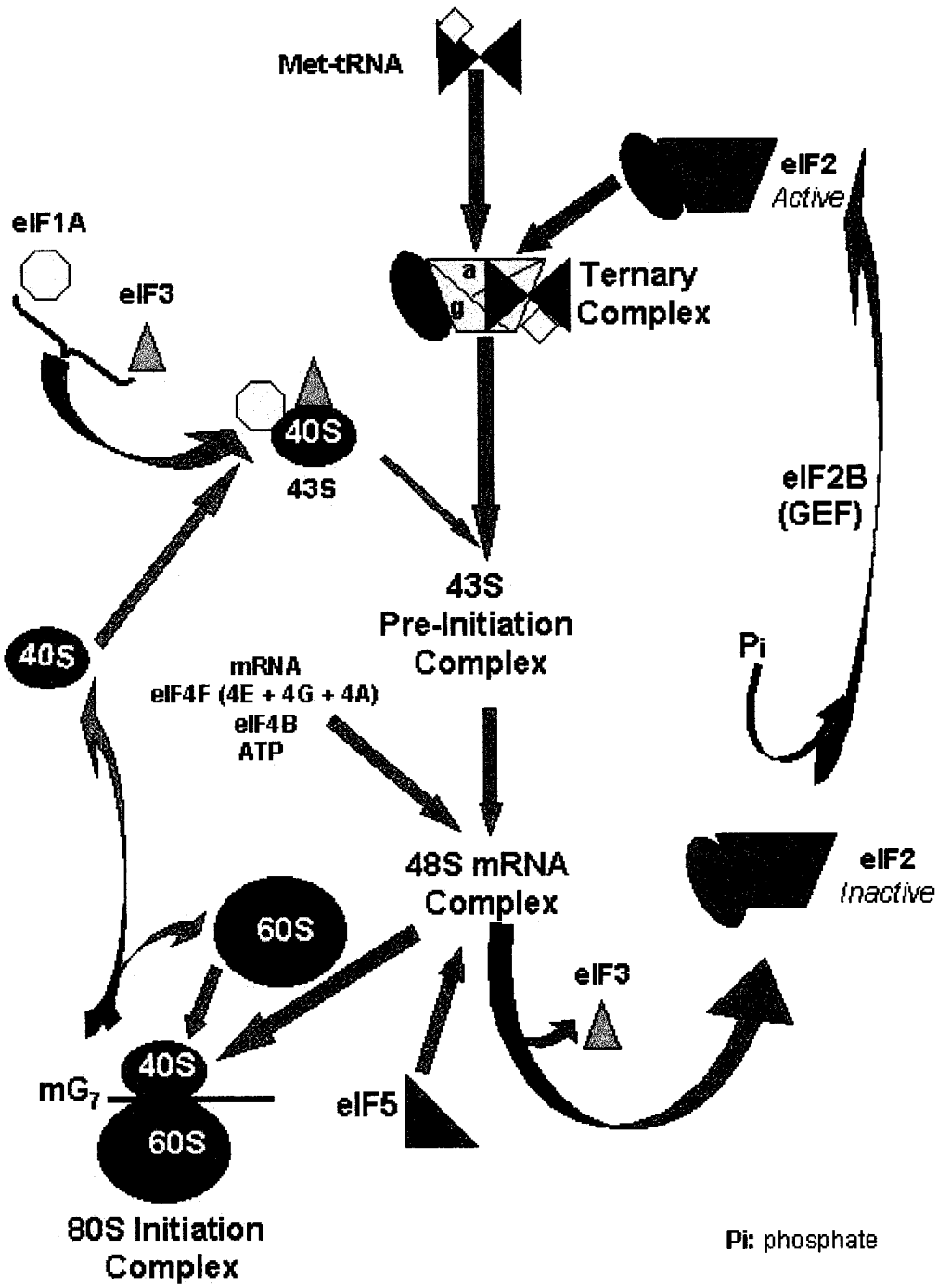


Fig.4. Cap-dependent initiation of protein translation in eukaryotes

II. Cap-independent initiation of translation

Cap-independent initiation is referred as "internal initiation" was first identified in picornavirus (120, 121). This mechanism involves an internal ribosomal entry site (IRES), a secondary structure within the 5' UTR (120, 121). IRES-mediated translation does not require a free 5' end, as demonstrated by the translation of circular IRES-containing RNAs (122). Viral IRESes can be divided into several functional groups based on their primary sequence and structure (121). IRESes can be functionally discriminated from other 5' UTR secondary structures by their ability to mediate translation of the downstream open reading frame (ORF) of bi-cistronic reporter mRNA, independent of the translational status of the first ORF (123, 124).

The majority of general initiation factors, including eIF4F, appear to be required for IRES-mediated translation (reviewed in 121). Exceptionally, the IRESes from hepatitis C, classical swine fever viruses and the cricket paralysis virus (CrPV) appear to bind the small ribosomal subunit and position it properly at the AUG, without the need for initiation factors (125, 126). Translation from other IRESes may require additional *trans*-acting factors, such as polypyrimidine tract-binding proteins (127, 128) and the La autoantigen (129).

Internal initiation allows picornavirus mRNA, which does not possess a cap structure, to escape the shut down of host translation machinery that occurs during infection. Translation of host mRNA is shut off, in part, by the cleavage of eIF4G. The cleavage separates the eIF4E binding domain of eIF4G (in the N-terminal portion of the protein) from proteins that interact with eIF4A and eIF3, located nearer the C terminus (130). This separates the cap-binding function of eIF4G from its RNA-helicase and ribosome-binding activities and thus inactivates translation of most cellular mRNAs. However, the C-terminal fragment of eIF4G can substitute for intact eIF4G in IRES-mediated translation and in fact may be more efficient (131, 132).

III. Components of the translational machinery regulated by phosphorylation

Changes in the rate or pattern of protein synthesis occur in response to stimuli initiated by heat shock, mitogenic factors, stress, or growth factors. Such changes in translation are normally mediated by changes in the activity or abundance of the translation initiation factors. Numerous components of the translational machinery are phosphoproteins that include at least 13 initiation factors, 3 elongation factor subunits, 3 ribosomal proteins, and a number of amino-acyl tRNA synthases (116). Although the phosphorylation state of eIF4B, eIF4E, eIF4G, eIF2, and eIF3 can all be modulated *in vivo* (reviewed by 115, 133), an understanding of how phosphorylation regulates the activity of these factors is mostly detailed only for eIF2 and eIF4E.

1. eIF2 complex

eIF2 is a multimeric initiation factor composed of three dissimilar subunits termed α, β, and γ, in order of increasing molecular mass. cDNAs for each of the subunits have been cloned and sequenced from a variety of species. The predicted amino acid sequences of the individual subunits are highly conserved among species. For example, the human eIF2α, β, and γ sequences are respectively, 58%, 47%, and 72%, identical to the corresponding subunits from *S. cerevisiae* (134).

eIF2α contains a serine residue at position 51 (Ser51), which is a phosphate acceptor for several protein kinases. HRI (135), GCN2 (136), PKR (137, 138) and PERK/PEK (139) were identified *in vivo* as eIF2α kinases for Ser51.

In contrast to eIF2α, the eIF2β amino acid sequence contains a greater number of interesting motifs including phosphorylation sites for CKII, PKC, and PKA (140). The N-terminal half of the protein includes three domains, each containing 6–8 lysine residues. The binding site for a second initiation factor eIF5 overlaps the second poly-lysine domain (141). The C-terminal portion of eIF2β contains the binding domain of the guanine nucleotide exchange factor, eIF2B (142), a zinc finger motif and a minimal proline-rich motif (P-X-X-P), which could potentially interact with SH3 domains. Finally, cross-linking of Met-tRNA$_i$ to eIF2β revealed four peptides distributed throughout the protein (143). Whether or not all of these sites are important for the binding of the Met-tRNA$_i$ to eIF2 is still unknown.

The primary amino acid sequence of eIF2γ contains all three consensus motifs for guanine nucleotide-binding domains. Binding of GTP to eIF2γ is greatly impaired by mutations in the guanine nucleotide binding domains of either the human (144) or yeast (134) protein. This establishes that eIF2γ is the primary guanine nucleotide binding site for the eIF2 complex. In addition, for eIF2γ,

cross-linking analysis has also implicated its N-terminus in the binding of the Met-$tRNA_i$ (143).

The primary role of eIF2 in initiation of translation (Fig.4), is to carry the Met-$tRNA_i$ to the 40S ribosomal subunit (reviewed in 117). Following association of mRNA with the 40S subunit and location of the subunit at the AUG start codon, eIF5 binds to eIF2 and stimulates the hydrolysis of GTP bound on eIF2. It has been proposed that either eIF5, the β- or the γ-subunits of eIF2 contains the GTPase activity responsible for the hydrolysis of GTP. However, eIF5 stimulates GTP hydrolysis only when eIF2 is bound to the 40S subunit as a ternary complex with GTP and Met-$tRNA_i$ (119), suggesting that either the GTPase is intrinsic to the 40S subunit or that an additional component of the 43S preinitiation complex is required. Interestingly, one of the two CKII phosphorylation sites on eIF2β, Ser67, is adjacent to the eIF5 binding site, suggesting that CKII might regulate the binding of eIF5 and influence GTP hydrolysis on eIF2. Following GTP hydrolysis, the eIF2-GDP complex is released from the 48S mRNA complex.

Phosphorylation of eIF2α on Ser51 results in an inhibition of translation and is a central control point in the initiation pathway (117). However, eIF2α phosphorylation does not inhibit its function but rather inhibits its recycling by the GDP/GTP exchange factor, eIF2B (117). Exchange of GDP to GTP on eIF2 is required for the next round of initiation (115). Phosphorylated eIF2α on Ser51 binds with high affinity to eIF2B and forms a stable complex in which the bound

GDP cannot be exchanged for GTP (142). As the eIF2 complex is normally in excess of eIF2B, this essentially sequesters the cellular eIF2B activity and leads to a general inhibition of translation.

2. eIF2B complex

As mentioned above, the initiation factor eIF2B mediates the recycling of inactive eIF2-GDP to active eIF2-GTP. Since eIF2 is required for every initiation event, modulating the activity of eIF2B provides a mechanism for controlling overall rates of peptide-chain initiation and is believed to play a key role in the regulation of translation in response to viral infection, amino acid deprivation and other 'stress' conditions (145, 146). eIF2B is a multimeric protein, having five non-identical subunits (α, β, γ. δ and ε) (146). The functions of these individual subunits have yet to be defined, but it seems likely that phosphorylation of the ε subunit of eIF2B plays a regulatory role (50). In addition, Kimball and colleagues demonstrated that eIF2 binds only to the δ and ε subunits of eIF2B, whereas eIF2B was shown to bind only to the β subunit of eIF2 (142).

Welsh and Proud identified an insulin-sensitive protein kinase that phosphorylates the ε subunit of eIF2B and was inactivated in response to insulin (and also serum or phorbol esters) (147). They showed that this kinase was the β isoform of the glycogen synthase kinase-3 (GSK-3) (147). eIF2B inactivation appeared to involve phosphorylation by GSK-3. Phosphorylation of eIF2B by

GSK-3 results in the inhibition of its guanine nucleotide exchange activity (148). The GSK-3 phosphorylation site in eIF2Bε was identified as Ser540 and is conserved in all mammalian species. GSK-3 is inactivated following insulin stimulation in cultured human muscle myoblasts (149) and rat fat cells (150, 151). Welsh et al. demonstrated using an antiserum to a peptide corresponding to the region around Ser540 and containing a phosphoserine residue at this position that insulin stimulation rapidly decreased phosphorylation of Ser540. This allowed them to draw the following model in which insulin causes inactivation of GSK-3 and hence decreased phosphorylation of eIF2B at this site, leading to activation of eIF2B and of translation initiation (152). The insulin-induced dephosphorylation of eIF2B was blocked by the two inhibitors of PI 3-kinase, wortmannin and LY294002 (152). This suggests a key role for PI 3-kinase in the regulation of both GSK-3 and eIF2B. Neither MAP kinase nor the mTOR pathway seems to be required for the activation of eIF2B following insulin stimulation (153).

3. eIF4F complex

As mentioned earlier, eIF4F is a molecular complex composed of eIF4E, the cap-binding protein, eIF4G, a large polypeptide with binding sites for a number of other proteins, including eIF4E, and the third component of eIF4F, the ATP-dependent RNA-helicase eIF4A.

a) eIF4E

In vivo, the cytoplasmic cap-binding protein eIF4E is suggested to be limiting for translation and appears to be a critical site of regulation during cell growth, development, and differentiation (154, 155). Elevated eIF4E levels are found in several transformed cell lines (156) and in virtually all breast carcinomas (157-159). *In vivo*, phosphorylation of eIF4E on Ser209 occurs in response to growth factors, hormones, and mitogens. Several protein kinases including PKCs and a kinase of the MAP-kinase cascade, possibly Mnk1, can achieve phosphorylation of eIF4E at this site (154, 155, 160). Conversely, dephosphorylation of eIF4E is triggered by serum deprivation, viral infection, and heat shock (154). However, phosphatase activity involved in dephosphorylating eIF4E remains to be identified.

Phosphorylation of eIF4E appears to enhance its recruitment to ribosomes, since its phosphorylated form is predominately found in the 48S pre-initiation complexes (161). However, a large proportion of phosphorylated eIF4E is also not associated with mRNAs (162). Nevertheless, phosphorylation of eIF4E increases its affinity to the cap (163) and its association with eIF4A and eIF4G to form the eIF4F complex (164-166). Since eIF4F has a greater affinity for the cap than eIF4E alone (164, 167), eIF4E phosphorylation may enhance translation by contributing to higher levels of eIF4F with enhanced cap-binding affinity. When eIF4E (and therefore eIF4F) is limiting, mRNAs with structure-rich 5' UTRs will be poorly translated. Increased availability of eIF4F will result in an increased

delivery of RNA helicase activity to the 5' UTR, disrupting the secondary structures. In agreement with this, mammalian cells overexpressing eIF4E showed an increased in translation efficiency of reporter mRNAs containing structured 5' UTRs (168).

eIF4E appears linked to cell growth (169). A number of approaches suggested that Ras is an upstream activator of eIF4E (170). For example, overexpression of Ras elevates eIF4E phosphorylation in absence of extracellular stimuli (171). In contrast, others reported that Ras could be downstream of eIF4E because eIF4E overexpression increases Ras activity and eIF4E's transforming ability is prevented by blocking the activation of Ras (172). In fact, these data may suggest the existence of a simple positive feedback loop, in which Ras acts upstream to activate eIF4E phosphorylation. This leads to increased translation of growth factors that are then secreted, and in turn bind cell surface receptors to induce further Ras activation (173).

b) eIF4G

eIF4G is a large multidomain protein, which serves as a scaffold on which initiation-factor complexes are assembled (174). It contains interaction sites for eIF4E, eIF4A and eIF3. It serves to bring together the cap-binding factor, eIF4E and the helicase, eIF4A, which acts to unwind regions of secondary structure in the 5′ UTR of the mRNA. Secondary structures in this region of the mRNAs are

known to reduce the translational efficiency of the mRNA probably by interfering with the movement of the 40S subunit and its associated factors during scanning. eIF4G is phosphorylated at unknown sites by S6 kinases ($p70^{S6K}$ and $p90^{S6K}$) (175). Once phosphorylated, eIF4G enhances *in vitro* protein synthesis and binding of mRNA to the 43S initiation complex.

4. 4EBP1/PHAS-I

eIF4E activity can be modulated by its interaction with a regulatory protein, designated as the eIF4E binding protein 1 (4EBP1 or PHAS-I) (176, 177). This 118-amino acid phosphoprotein competes with eIF4G for binding of eIF4E and prevents formation of eIF4F complex (178). 4EBP1 and eIF4G share a motif involved in binding of eIF4E and this explains their competitive action (178, 179). Under conditions such as serum deprivation and heat shock, 4EBP1 becomes dephosphorylated and sequesters eIF4E, globally limiting cap-dependent translation (155, 180). The effects of 4EBP1 may be most severe on specific subsets of mRNAs with secondary structure in their 5' UTR. Phosphorylation of 4EBP1 by the mTOR/FRAP protein kinase pathway, indicated by sensitivity to the immunosuppressant rapamycin, leads to its dissociation from eIF4E (180). Recent evidence suggests that mTOR may directly phosphorylate 4EBP1 (181).

5. Ribosomal protein S6

This ribosomal protein is phosphorylated on five Ser residues, located at 235, 236, 240, 244, and 247 by the $p70^{S6K}$ (182). However, the effect of S6 phosphorylation on protein synthesis is unclear. It has been postulated to be correlated with an enhancement of the rate of translation, especially at the level of initiation (183). Several studies have shown that increased S6 phosphorylation correlated with synthesis of a number of proteins whose levels were not affected by the transcription inhibitor actinomycin D, and were therefore argued to be controlled at the level of translation (184). eIF1α is one of these proteins (185). It is encoded by a 5'-TOP mRNA (186), whose translation is up-regulated by $p70^{S6K}$, in response to mitogenic stimuli (187).

IV. Signaling intermediates involved in protein synthesis

1. PI3-K

The mammalian PI3-Ks are a family of enzymes that phosphorylate the hydroxyl group on position 3 of the phosphatidyl-inositol (PtdIns) PI, PIP, and PIP_2. The PI3-K family plays a role in the regulation of many cellular processes, including proliferation, actin cytoskeletal remodeling, vesicular trafficking, apoptosis and protein synthesis (17, 188). In response to external stimuli, the p85 regulatory/adapter subunit binds tyrosine phosphorylated proteins and leads to the activation of the p110 catalytic subunit of PI-3K (17). In addition, upon the same stimuli, p85 translocates the p110 catalytic subunit to the membrane, placing it in close proximity to its lipid substrates (17). The involvement of PI3-K in protein translation has been demonstrated in several studies. The use of wortmannin and LY294002, potent inhibitors of PI3-K at low concentrations inhibits the phosphorylation of 4E-BP1, implicating this kinase in the regulation of 4E-BP1 activity (176, 180, 181, 189-191). Finally, overexpression of a constitutively activated p110 subunit of PI3-K targeted at the plasma membrane maintains 4E-BP1 in a constitutive hyperphosphorylated state (inactive) and enhances protein translation (192).

2. PDKs

These recently discovered kinases represented by at least four isoforms, through their PH domain, bind to and are believed to be activated by PtdIns(3,4,5)P_3 (193, 194). Once activated, PDK1 phosphorylates $p70^{S6K}$ on Thr 229, both *in vitro* and *in vivo* (195). In addition, it appears that PDK1 is the kinase responsible for the activation of PKB (Akt) following activation of PI-3K (194).

3. PKB (Akt, RAC-PK)

PKB exists in at least four isoforms (α,β_1, β_2, γ) and is activated by both RTKs and GPCRs. Its activation is PI3-K-dependent (196, 197), but in the case of GPCR, a PI3-K-independent mechanism also exists (151). PKB is targeted to the plasma membrane by direct binding to PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 mediated by its PH domain (198). Once translocated at the membrane, phosphorylation of PKB on Thr-308 by PDK1 contributes to its activation (194). The introduction of a Src myristoylation signal at the amino terminus of PKB creates a constitutively active membrane-targeted kinase. Overexpression of this membrane targeted PKB induces 4E-BP1 hyperphosphorylation on the same sites phosphorylated in response to serum stimulation (192). In addition, expression of activated PKB also enhances phosphorylation of 4E-BP1 (199),

indicating that regulation of 4E-BP1 phosphorylation is not only due to targeting of PKB at the membrane. Finally, dominant negative PKB prevented the increase of 4E-BP1 phosphorylation following insulin stimulation (192, 199).

4. mTOR (FRAP, RAFT-1)

mTOR is a 290-kDa protein kinase that is activated by PKB (200) and prefers to phosphorylate Ser/Thr-Pro motifs (201). mTOR kinase activity is inhibited by the immunosuppressant drug rapamycin (201). In mammalian cells, expression of a rapamycin-resistant mTOR mutant protein confers rapamycin resistance to 4E-BP1 and $p70^{S6K}$ phosphorylation (181, 202). As previously mentioned, mTOR, in response to amino acids and growth factors, controls the mammalian translation machinery via activation of the $p70^{S6K}$ protein kinase and via inhibition of the eIF4E regulator 4E-BP1 (203, 204).

5. GSK-3

Besides phosphorylation of glycogen synthase, GSK-3 regulates numerous cellular processes such as insulin signaling and specification of cell fate during embryogenesis (205). Phosphorylation of GSK-3 at Ser-9 inactivates the enzyme and is correlated with the activation of protein synthesis. *In vivo*, GSK-3 is directly phosphorylated by PKB, in a PI3-K dependent manner (205).

Phosphorylation of glycogen synthase by GSK-3 results in its inhibition (206). Glycogen synthase is the major regulatory enzyme of glycogen synthesis in mammals. This suggests that the regulation of GSK-3 may provide a way of coordinately regulating the activities of regulatory proteins in two major insulin-activated biosynthetic pathways, glycogen synthesis and protein synthesis (206).

6. $p70^{S6K}$

The major activity responsible for phosphorylation of ribosomal protein S6 is $p70^{S6K}$ (182), which is activated by sequential phosphorylation of seven Ser/Thr sites. Phosphorylation of the COOH-terminal sites occurs first, making Thr-389 available for phosphorylation by a kinase from the rapamycin-sensitive pathway (207). This culminates in phosphorylation of Thr-229 by the constitutively active PDK1 (195). It is well recognized that PI3-K acts upstream of $p70^{S6K}$. Nevertheless, the effect of PI3-K on the activation of $p70^{S6K}$ does not appear to be direct. Several signaling intermediates have been shown to be downstream of PI3-K and some of them play a role in the activation of $p70^{S6K}$. Among these, a constitutively membrane targeted PKB was shown to activate $p70^{S6K}$ (208). However, subsequent studies demonstrated that dominant-negative forms of PKB, effective in inhibiting insulin-mediated phosphorylation of 4E-BP1 and GSK-3 inactivation, did not affect $p70^{S6K}$ activation (199). This suggests that PKB and $p70^{S6K}$ lie on parallel pathways downstream of PI3-K.

7. Mnks protein kinases

These protein kinases, homologous to $p90^{S6K}$, were identified as binding partners of ERK1 and ERK2 (160) when screening for ERK1 substrates (209). Mnk1 is activated by phosphorylation at Thr-197 and Thr-202, both by a mitogen-activated pathway via ERK1 or ERK2 and also by a stress-activated pathway via p38 (210). Mnk1 efficiently phosphorylates eIF4E on Ser209 *in vitro* (160) and in vivo, following activation of either the ERK or p38 MAPK cascades (210, 211). Mnk1 does not interact directly with eIF4E. Rather Mnk1 binds the COOH terminus of eIF4G, in order to be recruited to its substrate eIF4E (210, 211).

V. Signal transduction pathways mediating protein translation

Insulin plays an important role in the overall regulation of protein synthesis. Some of the effects of this hormone involve changes in mRNA abundance, but insulin also has important effects on the translation process itself. Indeed, several initiation and elongation factors are regulated by insulin, often as a consequence of changes in their states of phosphorylation.

The binding of insulin to the insulin receptor (IR) results in the activation of the receptor tyrosine kinase activity (212). Following the IR autophosphorylation on specific tyrosine residues, the receptor phosphorylates a number of intracellular receptor kinase substrates (RKS) on specific tyrosine residues, and this initiates the intracellular signaling pathways. These substrates include Shc, Gab-1, $p62^{dok}$, IRS-1 (insulin receptor substrate-1) and its relatives IRS-2, IRS-3 and IRS-4 (reviewed in 213). Tyrosine phosphorylation sites in these proteins act as high affinity docking sites for other proteins containing SH2 domains. The two most well characterized SH2 domain-containing proteins recruited in insulin-activated transduction pathways are Grb2 and the p85 adapter of PI3-K, which are involved in the activation of Ras and PI 3-kinase respectively (Fig.5).

1. Effect of the Ras/MAPK pathway on protein synthesis

Activation of Ras, which is located on the inner face of the plasma membrane, appears to involve the guanine nucleotide exchange factor Sos, which is able to promote the exchange of GDP on Ras for GTP. Sos is bound to Grb-2 via its SH3 domains, and thus it is the Grb2–Sos complex, which binds to specific phosphotyrosine residues within IRSs when these proteins are phosphorylated by the insulin receptor. This binding is thought to bring the Grb2–Sos complex in close proximity to Ras. Activated Ras is able to initiate a cascade of activated protein kinases called the mitogen-activated protein kinase (MAP kinase) cascade (214). Ras-GTP, but not Ras-GDP, binds and contributes to the activation of the first kinase of the cascade, Raf-1. Activated Raf-1 phosphorylates another kinase MEK (also called MAP kinase kinase), which is a dual specificity kinase that activates extracellular-ligand-regulated kinase-1 and -2 (ERK-1 and ERK-2). ERK-1 and ERK-2 are very similar and are both expressed in most cells. They have a wide range of potential substrates in cells including transcription factors such as c-Jun and other protein kinases, including $p90^{S6K}$ (214). This kinase becomes activated following phosphorylation by MAP kinase and could now phosphorylate other proteins, including the ribosomal protein S6.

Insulin also activates mRNA translation by increasing the phosphorylation of eIF-4E itself (215). This is believed to increase the affinity of eIF4E for the Cap mRNA structure (163) and its affinity to associate with eIF4A and eIF4G (164-166). Indeed, eIF4E association with eIF4G is stimulated by insulin (216) as is eIF4G phosphorylation (175). Flynn and Proud have shown that the MEK inhibitor PD098059 blocks insulin's effect on eIF-4E phosphorylation in CHO cells overexpressing the insulin receptor (217) and Mnk1 is suggested as the kinase candidate mediating eIF-4E phosphorylation in response to insulin (Fig.5) (160).

2. Effect of the PI3-K dependent pathway on protein synthesis

PI 3-kinase is activated when its 85kDa subunit (p85) becomes bound to specific phosphotyrosine residues in IRSs, and this results in an increase in the product PtdIns(3,4,5)P_3 (16). The activation of PI 3-kinase appears to be necessary for many of the effects of insulin, including the protein kinase cascades involving PKB and $p70^{S6K}$ (16). Activation of PKB is associated with increased phosphorylation of Thr308 and Ser473, and phosphorylation of both sites seems to be required for full activation (193, 218). Thr308 can be phosphorylated, at least *in vitro*, by PDK1, which is activated *in vitro* by phosphoinositides phosphorylated in the 3' position (218). Thus PtdIns (3,4,5)P_3 appears to exert a dual effect on PKB. Activation involves both direct interaction with PKB and stimulation of the upstream kinase PDK1. PKB is able to phosphorylate GSK-3 and this decreases the activity of this kinase (219).

The guanine exchange factor eIF2B is activated by insulin (220) partly from low levels of phosphorylated eIF2α and partly from phosphorylation of its ε subunit (221). PI3-K appears to be required for the activation of eIF2B since both wortmannin and dominant negative mutants of the p85 regulatory subunit of PI3-K prevent eIF2B activation (153). It was also shown that *in vivo* activation of eIF2B by insulin correlates with the inactivation of GSK-3, but is independent of mTOR/p70 S6 kinase or the MAPK pathways (153). Interestingly, *in vitro* eIF2B

is a substrate of GSK-3 (147) suggesting that GSK-3, by phosphorylating eIF2B may negatively regulate eIF2B activity.

4E-BP1 is phosphorylated in response to insulin (176, 177). This takes place through a rapamycin-sensitive pathway (190, 222, 223) that includes PKB (192), suggesting that this pathway uses IRS-1 (Tyr-608, -628, and -658), PI3-K, PDK, PKB, mTOR (Fig.5). mTOR phosphorylates 4E-BP1 both *in vitro* and *in vivo* at serine and threonine residues identical to those phosphorylated in insulin-stimulated adipocytes (181). Although MAPK can phosphorylate 4E-BP1 *in vitro*, recent *in vivo* evidence has ruled out a role of the MAPK cascade in this reaction (190, 224).

The activation of a number of mRNAs encoding ribosomal proteins and elongation factors, is partially inhibited by rapamycin (225, 226). The translation of these mRNAs is controlled through sequences in their 5' UTRs, which typically contain short (8 nucleotides) polypyrimidine tracts (terminal oligopyrimidine tracts, TOP) (187, 225, 226). Alternatively, sensitivity to rapamycin raises the possibility that modification of ribosomal protein S6 by $p70^{S6K}$ directly enhances TOP mRNA translation (186, 227). This hypothesis was confirmed by using mutants of $p70^{S6K}$ (187). S6 is phosphorylated by both $p90^{S6K}$ (228) and $p70^{S6K}$ (229). $p90^{S6K}$ is activated in response to growth factors by forming complexes with ERK1 and ERK2 (230). This demonstrates two levels of regulation for S6 phosphorylation, one through the PI3-K/mTOR/$p70^{S6K}$ pathway and the other through the Ras/MAPK/ $p90^{S6K}$ pathway.

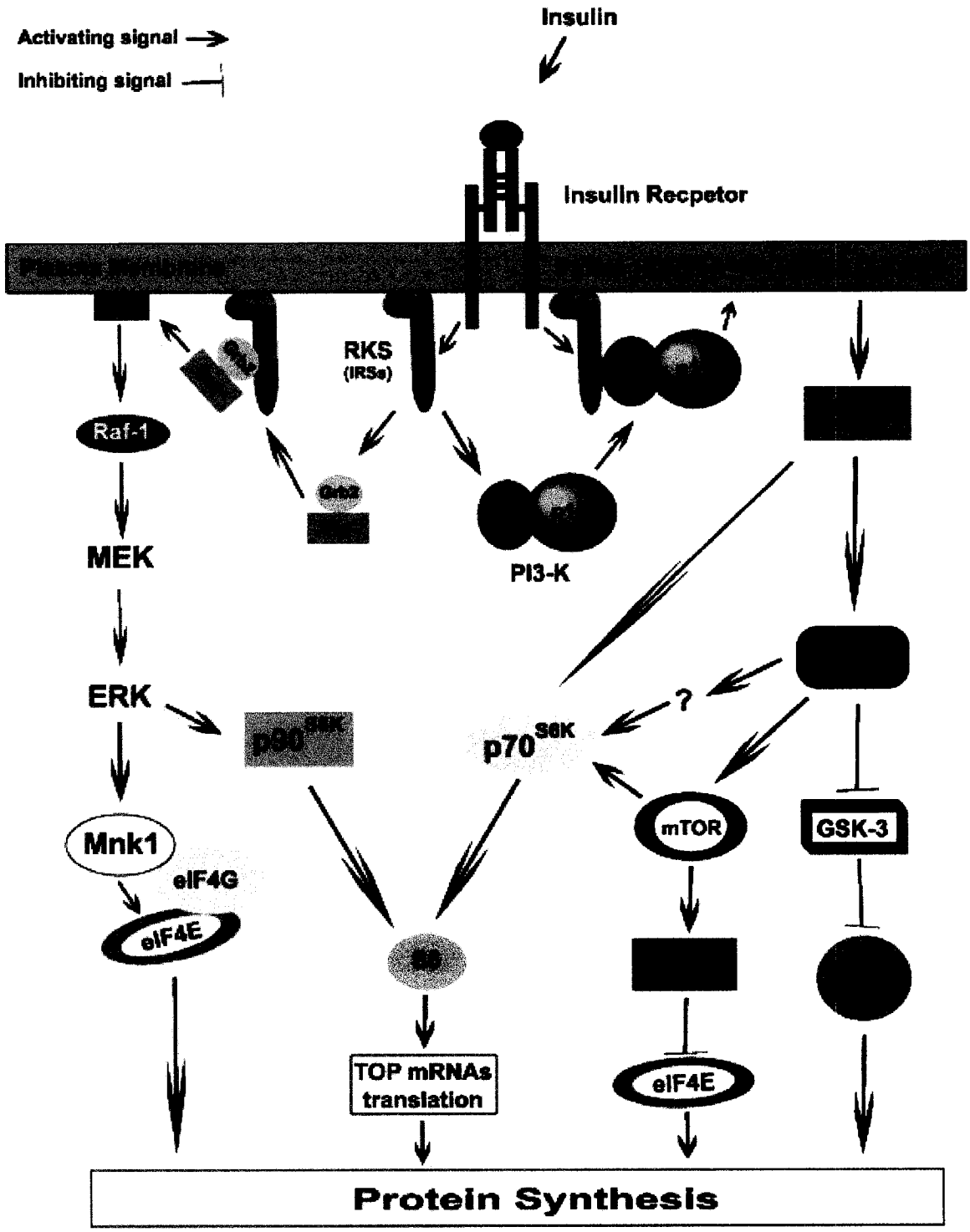


Fig.5. Signaling pathways modulating the function of initiation factors. (Modified from 231).

VI. Endoplasmic reticulum unfolded protein response

The endoplasmic reticulum (ER) is the subcellular compartment where proteins enter the secretory pathway and acquire their correct conformation. In addition, the ER is the most abundant cellular membrane network, a target of several signaling events, including post-translational modifications like phosphorylation (232). The ER contains intrinsic molecular signaling machines involved in the control of its general homeostasis and participates in ER-mediated membrane fusion (233), folding/degradation activity (234, 235) or Ca^{2+} fluxes (236, 237). The ER contains a high concentration of calcium and ATP and is a unique oxidizing environment that promotes protein modifications such as disulfide bond formation and protein glycosylation, features that are unique to this organelle (145). These processes are often perturbed when cells are deprived of essential nutrients or exposed to toxins (238). Conditions known to impair protein folding have dramatic effects on cellular survival (145). Therefore all eukaryotic cells have evolved specific mechanisms to maintain ER function under conditions of environmental stress (145, 239, 240). To date, two distinct ER-mediated signaling pathways are known to help maintain a proper folding environment in the ER. The first well characterized pathway mediates the transcriptional induction of genes encoding ER chaperones and disulfide exchange proteins (241). These are commonly used as markers for the state of the folding events in the ER (242). The second pathway consists of inhibition of the initiation of translation (243). This is achieved through the phosphorylation of eIF2α by the

ER-resident protein kinase PERK (139). Together, these pathways form what is defined as the Unfolded Protein Response (UPR) and is well conserved in all eukaryotes.

The UPR can be triggered by adverse physiological states. This includes hypoxia, glucose deprivation, heat shock, genetic defects that alter protein structure, and specific pharmacological compounds that affect the unique folding environment in the ER. Such drugs include tunicamycin, an antibiotic that blocks asparagine-N-linked glycosylation, the reducing agent dithiothreitol (DTT) and thapsigargin, an inhibitor of the ER calcium pump (145).

Accumulation of unfolded proteins in the ER lumen induces the transcription of a large set of genes, many of which encode proteins which function to increase the volume and capacity of protein folding, and/or to increase the degradation of misfolded proteins (244). In contrast to specific gene alteration, general translation is attenuated to decrease the protein-folding load on the ER (245). The cellular responses to ER stress is mediated by the activation of the ER transmembrane proteins: "inositol requiring 1" (IRE1), PERK and the activating transcription factor 6 (ATF6) (239) (Fig.6). In multicellular organisms, when these adaptive responses are not sufficient to relieve the unfolded protein load, cells enter the cell-death pathways.

Because of its simplicity, *Saccharomyces cerevisiae* has been extensively used to study multiple aspects of the UPR. In yeast, the only ER-stress-regulated transmembrane protein is the transmembrane receptor kinase/endoribonuclease, Ire1. It is similar to the ligand-activated type I transmembrane-receptor protein kinases activated following dimerization (246, 247). Ire1 consists of an ER-luminal, transmembrane, cytoplasmic Ser/Thr protein kinase domain and a carboxy-terminal RNase domain with strong similarity with the catalytic domain of RNAse L (145).

In physiological conditions, Ire1 is maintained in a monomeric and inactive state by binding of the ER-localized protein chaperone KAR2/BiP/GRP78 (248) (Fig.6). During ER stress, protein folding in the ER becomes less efficient and BiP, released from Ire1, binds to hydrophobic surfaces exposed on unfolded proteins. BiP binding to unfolded proteins reduces the amount of free BiP that is available for binding Ire1. In the absence of free BiP, Ire1 oligomerizes, undergoes *trans*-autophosphorylation and this activates its RNase function (Fig.6). Activated Ire1 cleaves the 5' and 3' exon–intron junctions of the *HAC1* messenger RNA encoding a basic leucine zipper (bZIP)-containing transcription factor (249, 250). The exons are then joined by transfer ligase RNA (251). The removal of the HAC1 intron enhances translation of the *HAC1* mRNA, so that Hac1 protein accumulates (249, 252). Activation of the UPR pathway is dependent on the cellular levels of Hac1 protein (145) and in the absence of ER stress, Hac1 protein is not detected, even though its mRNAs are produced and detected in polysomes (249).

The mammalian genome contains two homologues of the yeast IRE1, IREα and IREβ. IREα is expressed in most cells and tissues, with higher expression in the pancreas and placenta (253). IREβ expression, on the other hand, is primarily restricted to epithelial cells of the gastrointestinal tract (254). Both molecules respond to the accumulation of unfolded proteins in the ER by activation of their kinase and RNase activities. *In vitro* cleavage reactions of the yeast *HAC1*-mRNA by IREα and IREβ showed similar cleavage specificities. This suggests that IREs may share common sets of substrates and that specificity is rather established by their expression (255). Overexpression of either IREα and IREβ is enough to activate transcription from a BiP-promoter-reporter construct, and analysis of point mutations at key residues in the RNase domain showed that the RNase activity is required for this response, suggesting downstream processing activation of mRNAs (256).

Recently, a link between Presenilins, proteins localized to endomembrane, IRE1 activation and the UPR has been proposed. In fact, Presenilin-1 (PS1) mutants found in familial Alzheimer's patients inhibit ER-stress-induced IRE1α phosphorylation and BiP induction, when overexpressed in normal cells (257). PS1 is shown to be required for induction of BiP by the UPR (255) and in cells lacking PS1, IRE1α and IRE1β, the proteolytic cleavage in response to ER stress is prevented. Thus, PS1-dependent IRE1 processing correlates with activation of the UPR (Fig.6, scheme 2) (255).

The known IRE1 biological effector is the stress activated kinase JNK for which activation is mediated through binding of the adapter TRAF-2 to IRE1 (Fig.6, scheme 2) (258) and the mRNA encoding the bZIP transcription factor X-box binding protein 1, XBP1 (259, 260) (Fig.6, scheme 3). Upon activation of the UPR, *XBP1* mRNA is cleaved by IRE1 to remove a 26-nucleotide intron sequence (259, 261, 262) (Fig.6, scheme 3). The new carboxyl terminus on the product from spliced *XBP1* mRNA (XBP1-s) converts XBP1-u (unspliced) into a potent transcriptional activator, similar to that described for yeast *HAC*.

ATF6 is an ER transmembrane protein composed by two isoforms: ATF6α (90 kDa) and ATF6β (110 kDa, also known as CREB-RP) (262). Upon UPR activation, both forms of ATF6 are processed to generate 50–60-kDa cytosolic bZIP-containing fragments that migrate to the nucleus. ATF6 binds BiP in unstressed cells (263). Upon ER stress, BiP dissociates from ATF6 (263) and ATF6 translocates to the Golgi apparatus (264), where it is subjected to regulated intra-membrane proteolysis by site-1 protease (S1P) and site-2 protease (S2P). This leads to the production of an ATF6 UPR specific fragment with transcriptional activity (265-267) (Fig.6, scheme 3). The first cleavage of ATF6 is produced in its luminal domain by S1P, releasing ATF6 into two halves. The NH2-terminal half remains anchored at the membrane and is subjected to further proteolysis by S2P. On the other hand, the free cytosolic domain of ATF6 translocates to the nucleus to activate ER stress targeted genes (264, 268). When these stress signals are unable to rescue the cells, the apoptotic pathway

is activated. Caspase-12, which is located at the outer layer of the ER, is one of the apoptotic pathways of ER stress-mediated cell death (269). Recently, a model was proposed where stress activation of ATF6 increases the level of XBP-1 mRNA. Concomitant activation of IRE1α removes a non-conventional intron in XBP-1 mRNA. This increases the potential transactivation of specific UPR genes (260, 261).

BiP is an ER-localized protein that binds heavy-chain immunoglobulins and inhibits their secretion in the absence of the light chains in pre-B lymphocytes (270). In this manner, BiP prevents the secretion of incompletely assembled immunoglobulins. Independently, BiP is a member of a protein family expressed at a high level in virally transformed cells and upon conditions of glucose deprivation. For this reason, this protein family was named, glucose regulated proteins (GRPs). BiP interacts transiently with exposed hydrophobic patches on protein folding intermediates and is thought to prevent their aggregation while maintaining the protein in a folding competent state. BiP interaction ensures that only properly folded and assembled proteins exit the ER compartment, a process known as 'quality control' (271). Overexpression of BiP itself prevented transcriptional induction in response to ER stress and protected cells from death in response to calcium depletion from the ER (272, 273). This suggests that BiP could negatively regulate the UPR. In fact, in yeast (274) and mammalian cells (275), overexpression of BiP impedes the UPR.

In mammalian cells, UPR signaling could also lead to cell death. Upon ER stress, two death-inducing signals are generated. One is the IRE1-mediated transcriptional induction of CHOP/GADD153 and the second is translational inhibition mediated through phosphorylation of eIF2α. CHOP was originally identified as a gene induced upon DNA damage and growth arrest (276). However, subsequent studies have demonstrated a strong correlation between development of ER stress response and induction of CHOP (277-283). In addition, CHOP induction closely parallels the time course of BiP induction, where maximal induction occurs after several hours. Most evidence supports that CHOP negatively regulates cell growth and may induce apoptosis. In fact, overexpression of CHOP leads to cell cycle arrest and apoptosis (284, 285). Deletion of the CHOP gene in the mouse leads to a modest reduction in cell death upon activation of the UPR (286). Furthermore, expression of either the isolated luminal domain of IRE1 or PERK alone inhibited transcriptional upregulation of the CHOP promoter upon tunicamycin treatment (139). This shows that either the IRE1 or the PERK luminal domain can interfere with ER signaling for CHOP induction and suggests that both luminal domains respond to similar signals.

The COOH terminus of GADD34, also known as MYD116 (287) is related in sequence to the COOH terminus of HSV-encoded protein $_{\gamma 1}$34.5 (288). $_{\gamma 1}$34.5 plays an important role in evading the PKR-mediated shutdown of host protein synthesis in virally infected cells, and the COOH-terminal fragment of GADD34

can substitute for this activity of $_{\gamma 1}$34.5 (289). This activity of $_{\gamma 1}$34.5 is dependent on its ability to associate with the catalytic subunit of protein phosphatase 1 (PP1c), and correlates with an increase in a cellular phosphatase activity that dephosphorylates eIF2α (290, 291). It is shown that eIF2α phosphorylation and inhibition of protein synthesis are transient in stressed cells (245, 292). GADD34 is activated under stressful conditions (287, 293), and its profile of induction is similar to the other targets of UPR, CHOP and BiP. Moreover, phosphorylated eIF2α levels were markedly diminished in GADD34-overexpressing cells despite normal activity of PERK and GCN2. Mutations abolishing the interaction between GADD34 and PP1c prevented dephosphorylation of eIF2α and blocked attenuation of CHOP induction by GADD34. These findings implicate GADD34-mediated dephosphorylation of eIF2α in a negative feedback loop that relieves stress-induced gene expression, and promotes recovery from translational inhibition of the UPR (294) (Fig.6, scheme 1).

A cellular inhibitor of PKR, P58^{IPK} is activated after influenza virus infection (295-297). The activation of P58^{IPK} inhibits the PKR-mediated translational arrest by binding to and inactivating the kinase activity of PKR, thereby ensuring that the cellular protein-synthesis machinery remains available to synthesize viral proteins. The influenza virus has therefore found a way to co-opt a cellular activity to its purpose. Recently, a study has shown that P58^{IPK} also interacts with and inhibits PERK (Fig.6, scheme 1) (298). Levels of phosphorylated eIF2α were lower in ER-stressed P58^{IPK}-overexpressing cells and were enhanced in

$P58^{IPK}$ mutant cells (298). In addition, the absence of $P58^{IPK}$ resulted in increased expression levels of two ER stress-inducible genes, *BiP* and *Chop*, consistent with enhanced eIF2α phosphorylation in the $P58^{IPK}$ deleted cells (298). This suggests that $P58^{IPK}$ induction during the ER-stress response represses PERK activity and plays a functional role in the expression of downstream markers of PERK activity in the later phase of the ER-stress response.

One of the modifications used for translational control in response to environmental stress is the reversible phosphorylation of eIF2α on Ser51 and this dramatically inhibits translation (117). As pointed out earlier, the status of eIF2α is controlled by at least four known Ser/Thr protein kinases: HRI, which is activated upon iron or hemin deficiency in reticulocytes (135), GCN2, activated in amino acid-starved yeast cells (136), PKR, activated upon viral dsRNA infection (299) and finally PERK, which is uniquely localized to the ER and is activated by the accumulation of unfolded proteins in the ER lumen (139).

Activation of PERK under ER stress conditions was observed when agents causing ER stress induce slower migrating forms of PERK detected by sodium deodecyl-sulfate polyacrylamide gel electrophoresis. This was attributed to PERK hyperphosphorylation and resulted in an increase of its kinase activity (139). Moreover, induction of ER stress (reflected by induction of chaperones) was shown to activate PERK, whereas other stress such as amino acid deprivation and heatshock failed to activate this kinase (139). More importantly,

when exposed to agents that elicit ER stress, *Perk -/-* MEFs cells showed no eIF2α phosphorylation and translation inhibition (300). This demonstrates a unique role for PERK in coupling ER stress to eIF2α phosphorylation and translation inhibition.

VII. Concluding remarks

Signaling cascades that trigger protein translation or its inhibition are an active area of intensive research activities. However, in order to reach a complete understanding the mechanisms underlying these processes, identification and characterization of effector proteins that play an important role in modulating protein translation are still necessary. With the exception of the SH2 domain-containing p85 subunit of PI3-K, known to regulate protein synthesis downstream of RTKs, to date no other SH2/SH3 domain-containing protein has been linked to the control of the translational machinery.

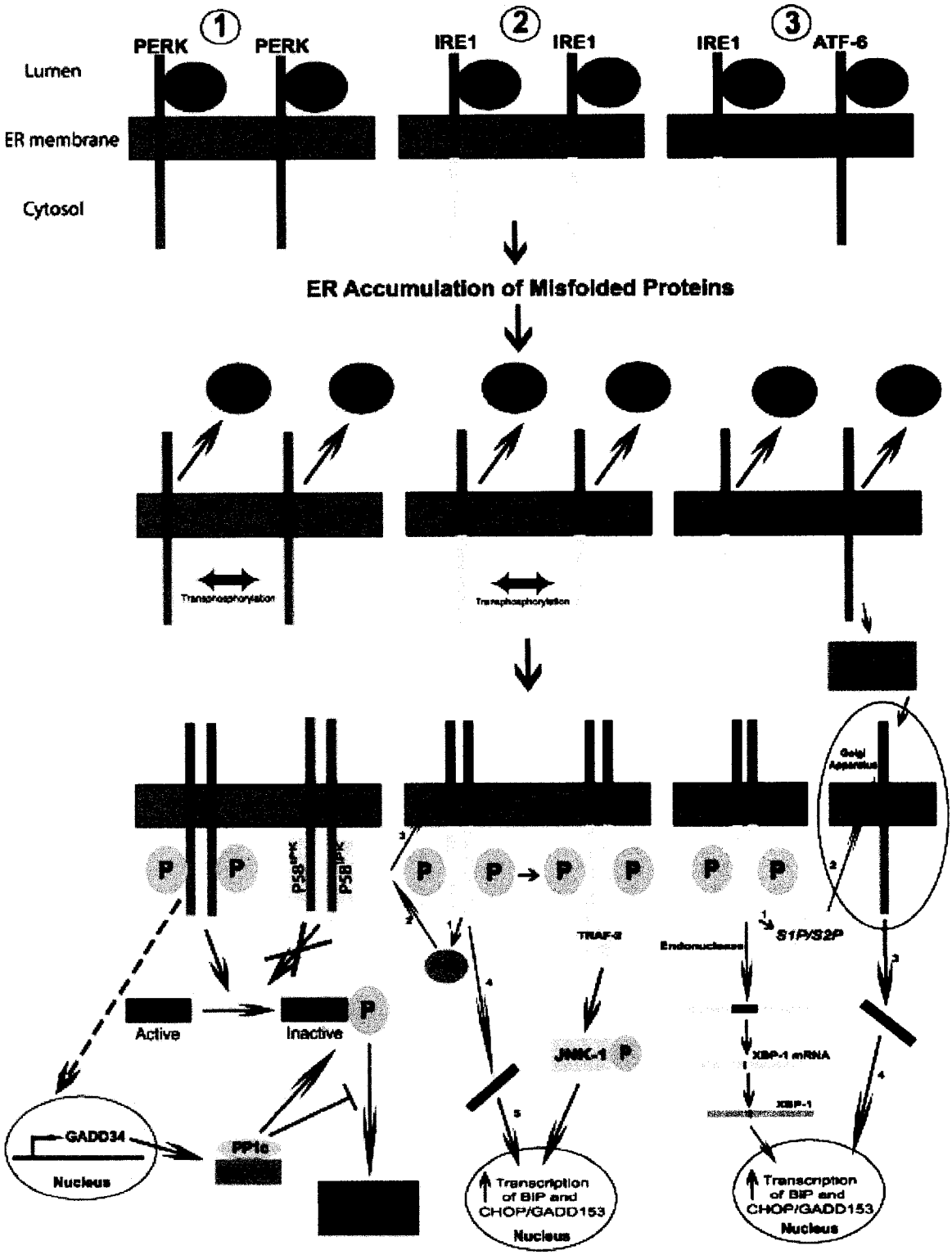


Fig.6. ER-stress Signaling pathways.

VII References

1. Schlessinger, J. (2000) Cell signaling by receptor tyrosine kinases. *Cell* 103, 211-225.

2. Blaikie, P., Immanuel, D., Wu, J., Li, N., Yajnik, V., and Margolis, B. (1994) A region in Shc distinct from the SH2 domain can bind tyrosine-phosphorylated growth factor receptors. *J Biol Chem* 269, 32031-32034.

3. Kavanaugh, W. M., and Williams, L. T. (1994) An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. *Science* 266, 1862-1865.

4. Zambrano, N., Buxbaum, J. D., Minopoli, G., Fiore, F., De Candia, P., De Renzis, S., Faraonio, R., Sabo, S., Cheetham, J., Sudol, M., and Russo, T. (1997) Interaction of the phosphotyrosine interaction/phosphotyrosine binding- related domains of Fe65 with wild-type and mutant Alzheimer's beta- amyloid precursor proteins. *J Biol Chem* 272, 6399-6405.

5. Borg, J. P., Ooi, J., Levy, E., and Margolis, B. (1996) The phosphotyrosine interaction domains of X11 and FE65 bind to distinct sites on the YENPTY motif of amyloid precursor protein. *Mol Cell Biol* 16, 6229-6241.

6. Forman-Kay, J. D., and Pawson, T. (1999) Diversity in protein recognition by PTB domains. *Curr Opin Struct Biol* 9, 690-695.

7. Birge, R. B., Knudsen, B. S., Besser, D., and Hanafusa, H. (1996) SH2 and SH3-containing adaptor proteins: redundant or independent mediators of intracellular signal transduction. *Genes Cells* 1, 595-613

8. Buday, L. (1999) Membrane-targeting of signalling molecules by SH2/SH3 domain-containing adaptor proteins. *Biochim Biophys Acta* 1422, 187-204.

9. Pawson, T. (1995) Protein modules and signalling networks. *Nature* 373, 573-580.

10. Feller, S. M. (2001) Crk family adaptors-signalling complex formation and biological roles. *Oncogene* 20, 6348-6371

11. Schlessinger, J. (1994) SH2/SH3 signaling proteins. *Curr Opin Genet Dev* 4, 25-30

12. Li, W., Fan, J., and Woodley, D. T. (2001) Nck/Dock: an adapter between cell surface receptors and the actin cytoskeleton. *Oncogene* 20, 6403-6417

13. McCarty, J. H. (1998) The Nck SH2/SH3 adaptor protein: a regulator of multiple intracellular signal transduction events. *Bioessays* 20, 913-921.

14. Li, W., and She, H. (2000) The SH2 and SH3 adapter Nck: a two-gene family and a linker between tyrosine kinases and multiple signaling networks [In Process Citation]. *Histol Histopathol* 15, 947-955

15. Morrione, A. (2000) Grb10 proteins in insulin-like growth factor and insulin receptor signaling (review). *Int J Mol Med* 5, 151-154

16. Shepherd, P. R., Withers, D. J., and Siddle, K. (1998) Phosphoinositide 3-kinase: the key switch mechanism in insulin signalling [published erratum appears in Biochem J 1998 Nov 1;335(Pt 3):711]. *Biochem J* 333, 471-490

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

18. Cohen, G. B., Ren, R., and Baltimore, D. (1995) Modular binding domains in signal transduction proteins. *Cell* 80, 237-248.

19. Cowburn, D. (1996) Adaptors and integrators. *Structure* 4, 1005-1008.

20. Lemmon, M. A., Ferguson, K. M., and Schlessinger, J. (1996) PH domains: diverse sequences with a common fold recruit signaling molecules to the cell surface. *Cell* 85, 621-624.

21. Sudol, M. (1996) The WW module competes with the SH3 domain? *Trends Biochem Sci* 21, 161-163.

22. Lehmann, J. M., Riethmuller, G., and Johnson, J. P. (1990) Nck, a melanoma cDNA encoding a cytoplasmic protein consisting of the src homology units SH2 and SH3. *Nucleic Acids Res* 18, 1048.

23. Margolis, B., Silvennoinen, O., Comoglio, F., Roonprapunt, C., Skolnik, E., Ullrich, A., and Schlessinger, J. (1992) High-efficiency expression/cloning of epidermal growth factor-receptor- binding proteins with Src homology 2 domains. *Proc Natl Acad Sci U S A* 89, 8894-8898.

24. Park, D., and Rhee, S. G. (1992) Phosphorylation of Nck in response to a variety of receptors, phorbol myristate acetate, and cyclic AMP. *Mol Cell Biol* 12, 5816-5823.

25. Li, W., Hu, P., Skolnik, E. Y., Ullrich, A., and Schlessinger, J. (1992) The SH2 and SH3 domain-containing Nck protein is oncogenic and a common target for phosphorylation by different surface receptors. *Mol Cell Biol* 12, 5824-5833

26. Meisenhelder, J., and Hunter, T. (1992) The SH2/SH3 domain-containing protein Nck is recognized by certain anti- phospholipase C-gamma 1 monoclonal antibodies, and its phosphorylation on tyrosine is stimulated by platelet-derived growth factor and epidermal growth factor treatment. *Mol Cell Biol* 12, 5843-5856.

27. Huebner, K., Kastury, K., Druck, T., Salcini, A. E., Lanfrancone, L., Pelicci, G., Lowenstein, E., Li, W., Park, S. H., Cannizzaro, L., and et al. (1994) Chromosome locations of genes encoding human signal transduction adapter proteins, Nck (NCK), Shc (SHC1), and Grb2 (GRB2). *Genomics* 22, 281-287.

28. Chen, M., She, H., Davis, E. M., Spicer, C. M., Kim, L., Ren, R., Le Beau, M. M., and Li, W. (1998) Identification of Nck family genes, chromosomal localization, expression, and signaling specificity. *J Biol Chem* 273, 25171-25178.

29. Vorobieva, N., Protopopov, A., Protopopova, M., Kashuba, V., Allikmets, R. L., Modi, W., Zabarovsky, E. R., Klein, G., Kisselev, L., and Graphodatsky, A. (1995) Localization of human ARF2 and NCK genes and 13 other NotI-linking clones to chromosome 3 by fluorescence in situ hybridization. *Cytogenet Cell Genet* 68, 91-94

30. Tanaka, M., Lu, W., Gupta, R., and Mayer, B. J. (1997) Expression of mutated Nck SH2/SH3 adaptor respecifies mesodermal cell fate in Xenopus laevis development. *Proc Natl Acad Sci U S A* 94, 4493-4498

31. Garrity, P. A., Rao, Y., Salecker, I., McGlade, J., Pawson, T., and Zipursky, S. L. (1996) Drosophila photoreceptor axon guidance and targeting requires the dreadlocks SH2/SH3 adapter protein. *Cell* 85, 639-650

32. Chou, M. M., Fajardo, J. E., and Hanafusa, H. (1992) The SH2- and SH3-containing Nck protein transforms mammalian fibroblasts in the absence of elevated phosphotyrosine levels. *Mol Cell Biol* 12, 5834-5842.

33. Nishimura, R., Li, W., Kashishian, A., Mondino, A., Zhou, M., Cooper, J., and Schlessinger, J. (1993) Two signaling molecules share a phosphotyrosine-containing binding site in the platelet-derived growth factor receptor. *Mol Cell Biol* 13, 6889-6896

34. Tang, J., Feng, G. S., and Li, W. (1997) Induced direct binding of the adapter protein Nck to the GTPase- activating protein-associated protein p62 by epidermal growth factor. *Oncogene* 15, 1823-1832.

35. Noguchi, T., Matozaki, T., Inagaki, K., Tsuda, M., Fukunaga, K., Kitamura, Y., Kitamura, T., Shii, K., Yamanashi, Y., and Kasuga, M. (1999) Tyrosine phosphorylation of p62(Dok) induced by cell adhesion and insulin: possible role in cell migration. *Embo J* 18, 1748-1760

36. Holland, S. J., Gale, N. W., Gish, G. D., Roth, R. A., Songyang, Z., Cantley, L. C., Henkemeyer, M., Yancopoulos, G. D., and Pawson, T. (1997) Juxtamembrane tyrosine residues couple the Eph family receptor EphB2/Nuk to specific SH2 domain proteins in neuronal cells. *Embo J* 16, 3877-3888.

37. Becker, E., Huynh-Do, U., Holland, S., Pawson, T., Daniel, T. O., and Skolnik, E. Y. (2000) Nck-interacting Ste20 kinase couples Eph receptors to c-Jun N-terminal kinase and integrin activation. *Mol Cell Biol* 20, 1537-1545

38. Stein, E., Huynh-Do, U., Lane, A. A., Cerretti, D. P., and Daniel, T. O. (1998) Nck recruitment to Eph receptor, EphB1/ELK, couples ligand activation to c-Jun kinase. *J Biol Chem* 273, 1303-1308

39. Lee, C. H., Li, W., Nishimura, R., Zhou, M., Batzer, A. G., Myers, M. G., Jr., White, M. F., Schlessinger, J., and Skolnik, E. Y. (1993) Nck associates with the SH2 domain-docking protein IRS-1 in insulin-stimulated cells. *Proc Natl Acad Sci U S A* 90, 11713-11717

40. Xu, P., Jacobs, A. R., and Taylor, S. I. (1999) Interaction of insulin receptor substrate 3 with insulin receptor, insulin receptor-related receptor, insulin-like growth factor-1 receptor, and downstream signaling proteins. *J Biol Chem* 274, 15262-15270

41. Schlaepfer, D. D., Broome, M. A., and Hunter, T. (1997) Fibronectin-stimulated signaling from a focal adhesion kinase-c-Src complex: involvement of the Grb2, p130cas, and Nck adaptor proteins. *Mol Cell Biol* 17, 1702-1713

42. Goicoechea, S. M., Tu, Y., Hua, Y., Chen, K., Shen, T. L., Guan, J. L., and Wu, C. (2002) Nck-2 interacts with focal adhesion kinase and modulates cell motility. *Int J Biochem Cell Biol* 34, 791-805.

43. Campellone, K. G., Giese, A., Tipper, D. J., and Leong, J. M. (2002) A tyrosine-phosphorylated 12-amino-acid sequence of enteropathogenic Escherichia coli Tir binds the host adaptor protein Nck and is required for Nck localization to actin pedestals. *Mol Microbiol* 43, 1227-1241.

44. Gruenheid, S., DeVinney, R., Blat, F., Goosney, D., Gelkops, S., Gish, G. D., Pawson, T., and Finlay, B. B. (2001) Enteropathogenic E.coli Tir bind Nck to initiate actin pedestal formation in host cells. *Nature Cell biology* 3, 856-859

45. Kenny, B., DeVinney, R., Stein, M., Reinscheid, D. J., Frey, E. A., and Finlay, B. B. (1997) Enteropathogenic E. coli (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell* 91, 511-520

46. Hu, Q., Milfay, D., and Williams, L. T. (1995) Binding of NCK to SOS and activation of ras-dependent gene expression. *Mol Cell Biol* 15, 1169-1174

47. Okada, S., and Pessin, J. E. (1996) Interactions between Src homology (SH) 2/SH3 adapter proteins and the guanylnucleotide exchange factor SOS are differentially regulated by insulin and epidermal growth factor. *J Biol Chem* 271, 25533-25538.

48. Gupta, R. W., and Mayer, B. J. (1998) Dominant-negative mutants of the SH2/SH3 adapters Nck and Grb2 inhibit MAP kinase activation and mesoderm-specific gene induction by eFGF in Xenopus. *Oncogene* 17, 2155-2165

49. Tanaka, M., Gupta, R., and Mayer, B. J. (1995) Differential inhibition of signaling pathways by dominant-negative SH2/SH3 adapter proteins. *Mol Cell Biol* 15, 6829-6837

50. Roche, S., McGlade, J., Jones, M., Gish, G. D., Pawson, T., and Courtneidge, S. A. (1996) Requirement of phospholipase C gamma, the tyrosine phosphatase Syp and the adaptor proteins Shc and Nck for PDGF-induced DNA synthesis: evidence for the existence of Ras-dependent and Ras-independent pathways. *Embo J* 15, 4940-4948

51. Rockow, S., Tang, J., Xiong, W., and Li, W. (1996) Nck inhibits NGF and basic FGF induced PC12 cell differentiation via mitogen-activated protein kinase-independent pathway. *Oncogene* 12, 2351-2359

52. Bagrodia, S., and Cerione, R. A. (1999) Pak to the future. *Trends Cell Biol* 9, 350-355

53. Lu, W., Katz, S., Gupta, R., and Mayer, B. J. (1997) Activation of Pak by membrane localization mediated by an SH3 domain from the adaptor protein Nck. *Curr Biol* 7, 85-94.

54. Galisteo, M. L., Chernoff, J., Su, Y. C., Skolnik, E. Y., and Schlessinger, J. (1996) The adaptor protein Nck links receptor tyrosine kinases with the serine- threonine kinase Pak1. *J Biol Chem* 271, 20997-21000.

55. Bokoch, G. M., Wang, Y., Bohl, B. P., Sells, M. A., Quilliam, L. A., and Knaus, U. G. (1996) Interaction of the Nck adapter protein with p21-activated kinase (PAK1). *J Biol Chem* 271, 25746-25749.

56. Bagrodia, S., Derijard, B., Davis, R. J., and Cerione, R. A. (1995) Cdc42 and PAK-mediated signaling leads to Jun kinase and p38 mitogen-activated protein kinase activation. *J Biol Chem* 270, 27995-27998.

57. Lu, W., and Mayer, B. J. (1999) Mechanism of activation of Pak1 kinase by membrane localization. *Oncogene* 18, 797-806

58. Quilliam, L. A., Lambert, Q. T., Mickelson-Young, L. A., Westwick, J. K., Sparks, A. B., Kay, B. K., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., and Der, C. J. (1996) Isolation of a NCK-associated kinase, PRK2, an SH3-binding protein and potential effector of Rho protein signaling. *J Biol Chem* 271, 28772-28776

59. Vojtek, A. B., and Cooper, J. A. (1995) Rho family members: activators of MAP kinase cascades. *Cell* 82, 527-529.

60. Kiosses, W. B., Hood, J., Yang, S., Gerritsen, M. E., Cheresh, D. A., Alderson, N., and Schwartz, M. A. (2002) A dominant-negative p65 PAK peptide inhibits angiogenesis. *Circ Res* 90, 697-702.

61. Su, Y. C., Han, J., Xu, S., Cobb, M., and Skolnik, E. Y. (1997) NIK is a new Ste20-related kinase that binds NCK and MEKK1 and activates the SAPK/JNK cascade via a conserved regulatory domain. *Embo J* 16, 1279-1290.

62. Hummel, T., Leifker, K., and Klambt, C. (2000) The Drosophila HEM-2/NAP1 homolog KETTE controls axonal pathfinding and cytoskeletal organization. *Genes Dev* 14, 863-873

63. Kitamura, Y., Kitamura, T., Sakaue, H., Maeda, T., Ueno, H., Nishio, S., Ohno, S., Osada, S., Sakaue, M., Ogawa, W., and Kasuga, M. (1997) Interaction of Nck-associated protein 1 with activated GTP-binding protein Rac. *Biochem J* 322 (Pt 3), 873-878

64. Kitamura, T., Kitamura, Y., Yonezawa, K., Totty, N. F., Gout, I., Hara, K., Waterfield, M. D., Sakaue, M., Ogawa, W., and Kasuga, M. (1996) Molecular cloning of p125Nap1, a protein that associates with an SH3 domain of Nck. *Biochem Biophys Res Commun* 219, 509-514

65. Suzuki, T., Nishiyama, K., Yamamoto, A., Inazawa, J., Iwaki, T., Yamada, T., Kanazawa, I., and Sakaki, Y. (2000) Molecular cloning of a novel apoptosis-related gene, human Nap1 (NCKAP1), and its possible relation to Alzheimer disease. *Genomics* 63, 246-254

66. Yamamoto, A., Suzuki, T., and Sakaki, Y. (2001) Isolation of hNap1BP which interacts with human Nap1 (NCKAP1) whose expression is down-regulated in Alzheimer's disease. *Gene* 271, 159-169

67. Li, X., Meriane, M., Triki, I., Shekarabi, M., Kennedy, T. E., Larose, L., and Lamarche-Vane, N. (2002) The adaptor protein Nck-1 couples the netrin-1 receptor DCC (deleted in colorectal cancer) to the activation of the small GTPase Rac1 through an atypical mechanism. *J Biol Chem* 30, 30

68. Tu, Y., Li, F., and Wu, C. (1998) Nck-2, a novel Src homology2/3-containing adaptor protein that interacts with the LIM-only protein PINCH and components of growth factor receptor kinase-signaling pathways. *Mol Biol Cell* 9, 3367-3382

69. Tu, Y., Li, F., Goicoechea, S., and Wu, C. (1999) The LIM-only protein PINCH directly interacts with integrin-linked kinase and is recruited to integrin-rich sites in spreading cells. *Mol Cell Biol* 19, 2425-2434.

70. Rivero-Lezcano, O. M., Marcilla, A., Sameshima, J. H., and Robbins, K. C. (1995) Wiskott-Aldrich syndrome protein physically associates with Nck through Src homology 3 domains. *Mol Cell Biol* 15, 5725-5731.

71. Derry, J. M., Ochs, H. D., and Francke, U. (1994) Isolation of a novel gene mutated in Wiskott-Aldrich syndrome. *Cell* 79, following 922

72. Mullins, R. D. (2000) How WASP-family proteins and the Arp2/3 complex convert intracellular signals into cytoskeletal structures. *Curr Opin Cell Biol* 12, 91-96

73. Machesky, L. M., and Insall, R. H. (1999) Signaling to actin dynamics. *J Cell Biol* 146, 267-272

74. Mayer, B. J. (2001) SH3 domains: complexity in moderation. *J Cell Sci* 114, 1253-1263

75. Takenawa, T., and Miki, H. (2001) WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin filaments and cell movement. *J Cell Sci* 114, 1801-1809.

76. Moreau, V., Frischknecht, F., Reckmann, I., Vincentelli, R., Rabut, G., Stewart, D., and Way, M. (2000) A complex of N-WASP and WIP integrates signalling cascades that lead to actin polymerization. *Nat Cell Biol* 2, 441-448.

77. Frischknecht, F., Moreau, V., Rottger, S., Gonfloni, S., Reckmann, I., Superti-Furga, G., and Way, M. (1999) Actin-based motility of vaccinia virus mimics receptor tyrosine kinase signalling. *Nature* 401, 926-929.

78. Scaplehorn, N., Holmstrom, A., Moreau, V., Frischknecht, F., Reckmann, I., and Way, M. (2002) Grb2 and nck act cooperatively to promote actin-based motility of vaccinia virus. *Curr Biol* 12, 740-745.

79. Eden, S., Rohatgi, R., Podtelejnikov, A. V., Mann, M., and Kirschner, M. W. (2002) Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck. *Nature* 418, 790-793.

80. Tu, Y., Kucik, D. F., and Wu, C. (2001) Identification and kinetic analysis of the interaction between Nck-2 and DOCK180. *FEBS Lett* 491, 193-199.

81. Cheresh, D. A., Leng, J., and Klemke, R. L. (1999) Regulation of cell contraction and membrane ruffling by distinct signals in migratory cells. *J Cell Biol* 146, 1107-1116

82. Kiyokawa, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kurata, T., and Matsuda, M. (1998) Activation of Rac1 by a Crk SH3-binding protein, DOCK180. *Genes Dev* 12, 3331-3336

83. Kiyokawa, E., Hashimoto, Y., Kurata, T., Sugimura, H., and Matsuda, M. (1998) Evidence that DOCK180 up-regulates signals from the CrkII-p130(Cas) complex. *J Biol Chem* 273, 24479-24484

84. Zhao, Z. S., Manser, E., Loo, T. H., and Lim, L. (2000) Coupling of PAK-interacting exchange factor PIX to GIT1 promotes focal complex disassembly. *Mol Cell Biol* 20, 6354-6363

85. Tu, Y., Liang, L., Frank, S. J., and Wu, C. (2001) Src homology 3 domain-dependent interaction of Nck-2 with insulin receptor substrate-1. *Biochem J* 354, 315-322.

86. Lussier, G., and Larose, L. (1997) A casein kinase I activity is constitutively associated with Nck. *J Biol Chem* 272, 2688-2694

87. Bioukar, E. B., Marricco, N. C., Zuo, D., and Larose, L. (1999) Serine phosphorylation of the ligand-activated beta-platelet-derived growth factor receptor by casein kinase I-gamma2 inhibits the receptor's autophosphorylating activity. *J Biol Chem* 274, 21457-21463

88. Huang, S. (2000) Review: perinucleolar structures. *J Struct Biol* 129, 233-240

89. Lawe, D. C., Hahn, C., and Wong, A. J. (1997) The Nck SH2/SH3 adaptor protein is present in the nucleus and associates with the nuclear protein SAM68. *Oncogene* 14, 223-231.

90. Sanchez-Margalet, V., and Najib, S. (1999) p68 Sam is a substrate of the insulin receptor and associates with the SH2 domains of p85 PI3K. *FEBS Lett* 455, 307-310.

91. Sanchez-Margalet, V., and Najib, S. (2001) Sam68 is a docking protein linking GAP and PI3K in insulin receptor signaling. *Mol Cell Endocrinol* 183, 113-121

92. Ren, R., Ye, Z. S., and Baltimore, D. (1994) Abl protein-tyrosine kinase selects the Crk adapter as a substrate using SH3-binding sites. *Genes Dev* 8, 783-795.

93. Smith, J. M., Katz, S., and Mayer, B. J. (1999) Activation of the Abl tyrosine kinase in vivo by Src homology 3 domains from the Src homology 2/Src homology 3 adaptor Nck. *J Biol Chem* 274, 27956-27962.

94. Adler, C. E., Miyoshi-Akiyama, T., Aleman, L. M., Tanaka, M., Smith, J. M., and Mayer, B. J. (2000) Abl family kinases and Cbl cooperate with the Nck adaptor to modulate Xenopus development. *J Biol Chem* 275, 36472-36478

95. Rivero-Lezcano, O. M., Sameshima, J. H., Marcilla, A., and Robbins, K. C. (1994) Physical association between Src homology 3 elements and the protein product of the c-cbl proto-oncogene. *J Biol Chem* 269, 17363-17366.

96. Langdon, W. Y. (1995) The cbl oncogene: a novel substrate of protein tyrosine kinases. *Aust N Z J Med* 25, 859-864.

97. Miyoshi-Akiyama, T., Aleman, L. M., Smith, J. M., Adler, C. E., and Mayer, B. J. (2001) Regulation of Cbl phosphorylation by the Abl tyrosine kinase and the Nck SH2/SH3 adaptor. *Oncogene* 20, 4058-4069.

98. Ming, X. F., Burgering, B. M., Wennstrom, S., Claesson-Welsh, L., Heldin, C. H., Bos, J. L., Kozma, S. C., and Thomas, G. (1994) Activation of p70/p85 S6 kinase by a pathway independent of p21ras. *Nature* 371, 426-429

99. Chou, M. M., and Blenis, J. (1996) The 70 kDa S6 kinase complexes with and is activated by the Rho family G proteins Cdc42 and Rac1. *Cell* 85, 573-583

100. Chou, M. M., and Blenis, J. (1995) The 70 kDa S6 kinase: regulation of a kinase with multiple roles in mitogenic signalling. *Curr Opin Cell Biol* 7, 806-814

101. Rao, Y., and Zipursky, S. L. (1998) Domain requirements for the Dock adapter protein in growth- cone signaling. *Proc Natl Acad Sci U S A* 95, 2077-2082.

102. Hing, H., Xiao, J., Harden, N., Lim, L., and Zipursky, S. L. (1999) Pak functions downstream of Dock to regulate photoreceptor axon guidance in Drosophila. *Cell* 97, 853-863.

103. Newsome, T. P., Schmidt, S., Dietzl, G., Keleman, K., Asling, B., Debant, A., and Dickson, B. J. (2000) Trio combines with dock to regulate Pak activity during photoreceptor axon pathfinding in Drosophila. *Cell* 101, 283-294

104. Yablonski, D., Kane, L. P., Qian, D., and Weiss, A. (1998) A Nck-Pak1 signaling module is required for T-cell receptor-mediated activation of NFAT, but not of JNK. *Embo J* 17, 5647-5657

105. Wunderlich, L., Goher, A., Farago, A., Downward, J., and Buday, L. (1999) Requirement of multiple SH3 domains of Nck for ligand binding. *Cell Signal* 11, 253-262.

106. Bubeck Wardenburg, J., Pappu, R., Bu, J. Y., Mayer, B., Chernoff, J., Straus, D., and Chan, A. C. (1998) Regulation of PAK activation and the T cell cytoskeleton by the linker protein SLP-76. *Immunity* 9, 607-616

107. Ku, G. M., Yablonski, D., Manser, E., Lim, L., and Weiss, A. (2001) A PAK1-PIX-PKL complex is activated by the T-cell receptor independent of Nck, Slp-76 and LAT. *Embo J* 20, 457-465

108. Gil, D., Schamel, W. W., Montoya, M., Sanchez-Madrid, F., and Alarcon, B. (2002) Recruitment of Nck by CD3 epsilon reveals a ligand-induced conformational change essential for T cell receptor signaling and synapse formation. *Cell* 109, 901-912.

109. Mizuno, K., Tagawa, Y., Mitomo, K., Watanabe, N., Katagiri, T., Ogimoto, M., and Yakura, H. (2002) Src homology region 2 domain-containing phosphatase 1 positively regulates B cell receptor-induced apoptosis by modulating association of B cell linker protein with Nck and activation of c-Jun NH2-terminal kinase. *J Immunol* 169, 778-786.

110. Bonini, J. A., Colca, J., and Hofmann, C. (1995) Altered expression of insulin signaling components in streptozotocin- treated rats. *Biochem Biophys Res Commun* 212, 933-938.

111. Bonini, J. A., Colca, J. R., Dailey, C., White, M., and Hofmann, C. (1995) Compensatory alterations for insulin signal transduction and glucose transport in insulin-resistant diabetes. *Am J Physiol* 269, E759-765.

112. Kim, Y. B., Inoue, T., Nakajima, R., Shirai-Morishita, Y., Tokuyama, K., and Suzuki, M. (1999) Effect of long-term exercise on gene expression of insulin signaling pathway intermediates in skeletal muscle. *Biochem Biophys Res Commun* 254, 720-727

113. Chen, M., She, H., Kim, A., Woodley, D. T., and Li, W. (2000) Nckbeta adapter regulates actin polymerization in NIH 3T3 fibroblasts in response to platelet-derived growth factor bb. *Mol Cell Biol* 20, 7867-7880.

114. Stoletov, K. V., Ratcliffe, K. E., Spring, S. C., and Terman, B. I. (2001) NCK and PAK participate in the signaling pathway by which vascular endothelial growth factor stimulates the assembly of focal adhesions. *J Biol Chem* 276, 22748-22755.

115. Merrick, W. C. (1992) Mechanism and regulation of eukaryotic protein synthesis. *Microbiol Rev* 56, 291-315

116. Hershey, J. W. (1991) Translational control in mammalian cells. *Annu Rev Biochem* 60, 717-755

117. Pain, V. M. (1996) Initiation of protein synthesis in eukaryotic cells. *Eur J Biochem* 236, 747-771

118. Rhoads, R. E. (1993) Regulation of eukaryotic protein synthesis by initiation factors. *J Biol Chem* 268, 3017-3020

119. Chakrabarti, A., and Maitra, U. (1991) Function of eukaryotic initiation factor 5 in the formation of an 80 S ribosomal polypeptide chain initiation complex. *J Biol Chem* 266, 14039-14045

120. Jackson, R. J., Hunt, S. L., Reynolds, J. E., and Kaminski, A. (1995) Cap-dependent and cap-independent translation: operational distinctions and mechanistic interpretations. *Curr Top Microbiol Immunol* 203, 1-29

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

122. Chen, C. Y., and Sarnow, P. (1995) Initiation of protein synthesis by the eukaryotic translational apparatus on circular RNAs. *Science* 268, 415-417.

123. Jang, S. K., Krausslich, H. G., Nicklin, M. J., 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.

124. 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.

125. 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.

126. Thompson, S. R., Gulyas, K. D., and Sarnow, P. (2001) Internal initiation in Saccharomyces cerevisiae mediated by an initiator tRNA/eIF2-independent internal ribosome entry site element. *Proc Natl Acad Sci U S A* 98, 12972-12977.

127. Kaminski, A., Hunt, S. L., Patton, J. G., and Jackson, R. J. (1995) Direct evidence that polypyrimidine tract binding protein (PTB) is essential for internal initiation of translation of encephalomyocarditis virus RNA. *Rna* 1, 924-938.

128. Hellen, C. U., Witherell, G. W., Schmid, M., Shin, S. H., Pestova, T. V., Gil, A., and Wimmer, E. (1993) A cytoplasmic 57-kDa protein that is required for translation of picornavirus RNA by internal ribosomal entry is identical to the nuclear pyrimidine tract-binding protein. *Proc Natl Acad Sci U S A* 90, 7642-7646.

129. Meerovitch, K., Svitkin, Y. V., Lee, H. S., Lejbkowicz, F., Kenan, D. J., Chan, E. K., Agol, V. I., Keene, J. D., and Sonenberg, N. (1993) La autoantigen enhances and corrects aberrant translation of poliovirus RNA in reticulocyte lysate. *J Virol* 67, 3798-3807.

130. 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.

131. Ziegler, E., Borman, A. M., Kirchweger, R., Skern, T., and Kean, K. M. (1995) Foot-and-mouth disease virus Lb proteinase can stimulate rhinovirus and enterovirus IRES-driven translation and cleave several proteins of cellular and viral origin. *J Virol* 69, 3465-3474.

132. Pestova, T. V., Shatsky, I. N., and Hellen, C. U. (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.

133. Morley, S. J. (1994) Signal transduction mechanisms in the regulation of protein synthesis. *Mol Biol Rep* 19, 221-231.

134. Erickson, F. L., Harding, L. D., Dorris, D. R., and Hannig, E. M. (1997) Functional analysis of homologs of translation initiation factor 2gamma in yeast. *Mol Gen Genet* 253, 711-719

135. Chen, J. J., and London, I. M. (1995) Regulation of protein synthesis by heme-regulated eIF-2 alpha kinase. *Trends Biochem Sci* 20, 105-108

136. Hinnebusch, A. G. (1994) The eIF-2 alpha kinases: regulators of protein synthesis in starvation and stress. *Semin Cell Biol* 5, 417-426.

137. Clemens, M. J., and Elia, A. (1997) The double-stranded RNA-dependent protein kinase PKR: structure and function. *J Interferon Cytokine Res* 17, 503-524.

138. Clemens, M. J. (1997) PKR--a protein kinase regulated by double-stranded RNA. *Int J Biochem Cell Biol* 29, 945-949.

139. Harding, H. P., Zhang, Y., and Ron, D. (1999) Protein translation and folding are coupled by an endoplasmic-reticulum- resident kinase. *Nature* 397, 271-274.

140. Welsh, G. I., Price, N. T., Bladergroen, B. A., Bloomberg, G., and Proud, C. G. (1994) Identification of novel phosphorylation sites in the beta-subunit of translation initiation factor eIF-2. *Biochem Biophys Res Commun* 201, 1279-1288

141. Das, S., Maiti, T., Das, K., and Maitra, U. (1997) Specific interaction of eukaryotic translation initiation factor 5 (eIF5) with the beta-subunit of eIF2. *J Biol Chem* 272, 31712-31718

142. Kimball, S. R., Heinzinger, N. K., Horetsky, R. L., and Jefferson, L. S. (1998) Identification of interprotein interactions between the subunits of eukaryotic initiation factors eIF2 and eIF2B. *J Biol Chem* 273, 3039-3044

143. Gaspar, N. J., Kinzy, T. G., Scherer, B. J., Humbelin, M., Hershey, J. W., and Merrick, W. C. (1994) Translation initiation factor eIF-2. Cloning and expression of the human cDNA encoding the gamma-subunit. *J Biol Chem* 269, 3415-3422

144. Naranda, T., Sirangelo, I., Fabbri, B. J., and Hershey, J. W. (1995) Mutations in the NKXD consensus element indicate that GTP binds to the gamma-subunit of translation initiation factor eIF2. *FEBS Lett* 372, 249-252

145. Kaufman, R. J. (1999) Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev* 13, 1211-1233.

146. Price, N., and Proud, C. (1994) The guanine nucleotide-exchange factor, eIF-2B. *Biochimie* 76, 748-760

147. Welsh, G. I., and Proud, C. G. (1993) Glycogen synthase kinase-3 is rapidly inactivated in response to insulin and phosphorylates eukaryotic initiation factor eIF-2B. *Biochem J* 294, 625-629.

148. Singh, L. P., Denslow, N. D., and Wahba, A. J. (1996) Modulation of rabbit reticulocyte guanine nucleotide exchange factor activity by casein kinases 1 and 2 and glycogen synthase kinase 3. *Biochemistry* 35, 3206-3212

149. Borthwick, A. C., Wells, A. M., Rochford, J. J., Hurel, S. J., Turnbull, D. M., and Yeaman, S. J. (1995) Inhibition of glycogen synthase kinase-3 by insulin in cultured human skeletal muscle myoblasts. *Biochem Biophys Res Commun* 210, 738-745

150. Moule, S. K., Edgell, N. J., Welsh, G. I., Diggle, T. A., Foulstone, E. J., Heesom, K. J., Proud, C. G., and Denton, R. M. (1995) Multiple signalling pathways involved in the stimulation of fatty acid and glycogen synthesis by insulin in rat epididymal fat cells. *Biochem J* 311 (Pt 2), 595-601

151. Moule, S. K., Welsh, G. I., Edgell, N. J., Foulstone, E. J., Proud, C. G., and Denton, R. M. (1997) Regulation of protein kinase B and glycogen synthase kinase-3 by insulin and beta-adrenergic agonists in rat epididymal fat cells. Activation of protein kinase B by wortmannin-sensitive and -insensitive mechanisms. *J Biol Chem* 272, 7713-7719

152. Welsh, G. I., Miller, C. M., Loughlin, A. J., Price, N. T., and Proud, C. G. (1998) Regulation of eukaryotic initiation factor eIF2B: glycogen synthase kinase-3 phosphorylates a conserved serine which undergoes dephosphorylation in response to insulin. *FEBS Lett* 421, 125-130

153. Welsh, G. I., Stokes, C. M., Wang, X., Sakaue, H., Ogawa, W., Kasuga, M., and Proud, C. G. (1997) Activation of translation initiation factor eIF2B by insulin requires phosphatidyl inositol 3-kinase. *FEBS Lett* 410, 418-422

154. Morley, S. J. (1997) Intracellular signalling pathways regulating initiation factor eIF4E phosphorylation during the activation of cell growth. *Biochem Soc Trans* 25, 503-509.

155. Flynn, A., and Proud, C. G. (1996) The role of eIF4 in cell proliferation. *Cancer Surv* 27, 293-310

156. Miyagi, Y., Sugiyama, A., Asai, A., Okazaki, T., Kuchino, Y., and Kerr, S. J. (1995) Elevated levels of eukaryotic translation initiation factor eIF-4E, mRNA in a broad spectrum of transformed cell lines. *Cancer Lett* 91, 247-252

157. Li, B. D., Liu, L., Dawson, M., and De Benedetti, A. (1997) Overexpression of eukaryotic initiation factor 4E (eIF4E) in breast carcinoma. *Cancer* 79, 2385-2390.

158. Nathan, C. A., Carter, P., Liu, L., Li, B. D., Abreo, F., Tudor, A., Zimmer, S. G., and De Benedetti, A. (1997) Elevated expression of eIF4E and FGF-2 isoforms during vascularization of breast carcinomas. *Oncogene* 15, 1087-1094.

159. Kerekatte, V., Smiley, K., Hu, B., Smith, A., Gelder, F., and De Benedetti, A. (1995) The proto-oncogene/translation factor eIF4E: a survey of its expression in breast carcinomas. *Int J Cancer* 64, 27-31.

160. 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.

161. Joshi-Barve, S., Rychlik, W., and Rhoads, R. E. (1990) Alteration of the major phosphorylation site of eukaryotic protein synthesis initiation factor 4E prevents its association with the 48 S initiation complex. *J Biol Chem* 265, 2979-2983.

162. Rau, M., Ohlmann, T., Morley, S. J., and Pain, V. M. (1996) A reevaluation of the cap-binding protein, eIF4E, as a rate-limiting factor for initiation of translation in reticulocyte lysate. *J Biol Chem* 271, 8983-8990.

163. Minich, W. B., Balasta, M. L., Goss, D. J., and Rhoads, R. E. (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.

164. 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.

165. Lamphear, B. J., and Panniers, R. (1990) Cap binding protein complex that restores protein synthesis in heat- shocked Ehrlich cell lysates contains highly phosphorylated eIF-4E. *J Biol Chem* 265, 5333-5336.

166. Morley, S. J., Rau, M., Kay, J. E., and Pain, V. M. (1993) Increased phosphorylation of eukaryotic initiation factor 4 alpha during activation of T lymphocytes correlates with increased eIF-4F complex formation. *Biochem Soc Trans* 21, 397S.

167. Haghighat, A., and Sonenberg, N. (1997) eIF4G dramatically enhances the binding of eIF4E to the mRNA 5'-cap structure. *J Biol Chem* 272, 21677-21680.

168. 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.

169. Lazaris-Karatzas, A., and Sonenberg, N. (1992) The mRNA 5' cap-binding protein, eIF-4E, cooperates with v-myc or E1A in the transformation of primary rodent fibroblasts. *Mol Cell Biol* 12, 1234-1238.

170. 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.

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. Lazaris-Karatzas, A., Smith, M. R., Frederickson, R. M., Jaramillo, M. L., Liu, Y. L., Kung, H. F., and Sonenberg, N. (1992) Ras mediates translation initiation factor 4E-induced malignant transformation. *Genes Dev* 6, 1631-1642.

173. Rhoads, R. E. (1991) Protein synthesis, cell growth and oncogenesis. *Curr Opin Cell Biol* 3, 1019-1024.

174. Hentze, M. W. (1997) eIF4G: a multipurpose ribosome adapter? *Science* 275, 500-501.

175. Morley, S. J., Dever, T. E., Etchison, D., and Traugh, J. A. (1991) Phosphorylation of eIF-4F by protein kinase C or multipotential S6 kinase stimulates protein synthesis at initiation. *J Biol Chem* 266, 4669-4672

176. 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.

177. Pause, A., Belsham, G. J., Gingras, A. C., Donze, 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 [see comments]. *Nature* 371, 762-767

178. 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

179. 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.

180. 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.

181. 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.

182. Proud, C. G. (1996) p70 S6 kinase: an enigma with variations. *Trends Biochem Sci* 21, 181-185

183. Thomas, G., Martin-Perez, J., Siegmann, M., and Otto, A. M. (1982) The effect of serum, EGF, PGF2 alpha and insulin on S6 phosphorylation and the initiation of protein and DNA synthesis. *Cell* 30, 235-242

184. Thomas, G., Siegmann, M., Kubler, A. M., Gordon, J., and Jimenez de Asua, L. (1980) Regulation of 40S ribosomal protein S6 phosphorylation in Swiss mouse 3T3 cells. *Cell* 19, 1015-1023

185. Thomas, G. (1986) Translational control of mRNA expression during the early mitogenic response in Swiss mouse 3T3 cells: identification of specific proteins. *J Cell Biol* 103, 2137-2144

186. Jefferies, H. B., and Thomas, G. (1994) Elongation factor-1 alpha mRNA is selectively translated following mitogenic stimulation. *J Biol Chem* 269, 4367-4372

187. 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

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

189. 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

190. 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

191. 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

192. 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

193. Stephens, L., Anderson, K., Stokoe, D., Erdjument-Bromage, H., Painter, G. F., Holmes, A. B., Gaffney, P. R., Reese, C. B., McCormick, F., Tempst, P., Coadwell, J., and Hawkins, P. T. (1998) Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. *Science* 279, 710-714

194. Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reese, C. B., Painter, G. F., Holmes, A. B., McCormick, F., and Hawkins, P. T. (1997) Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science* 277, 567-570

195. 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

196. Wijkander, J., Holst, L. S., Rahn, T., Resjo, S., Castan, I., Manganiello, V., Belfrage, P., and Degerman, E. (1997) Regulation of protein kinase B in rat adipocytes by insulin, vanadate, and peroxovanadate. Membrane translocation in response to peroxovanadate. *J Biol Chem* 272, 21520-21526

197. Murga, C., Laguinge, L., Wetzker, R., Cuadrado, A., and Gutkind, J. S. (1998) Activation of Akt/protein kinase B by G protein-coupled receptors. A role for alpha and beta gamma subunits of heterotrimeric G proteins acting through phosphatidylinositol-3-OH kinasegamma. *J Biol Chem* 273, 19080-19085

198. Sable, C. L., Filippa, N., Filloux, C., Hemmings, B. A., and Van Obberghen, E. (1998) Involvement of the pleckstrin homology domain in the insulin-stimulated activation of protein kinase B. *J Biol Chem* 273, 29600-29606

199. 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

200. Scott, P. H., Brunn, G. J., Kohn, A. D., Roth, R. A., and Lawrence, J. C., Jr. (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

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

202. 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.

203. Thomas, G., and Hall, M. N. (1997) TOR signalling and control of cell growth. *Curr Opin Cell Biol* 9, 782-787.

204. 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

205. Frame, S., and Cohen, P. (2001) GSK3 takes centre stage more than 20 years after its discovery. *Biochem J* 359, 1-16

206. Cohen, P., Alessi, D. R., and Cross, D. A. (1997) PDK1, one of the missing links in insulin signal transduction? *FEBS Lett* 410, 3-10

207. 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

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

209. 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

210. 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

211. 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

212. White, M. F., and Kahn, C. R. (1994) The insulin signaling system. *J Biol Chem* 269, 1-4

213. Guy, G. R., Yusoff, P., Bangarusamy, D., Fong, C. W., and Wong, E. S. (2002) Dockers at the crossroads. *Cell Signal* 14, 11-20

214. Denton, R. M., and Tavare, J. M. (1995) Does mitogen-activated-protein kinase have a role in insulin action? The cases for and against. *Eur J Biochem* 227, 597-611

215. 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.

216. Sinaud, S., Balage, M., Bayle, G., Dardevet, D., Vary, T. C., Kimball, S. R., Jefferson, L. S., and Grizard, J. (1999) Diazoxide-induced insulin deficiency greatly reduced muscle protein synthesis in rats: involvement of eIF4E. *Am J Physiol* 276, E50-61

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

218. Vanhaesebroeck, B., and Alessi, D. R. (2000) The PI3K-PDK1 connection: more than just a road to PKB. *Biochem J* 346 Pt 3, 561-576.

219. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378, 785-789

220. Welsh, G. I., and Proud, C. G. (1992) Regulation of protein synthesis in Swiss 3T3 fibroblasts. Rapid activation of the guanine-nucleotide-exchange factor by insulin and growth factors. *Biochem J* 284, 19-23

221. Kimball, S. R., Horetsky, R. L., and Jefferson, L. S. (1998) Implication of eIF2B rather than eIF4E in the regulation of global protein synthesis by amino acids in L6 myoblasts. *J Biol Chem* 273, 30945-30953

222. Mendez, R., Myers, M. G., Jr., White, M. F., and Rhoads, R. E. (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

223. Azpiazu, I., Saltiel, A. R., DePaoli-Roach, A. A., and Lawrence, J. C. (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

224. 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.

225. 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.

226. 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.

227. 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.

228. Chang, Y. W., and Traugh, J. A. (1997) Phosphorylation of elongation factor 1 and ribosomal protein S6 by multipotential S6 kinase and insulin stimulation of translational elongation. *J Biol Chem* 272, 28252-28257.

229. Myers, M. G., Jr., Grammer, T. C., Wang, L. M., Sun, X. J., Pierce, J. H., Blenis, J., and White, M. F. (1994) Insulin receptor substrate-1 mediates phosphatidylinositol 3'-kinase and p70S6k signaling during insulin, insulin-like growth factor-1, and interleukin-4 stimulation. *J Biol Chem* 269, 28783-28789

230. Smith, J. A., Poteet-Smith, C. E., Malarkey, K., and Sturgill, T. W. (1999) Identification of an extracellular signal-regulated kinase (ERK) docking site in ribosomal S6 kinase, a sequence critical for activation by ERK in vivo. *J Biol Chem* 274, 2893-2898

231. Rhoads, R. E. (1999) Signal transduction pathways that regulate eukaryotic protein synthesis. *J Biol Chem* 274, 30337-30340.

232. Wada, I., Rindress, D., Cameron, P. H., Ou, W. J., Doherty, J. J., 2nd, Louvard, D., Bell, A. W., Dignard, D., Thomas, D. Y., and Bergeron, J. J. (1991) SSR alpha and associated calnexin are major calcium binding proteins of the endoplasmic reticulum membrane. *J Biol Chem* 266, 19599-19610

233. Lavoie, C., Chevet, E., Roy, L., Tonks, N. K., Fazel, A., Posner, B. I., Paiement, J., and Bergeron, J. J. (2000) Tyrosine phosphorylation of p97 regulates transitional endoplasmic reticulum assembly in vitro. *Proc Natl Acad Sci U S A* 97, 13637-13642

234. Chevet, E., Jakob, C. A., Thomas, D. Y., and Bergeron, J. J. (1999) Calnexin family members as modulators of genetic diseases. *Semin Cell Dev Biol* 10, 473-480

235. Cabral, C. M., Choudhury, P., Liu, Y., and Sifers, R. N. (2000) Processing by endoplasmic reticulum mannosidases partitions a secretion-impaired glycoprotein into distinct disposal pathways. *J Biol Chem* 275, 25015-25022

236. Schlossmann, J., Ammendola, A., Ashman, K., Zong, X., Huber, A., Neubauer, G., Wang, G. X., Allescher, H. D., Korth, M., Wilm, M., Hofmann, F., and Ruth, P. (2000) Regulation of intracellular calcium by a signalling complex of IRAG, IP3 receptor and cGMP kinase Ibeta. *Nature* 404, 197-201

237. Roderick, H. L., Lechleiter, J. D., and Camacho, P. (2000) Cytosolic phosphorylation of calnexin controls intracellular Ca(2+) oscillations via an interaction with SERCA2b. *J Cell Biol* 149, 1235-1248

238. Kaufman, R. J., Scheuner, D., Schroder, M., Shen, X., Lee, K., Liu, C. Y., and Arnold, S. M. (2002) The unfolded protein response in nutrient sensing and differentiation. *Nat Rev Mol Cell Biol* 3, 411-421.

239. Mori, K. (2000) Tripartite management of unfolded proteins in the endoplasmic reticulum. *Cell* 101, 451-454

240. Sidrauski, C., Chapman, R., and Walter, P. (1998) The unfolded protein response: an intracellular signalling pathway with many surprising features. *Trends Cell Biol* 8, 245-249

241. Gething, M. J., and Sambrook, J. (1992) Protein folding in the cell. *Nature* 355, 33-45.

242. Kozutsumi, Y., Segal, M., Normington, K., Gething, M. J., and Sambrook, J. (1988) The presence of malfolded proteins in the endoplasmic reticulum signals the induction of glucose-regulated proteins. *Nature* 332, 462-464.

243. Brostrom, C. O., and Brostrom, M. A. (1998) Regulation of translational initiation during cellular responses to stress. *Prog Nucleic Acid Res Mol Biol* 58, 79-125

244. Travers, K. J., Patil, C. K., Wodicka, L., Lockhart, D. J., Weissman, J. S., and Walter, P. (2000) Functional and genomic analyses reveal an essential coordination between the unfolded protein response and ER-associated degradation. *Cell* 101, 249-258

245. Prostko, C. R., Brostrom, M. A., and Brostrom, C. O. (1993) Reversible phosphorylation of eukaryotic initiation factor 2 alpha in response to endoplasmic reticular signaling. *Mol Cell Biochem* 127-128, 255-265

246. Welihinda, A. A., and Kaufman, R. J. (1996) The unfolded protein response pathway in Saccharomyces cerevisiae. Oligomerization and trans-phosphorylation of Ire1p (Ern1p) are required for kinase activation. *J Biol Chem* 271, 18181-18187

247. Shamu, C. E., and Walter, P. (1996) Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus. *Embo J* 15, 3028-3039

248. Bertolotti, A., Zhang, Y., Hendershot, L. M., Harding, H. P., and Ron, D. (2000) Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat Cell Biol* 2, 326-332

249. Kawahara, T., Yanagi, H., Yura, T., and Mori, K. (1997) Endoplasmic reticulum stress-induced mRNA splicing permits synthesis of transcription factor Hac1p/Ern4p that activates the unfolded protein response. *Mol Biol Cell* 8, 1845-1862

250. Cox, J. S., and Walter, P. (1996) A novel mechanism for regulating activity of a transcription factor that controls the unfolded protein response. *Cell* 87, 391-404

251. Sidrauski, C., Cox, J. S., and Walter, P. (1996) tRNA ligase is required for regulated mRNA splicing in the unfolded protein response. *Cell* 87, 405-413

252. Chapman, R. E., and Walter, P. (1997) Translational attenuation mediated by an mRNA intron. *Curr Biol* 7, 850-859.

253. Tirasophon, W., Welihinda, A. A., and Kaufman, R. J. (1998) A stress response pathway from the endoplasmic reticulum to the nucleus requires a novel bifunctional protein kinase/endoribonuclease (Ire1p) in mammalian cells. *Genes Dev* 12, 1812-1824

254. Wang, X. Z., Harding, H. P., Zhang, Y., Jolicoeur, E. M., Kuroda, M., and Ron, D. (1998) Cloning of mammalian Ire1 reveals diversity in the ER stress responses. *Embo J* 17, 5708-5717

255. Niwa, M., Sidrauski, C., Kaufman, R. J., and Walter, P. (1999) A role for presenilin-1 in nuclear accumulation of Ire1 fragments and induction of the mammalian unfolded protein response. *Cell* 99, 691-702

256. Tirasophon, W., Lee, K., Callaghan, B., Welihinda, A., and Kaufman, R. J. (2000) The endoribonuclease activity of mammalian IRE1 autoregulates its mRNA and is required for the unfolded protein response. *Genes Dev* 14, 2725-2736

257. Katayama, T., Imaizumi, K., Sato, N., Miyoshi, K., Kudo, T., Hitomi, J., Morihara, T., Yoneda, T., Gomi, F., Mori, Y., Nakano, Y., Takeda, J., Tsuda, T., Itoyama, Y., Murayama, O., Takashima, A., St George-Hyslop, P., Takeda, M., and Tohyama, M. (1999) Presenilin-1 mutations downregulate the signalling pathway of the unfolded-protein response. *Nat Cell Biol* 1, 479-485.

258. Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H. P., and Ron, D. (2000) Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* 287, 664-666

259. Calfon, M., Zeng, H., Urano, F., Till, J. H., Hubbard, S. R., Harding, H. P., Clark, S. G., and Ron, D. (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* 415, 92-96

260. Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., and Mori, K. (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* 107, 881-891

261. Lee, K., Tirasophon, W., Shen, X., Michalak, M., Prywes, R., Okada, T., Yoshida, H., Mori, K., and Kaufman, R. J. (2002) IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. *Genes Dev* 16, 452-466

262. Yoshida, H., Haze, K., Yanagi, H., Yura, T., and Mori, K. (1998) Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. *J Biol Chem* 273, 33741-33749

263. Shen, J., Chen, X., Hendershot, L., and Prywes, R. (2002) ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. *Dev Cell* 3, 99-111.

264. Chen, X., Shen, J., and Prywes, R. (2002) The luminal domain of ATF6 senses endoplasmic reticulum (ER) stress and causes translocation of ATF6 from the ER to the Golgi. *J Biol Chem* 277, 13045-13052

265. Haze, K., Okada, T., Yoshida, H., Yanagi, H., Yura, T., Negishi, M., and Mori, K. (2001) Identification of the G13 (cAMP-response-element-binding protein-related protein) gene product related to activating transcription factor 6 as a transcriptional activator of the mammalian unfolded protein response. *Biochem J* 355, 19-28

266. Haze, K., Yoshida, H., Yanagi, H., Yura, T., and Mori, K. (1999) Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. *Mol Biol Cell* 10, 3787-3799

267. Yoshida, H., Okada, T., Haze, K., Yanagi, H., Yura, T., Negishi, M., and Mori, K. (2000) ATF6 activated by proteolysis binds in the presence of NF-Y (CBF) directly to the cis-acting element responsible for the mammalian unfolded protein response. *Mol Cell Biol* 20, 6755-6767

268. Ye, J., Rawson, R. B., Komuro, R., Chen, X., Dave, U. P., Prywes, R., Brown, M. S., and Goldstein, J. L. (2000) ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs. *Mol Cell* 6, 1355-1364.

269. Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B. A., and Yuan, J. (2000) Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. *Nature* 403, 98-103

270. Haas, I. G., and Wabl, M. (1983) Immunoglobulin heavy chain binding protein. *Nature* 306, 387-389

271. Hurtley, S. M., and Helenius, A. (1989) Protein oligomerization in the endoplasmic reticulum. *Annu Rev Cell Biol* 5, 277-307

272. Morris, J. A., Dorner, A. J., Edwards, C. A., Hendershot, L. M., and Kaufman, R. J. (1997) Immunoglobulin binding protein (BiP) function is required to protect cells from endoplasmic reticulum stress but is not required for the secretion of selective proteins. *J Biol Chem* 272, 4327-4334

273. Dorner, A. J., Wasley, L. C., and Kaufman, R. J. (1992) Overexpression of GRP78 mitigates stress induction of glucose regulated proteins and blocks secretion of selective proteins in Chinese hamster ovary cells. *Embo J* 11, 1563-1571

274. Kohno, K., Normington, K., Sambrook, J., Gething, M. J., and Mori, K. (1993) The promoter region of the yeast KAR2 (BiP) gene contains a regulatory domain that responds to the presence of unfolded proteins in the endoplasmic reticulum. *Mol Cell Biol* 13, 877-890

275. Malmstrom, B. G. (1982) Enzymology of oxygen. *Annu Rev Biochem* 51, 21-59

276. Fornace, A. J., Jr., Alamo, I., Jr., and Hollander, M. C. (1988) DNA damage-inducible transcripts in mammalian cells. *Proc Natl Acad Sci U S A* 85, 8800-8804

277. Chen, Q., Yu, K., Holbrook, N. J., and Stevens, J. L. (1992) Activation of the growth arrest and DNA damage-inducible gene gadd 153 by nephrotoxic cysteine conjugates and dithiothreitol. *J Biol Chem* 267, 8207-8212

278. Brewer, J. W., Cleveland, J. L., and Hendershot, L. M. (1997) A pathway distinct from the mammalian unfolded protein response regulates expression of endoplasmic reticulum chaperones in non-stressed cells. *Embo J* 16, 7207-7216

279. Bartlett, J. D., Luethy, J. D., Carlson, S. G., Sollott, S. J., and Holbrook, N. J. (1992) Calcium ionophore A23187 induces expression of the growth arrest and DNA damage inducible CCAAT/enhancer-binding protein (C/EBP)-related gene, gadd153. Ca2+ increases transcriptional activity and mRNA stability. *J Biol Chem* 267, 20465-20470

280. Carlberg, M., Dricu, A., Blegen, H., Kass, G. E., Orrenius, S., and Larsson, O. (1996) Short exposures to tunicamycin induce apoptosis in SV40-transformed but not in normal human fibroblasts. *Carcinogenesis* 17, 2589-2596

281. Dricu, A., Carlberg, M., Wang, M., and Larsson, O. (1997) Inhibition of N-linked glycosylation using tunicamycin causes cell death in malignant cells: role of down-regulation of the insulin-like growth factor 1 receptor in induction of apoptosis. *Cancer Res* 57, 543-548

282. Price, B. D., and Calderwood, S. K. (1992) Gadd45 and Gadd153 messenger RNA levels are increased during hypoxia and after exposure of cells to agents which elevate the levels of the glucose-regulated proteins. *Cancer Res* 52, 3814-3817

283. Sunnerhagen, M., Forsen, S., Hoffren, A. M., Drakenberg, T., Teleman, O., and Stenflo, J. (1995) Structure of the Ca(2+)-free Gla domain sheds light on membrane binding of blood coagulation proteins. *Nat Struct Biol* 2, 504-509

284. Zhan, Q., Lord, K. A., Alamo, I., Jr., Hollander, M. C., Carrier, F., Ron, D., Kohn, K. W., Hoffman, B., Liebermann, D. A., and Fornace, A. J., Jr. (1994) The gadd and MyD genes define a novel set of mammalian genes encoding acidic proteins that synergistically suppress cell growth. *Mol Cell Biol* 14, 2361-2371

285. Barone, M. V., Crozat, A., Tabaee, A., Philipson, L., and Ron, D. (1994) CHOP (GADD153) and its oncogenic variant, TLS-CHOP, have opposing effects on the induction of G1/S arrest. *Genes Dev* 8, 453-464

286. Zinszner, H., Kuroda, M., Wang, X., Batchvarova, N., Lightfoot, R. T., Remotti, H., Stevens, J. L., and Ron, D. (1998) CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev* 12, 982-995

287. Fornace, A. J., Jr., Nebert, D. W., Hollander, M. C., Luethy, J. D., Papathanasiou, M., Fargnoli, J., and Holbrook, N. J. (1989) Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents. *Mol Cell Biol* 9, 4196-4203

288. Chou, J., and Roizman, B. (1994) Herpes simplex virus 1 gamma(1)34.5 gene function, which blocks the host response to infection, maps in the homologous domain of the genes expressed during growth arrest and DNA damage. *Proc Natl Acad Sci U S A* 91, 5247-5251

289. He, B., Chou, J., Liebermann, D. A., Hoffman, B., and Roizman, B. (1996) The carboxyl terminus of the murine MyD116 gene substitutes for the corresponding domain of the gamma(1)34.5 gene of herpes simplex virus to preclude the premature shutoff of total protein synthesis in infected human cells. *J Virol* 70, 84-90

290. He, B., Gross, M., and Roizman, B. (1998) The gamma134.5 protein of herpes simplex virus 1 has the structural and functional attributes of a protein phosphatase 1 regulatory subunit and is present in a high molecular weight complex with the enzyme in infected cells. *J Biol Chem* 273, 20737-20743

291. He, B., Gross, M., and Roizman, B. (1997) The gamma(1)34.5 protein of herpes simplex virus 1 complexes with protein phosphatase 1alpha to dephosphorylate the alpha subunit of the eukaryotic translation initiation factor 2 and preclude the shutoff of protein synthesis by double-stranded RNA-activated protein kinase. *Proc Natl Acad Sci U S A* 94, 843-848

292. Prostko, C. R., Brostrom, M. A., Malara, E. M., and Brostrom, C. O. (1992) Phosphorylation of eukaryotic initiation factor (eIF) 2 alpha and inhibition of eIF-2B in GH3 pituitary cells by perturbants of early protein processing that induce GRP78. *J Biol Chem* 267, 16751-16754

293. Doutheil, J., Althausen, S., Gissel, C., and Paschen, W. (1999) Activation of MYD116 (gadd34) expression following transient forebrain ischemia of rat: implications for a role of disturbances of endoplasmic reticulum calcium homeostasis. *Brain Res Mol Brain Res* 63, 225-232

294. Novoa, I., Zeng, H., Harding, H. P., and Ron, D. (2001) Feedback inhibition of the unfolded protein response by GADD34-mediated dephosphorylation of eIF2alpha. *J Cell Biol* 153, 1011-1022

295. Lee, T. G., Tang, N., Thompson, S., Miller, J., and Katze, M. G. (1994) The 58,000-dalton cellular inhibitor of the interferon-induced double-stranded RNA-activated protein kinase (PKR) is a member of the tetratricopeptide repeat family of proteins. *Mol Cell Biol* 14, 2331-2342.

296. Lee, T. G., Tomita, J., Hovanessian, A. G., and Katze, M. G. (1992) Characterization and regulation of the 58,000-dalton cellular inhibitor of the interferon-induced, dsRNA-activated protein kinase. *J Biol Chem* 267, 14238-14243.

297. Lee, T. G., Tomita, J., Hovanessian, A. G., and Katze, M. G. (1990) Purification and partial characterization of a cellular inhibitor of the interferon-induced protein kinase of Mr 68,000 from influenza virus-infected cells. *Proc Natl Acad Sci U S A* 87, 6208-6212.

298. Yan, W., Frank, C. L., Korth, M. J., Sopher, B. L., Novoa, I., Ron, D., and Katze, M. G. (2002) Control of PERK eIF2{alpha} kinase activity by the endoplasmic reticulum stress-induced molecular chaperone P58IPK. *Proc Natl Acad Sci U S A* 22, 22

299. Brostrom, C. O., Prostko, C. R., Kaufman, R. J., and Brostrom, M. A. (1996) Inhibition of translational initiation by activators of the glucose-regulated stress protein and heat shock protein stress response systems. Role of the interferon-inducible double-stranded RNA-activated eukaryotic initiation factor 2alpha kinase. *J Biol Chem* 271, 24995-25002

300. Harding, H. P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000) Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol Cell* 6, 1099-1108

CHAPTER II

Modulation of Protein Translation by Nck-1

Preface

The β subunit of the eukaryotic initiation factor 2 was found interacting with the SH3 domains of Nck-1 in a yeast two-hybrid screen. My objective was to validate and characterize the functional significance of a potential interaction between the SH2/SH3 adapter protein Nck and a subunit of a factor regulating protein translation.

Abbreviations

eIF : eukaryotic initiation factor

PI-3K : phosphatidylinositol 3' kinase

SH : Src homology domain

IRES : internal ribosomal entry site

NRS : normal rabbit serum

TCL : total cell lysate

IP: : immunoprecipitation

WB : immunoblotting

GST : glutathione-S-transferase

HA : hemagglutinin

Abstract

In mammals, Nck represented by two genes, is a 47kDa SH2/SH3 domain-containing protein lacking intrinsic enzymatic function. Here, we reported that the first and the third SH3 domains of Nck-1 interact with the C-terminal region of the β subunit of the eukaryotic initiation factor 2 (eIF2β). Binding of eIF2β was specific to the SH3 domains of Nck-1 and *in vivo*, the interaction Nck/eIF2β was demonstrated by reciprocal coimmunoprecipitations. In addition, Nck was detected in a molecular complex with eIF2β in an enriched ribosomal fraction, whereas no other SH2/SH3 domain-containing adapters were found. Cell fractionation studies demonstrated that the presence of Nck in purified ribosomal fractions was enhanced after insulin stimulation, suggesting that growth factors dynamically regulate translocation of Nck to ribosomes. In HEK293 cells, we observed that transient overexpression of Nck-1 significantly enhanced Cap-dependent and -independent protein translation. This effect of Nck-1 required the integrity of its first and third SH3 domains originally found to interact with eIF2β. Finally, *in vitro*, Nck-1 also increased protein translation, revealing a direct role for Nck-1 in this process. Our study demonstrates that in addition to mediate receptor tyrosine kinase signaling, Nck-1 modulates protein translation potentially through its direct interaction with an intrinsic component of the protein translation machinery.

Introduction

Translation initiation is a complex process in which, initiator tRNA (Met-tRNA$_i$), the 40S and 60S ribosomal subunits are assembled into 80S ribosomes at the initiation codon of mRNA by the coordinated action of the eukaryotic initiation factors (eIFs). Thus far, signaling-dependent events regulating eIFs have involved changes in their intrinsic activity or protein interacting properties, resulting from their phosphorylation/dephosphorylation and/or sequestration into inactive complex. For example, eIF2 is a molecular complex of three subunits (α, β and γ) responsible for one of the earliest steps in the initiation of protein synthesis (1). eIF2 forms a ternary complex with the Met-tRNA and GTP, and in collaboration with other initiation factors binds the 40S ribosomal subunit to give rise to the pre-initiation 43S complex (2). Inhibition of protein synthesis correlates with the phosphorylation of the eIF2α subunit (1) by the heme regulated eIF2α kinase (HRI) (3), the IFN-inducible RNA-dependent protein kinase (PKR) (4), the serum starvation kinase (GCN2) (5) and the endoplasmic reticulum stress kinase (PERK) (6). Phosphorylation of eIF2α on Ser^{51} by these eIF2α kinases inhibits the early steps of translation by blocking on eIF2 the exchange of GDP for GTP, a reaction under the control of the guanine exchange factor, eIF2B (7, 8). Thus, growth factors may enhance initiation of protein translation by preventing activation of eIF2α kinases or by activating specific phosphatases to maintain low levels of eIF2α phosphorylation. In contrast to eIF2α, the β and γ subunits of eIF2 known to interact with critical components of the translational initiation

machinery such as eIF2B, eIF5, mRNA, GTP and Met-tRNA (9-11), have not yet been reported to be regulated by signaling molecules.

To date, with the exception of the p85 adapter subunit of the phosphatidylinositol 3' kinase (PI-3K), no SH2/SH3 domain-containing adapters have been directly implicated in the regulation of the initiation of protein synthesis by growth factors. Here, we demonstrate a direct interaction between the adapter protein Nck-1 and the translation initiation factor subunit eIF2β in an enriched ribosomal fraction. Given these observations and the striking finding that the presence of Nck in ribosomes was increased upon insulin treatment, we investigated the role of Nck-1 in protein translation.

Materials and Methods

Antibodies

Nck antiserum was prepared as described (12). eIF2β antisera were obtained after rabbit immunization with a glutathione-S-transferase (GST) chimera of the C-terminal region of mouse eIF2β (residues 133-333). L4 antibody was provided by Dr C. Nicchitta (Duke University Medical Center, Durham, NC). p85, CrkII, Grb2 and hemagglutinin (HA) antibodies were purchased from Santa Cruz Biotechnology.

Cell Culture

Transformed rat hepatocytes overexpressing the human insulin receptor (HTC-IR) cells were grown in DMEM (Invitrogen) containing 10% Fetal Bovine Serum (FBS) (Invitrogen) and Geneticin (G418) at 40 μg/ml (Invitrogen). Human embryonic kidney 293 (HEK293) cells were grown in DMEM containing 10% FBS.

Yeast Two-Hybrid Assays

Yeast two-hybrid screen was performed as described (13). pACII vector encoding the transcriptional activation domain of Gal4 fused to mouse T cell cDNA library fragments were introduced into *Saccharomyces cerevisiae* strains already transfected with the pASI vector encoding a fusion between the DNA binding domain of Gal4 and the three SH3 domains of Nck-1 (residues 1-251).

Immunoprecipitation

Mouse tissues were homogenized in 5 mM Tris-HCl pH 7.4/ 1 mM $MgCl_2$/ 0.25 M sucrose/ 2 mM $NaVO_4$/ 1 mM PMSF/ 1 mM benzamidine/ 1 mM NaF and centrifuged at 200,000 x g for 30 min. The resulting supernatants were submitted to Nck or eIF2β immunoprecipitation. HTC-IR lysates were prepared as described (12). Clarified lysates were submitted to either Nck or eIF2β immunoprecipitation and analyzed by immunoblotting.

Nck-1 Constructs and Transfection

Human Nck-1 mutants were produced by overlapping PCR with specific primers containing appropriate mutated sites (14-16), subcloned into pcDNA 3.1Myc/His plasmid (Invitrogen) and sequenced. The HA-tagged Nck-1 constructs subcloned into pRK5 were provided by Dr W. Li (Norris Cancer Center, University of Southern California, Los Angeles). Transient transfections of HEK293 cells were performed by using calcium phosphate precipitation.

GST Fusion Proteins

Wild type Nck-1 and eIF2β cDNAs were subcloned into pGEX-2TK and GST fusion proteins were expressed, purified as recommended by the manufacturer (Amersham-Phamarcia) and used for binding assay experiments. GST and GST-Nck-1 were eluted from the glutathione beads before their use in overlay assays or in the *in vitro* translation system.

Overlay Assay

Recombinant GST and GST-Nck-1 proteins (5 μg) were spotted onto nitrocellulose and dried. Membranes were blocked in Tris-buffered saline containing 0.05% Tween-20 (TBST) and 10% nonfat dry milk and probed overnight at 4°C with ^{32}P-labeled GST-eIF2β. Membranes were washed in TBST and exposed for autoradiography.

Binding Assay

HEK293 cells transiently transfected with empty vector or plasmid encoding either HA-tagged Nck-1 wild type or mutated in its individual SH3 domains were lysed (10mM Hepes pH7.4/ 1% (vol/vol) Triton X-100/ 10 μg/ml aprotinin/ 10 μg/ml leupeptin and 1 mM PMSF). After preclearing with glutathione immobilized on beads, lysates were mixed with 25 μg of GST or GST-eIF2β recombinant protein for 2 h at 4°C. Samples were analyzed by immunoblotting with Nck (Transduction Laboratories) or HA antibodies.

Sucrose Cushion Sedimentation

Confluent cells were prepared for sucrose cushion sedimentation as described (17). Briefly, HTC-IR and HEK293 cells were lysed in 20mM Tris-HCl pH 8.0, 150mM NaCl, 1mM NaF, 1.5% CHAPS, 0.5mM EDTA, 1mM $NaVO_4$, 1mM β-glycerophosphate, 1mM PMSF, 1mM Iodoacetamide, 10μg/ml Leupetin and 10μg/ml Aprotinin. Cell lysates were passed through $25^{1/2}$ G needles, sonicated and centrifuged at 1500g for 15min. Clarified lysates were centrifuged at 110,000

x g in TLA 100.2 rotor at 4°C for 30min. The resulting supernatants were layered onto a 1.5M Sucrose cushion containing 0.1% CHAPS to be further centrifuged at 400,000g in TLA 100.2 at 4°C for 3 hrs. Supernatants were TCA precipitated and resuspended in Laemmli sample buffer (18) or subjected to immunoprecipitation with either anti-Nck or anti-eIF2β antibodies. Pellets were resuspended in Laemmli sample buffer or solubilized in 30mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton-X-100 containing protease inhibitors (Leupeptin, Aprotinin, PMSF) and heated at 65 °C for 10 min to facilitate protein resuspension before immunoprecipitation. Samples were analyzed by immunoblotting for Nck, eIF2β, p85, Grb2, CrkII and L4.

Subcellular Fractionation

Insulin stimulated and nonstimulated HEK293 cell lysates were prepared and layered onto a 15-35% linear sucrose gradient as described (19) with minor modifications. Briefly, upon treatments, cells were washed with cold Phosphate-Buffered Saline (PBS) and buffer A (110 mM Potassium Acetate, 2 mM Magnesium Acetate, 2 mM Dithiothreitol (DTT), 10 mM HEPES [N-2-Hydroxyeth-ylpiperazine-N9-2-Ethanesulfonic Acid] [pH 7.3]) and recovered in buffer B (10mM Potassium Acetate, 2mM Magnesium Acetate, 2mM DTT, 5mM HEPES [pH 7.3], 10μg/ml of Leupeptin, 10μg/ml Aprotinin). Cell extract were incubated on ice for 10 min, and disrupted by passage through $25^{½}$ G needles. The KCl concentration was adjusted to 100mM at this point. Centrifugation at 1500*g* for 15 min resulted in a nuclear pellet and a supernatant fraction designated as the

cytoplasmic content, which was then layered on a 15-35% sucrose gradient and centrifuged at 4°C in a Beckman SW40 Ti rotor at 40,000*g* for 120 min. Fractions were bottom collected and analyzed by immunoblotting.

Luciferase Assay

The bicistronic reporter plasmid pcDNA3-RLUC-POLIRES-FLUC was kindly provided by Dr. N. Sonenberg (McGill University, Montreal) (20). HEK293 cells were transiently transfected with 0.5 µg of the reporter vector and either 0.5 µg of plasmids encoding various Myc-Nck-1 molecules (Fig.1A). *Renilla reniformis* luciferase (RLUC) and firefly luciferase (FLUC) activities were measured using a dual-luciferase reporter assay system (Promega) in a luminometer (LUMAT), 36 hrs after transfection.

In vitro Transcription and Translation Assay

Linearized pcDNA3-RLUC-POLIRES-FLUC vector was *in vitro* transcribed and translated in the TNT-coupled wheat germ Extract System (Promega) supplemented with [^{35}S] methionine and increasing amounts of recombinant GST-Nck-1. Samples were prepared for [^{35}S] methionine incorporation assay according to the manufacturer's instructions (Promega) and quantified by scintillation counting.

Quantitative RNA Analysis

Trizol (Invitrogen) prepared RNAs (5 μg) from HEK293 cells transiently cotransfected with the luciferase reporter vector and other various Myc-Nck-1 plasmids (Fig.1A) were converted into single-stranded cDNAs (SuperScript II Kit, Invitrogen) followed by PCR reactions with the primers FLUC: 5'-TACAATTTGGACTTTCCGCC-3'and 5'-TTCTTCGCCAAAAGCACTCT-3', RLUC: 5'-AACGCGGCCTCTTCTTATTT-3', and 5'-TATCAGGCCATTCATCCCAT-3' and classic 18S internal standard primers (QuantumRNA, Ambion, Austin, TX). PCR reactions were supplemented with 10 μCi of 3000 Ci/nM of [α-^{32}P] dATP. Size markers were prepared by 5' end-labeling of 1kb ladder marker (Invitrogen). Samples were submitted to electrophoresis on agarose, transferred onto nylon membranes, and analyzed by autoradiography; reciprocal bands quantified by densitometry.

Results

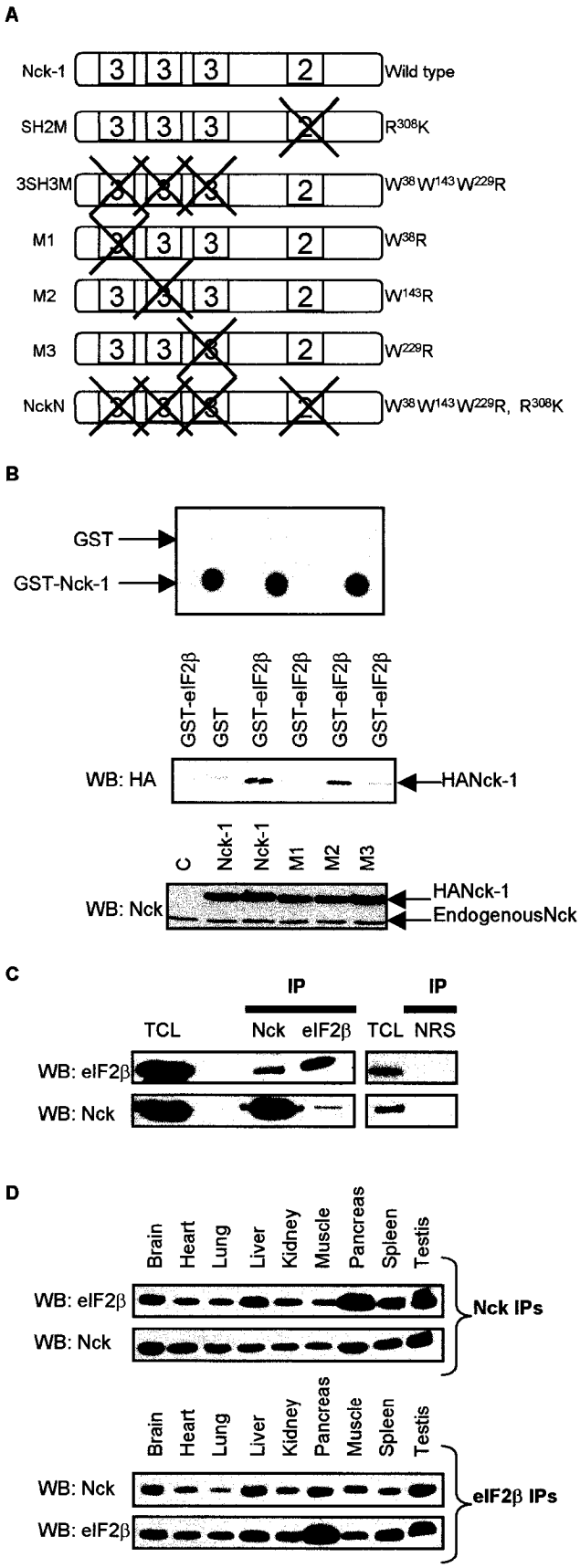
Proteins Interacting with the SH3 Domains of Nck-1. Two clones encoding a fusion protein containing the C-terminal region of the *β* subunit of the eukaryotic initiation factor 2 (eIF2β) were identified interacting with the SH3 domains of Nck-1 in the yeast two-hybrid system. Further characterization of this interaction in the same system revealed that the first and the third SH3 domains of Nck-1 were positive, while neither the second SH3 domain of Nck-1 nor the SH3 domains of Abl, Grb2, CrkII or PLCγ-1 interacted with eIF2β (Table 1). By overlay assays, we observed a direct interaction of GST-eIF2β with GST-Nck-1 (Fig.1B. *Top*). Furthermore, *in vitro* binding assays (Fig.1B *Middle* and *Bottom*) also showed that Nck-1 interacts with eIF2β and that this interaction required the first and the third SH3 domains of Nck-1.

***In vivo* Nck/eIF2β Interaction.** Reciprocal coimmunoprecipitations with lysates from cultured cells (Fig.1C) and mouse tissue homogenates (Fig.1D) were performed. As previously reported by others for Nck (21, 22) and expected for eIF2β, the two proteins are expressed in all tissues with high levels of expression in brain, pancreas, spleen and testis (Fig.1D). Furthermore, Nck and eIF2β coimmunoprecipitated in HTC-IR (Fig.1C), HEK293 (data not shown) cell lysates and tissue homogenates (Fig.1D), demonstrating *in vivo* their interaction.

Table.1. Yeast Two-Hybrid Assay. Individual Nck SH3 domains and SH3 domains of Grb2, CrkII, p85 of PI-3K, Abl and PLCγ-1 were used in yeast two-hybrid assays to characterize their interaction with eIF2β. The 3SH3 construct is the region of Nck comprising the three Src homology 3 domains (residues 1-251). The SH3-1 (residues 1-56), SH3-2 (residues 113-160) and SH3-3 (residues 197-251) constructs denote the first or second or third SH3 domain of Nck, respectively. SH3-C and SH3-N denote the C-terminal and N-terminal SH3 domain of Grb2, respectively.

Molecules	Interaction with eIF2β
Nck 3SH3	+
Nck SH3-1	+
Nck SH3-2	-
Nck SH3-3	+
Abl	-
Grb2 SH3-N	-
Grb2 SH3-C	-
CrkII SH3-C	-
PLCγ1 SH3	-

Fig.1. Nck and eIF2β Interaction. (A) Schematic representation of Nck-1 molecules. X represents functional mutation of the SH domain. (B) **Overlay assay** (*Top*). Autoradiography of GST or GST-Nck-1 (5 μg) triplicates immobilized on nitrocellulose and overlayed with ^{32}P-labeled GST-eIF2β. **Binding assay** (*Middle* and *Bottom*). *In vitro* binding by GST or GST-eIF2β (25 μg) of HA-Nck-1 molecules transiently expressed in HEK293 cells (*Middle*). Expression of endogenous and HA-tagged Nck-1 (*Bottom*). (C) HTC-IR cell lysates subjected to Nck, eIF2β or NRS immunoprecipitation. (D) Tissue homogenates from adult male BalbC mice. Four milligrams of proteins used for coimmunoprecipitations and 1 mg for protein expression. Results are typical of two (tissues) and three (cultured cells) experiments.


A
Nck-1
Wild type
SH2M
R308K
3SH3M
W38W143W229R
M1
W38R
M2
W143R
M3
W229R
NckN
W38W143W229R, R308K
B
GST
GST-Nck-1
GST-eIF2β
GST
GST-eIF2β
GST-eIF2β
GST-eIF2β
GST-eIF2β
WB: HA
HANck-1
C
Nck-1
Nck-1
M1
M2
M3
WB: Nck
HANck-1
EndogenousNck
C
IP
IP
TCL
Nck
eIF2β
TCL
NRS
WB: eIF2β
WB: Nck
D
Brain
Heart
Lung
Liver
Kidney
Muscle
Pancreas
Spleen
Testis
WB: eIF2β
WB: Nck
Nck IPs
Brain
Heart
Lung
Liver
Kidney
Pancreas
Muscle
Spleen
Testis
WB: Nck
WB: eIF2β
eIF2β IPs

Nck and eIF2β Colocalize in a Ribosome-Enriched Fraction. By using the sucrose cushion sedimentation, a ribosome-enriched pellet (P) and supernatant contained soluble cytosolic components (S) were generated from HEK293 and HTC-IR cells. In both cell lines eIF2β as expected, and Nck are detected in the enriched ribosomal fraction (Fig.2A). Other SH2/SH3 domain-containing adapter molecules such as Grb2, CrkII (Fig.2A, *Lower*) or the p85 subunit of PI-3K (data not shown) were not detected in the ribosomal enriched fraction. As control, immunoblot for the ribosomal protein L4 showed that the pellets were enriched in ribosomes (Fig.2A).

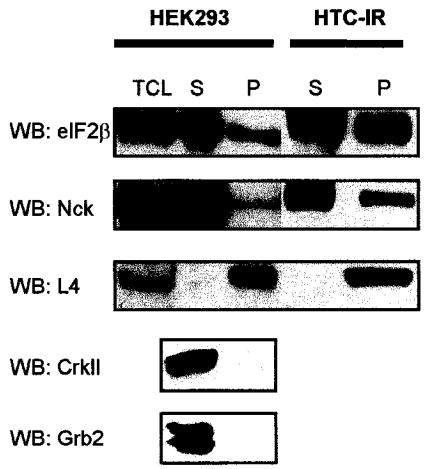
Nck and eIF2β Coimmunoprecipitate from the Ribosome-Enriched Fraction. To determine whether Nck and eIF2β interact in the ribosomal-enriched fraction, endogenous Nck and eIF2β were coimmunoprecipited by using supernatant and pellet fractions, after sucrose cushion sedimentation. As shown in Fig.2B, Nck was detected in eIF2β immunoprecipitates in pellet and supernatant fractions, and reciprocal results were also obtained for eIF2β in Nck immunoprecipitates. Taken together, these results demonstrate that Nck and eIF2β are part of the same molecular complex in this compartment.

Nck Translocation to Ribosomes is Independent of The Functional Integrity of Its SH Domains. To determine whether the SH domains of Nck were involved in mediating its ribosomal localization, enriched ribosomal fractions were prepared from HEK293 cells transiently transfected with various Myc-Nck-1

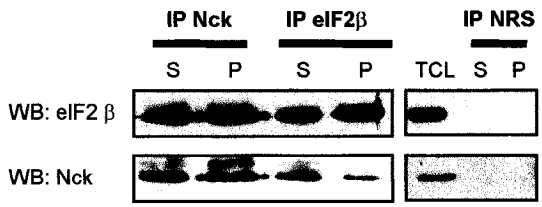
constructs (Fig.1A). All Nck-1 mutants were detected in the ribosomal-enriched fraction (Fig.2D), demonstrating that the localization of Nck into ribosomes is modulated by a mechanism independent of its Src homology domains.

Fig.2. Nck is Detected and Interacts with eIF2β in an Enriched Ribosomal Fraction and Nck Ribosomal Localization is Independent of Its Functional SH Domains. Sucrose cushion sedimentation. (A) Immunoblot analysis of an aliquot of the supernatant (S) and the entire pellet (P). (B) Sucrose cushion supernatants (S) and resuspended pellets (P) were immunoprecipitated in duplicate by using Nck, eIF2β antibodies, or NRS. One tenth of the immunoprecipitated supernatant and the entire immunoprecipitated pellet were used for immunoblotting. Results are representative of three independent experiments. (C and D) Immunoblot analysis of one tenth of the supernatants (C) and the total pellets (D) from HEK293 cells transiently transfected with various Myc-Nck-1 constructs. Results shown are typical of two independent experiments.

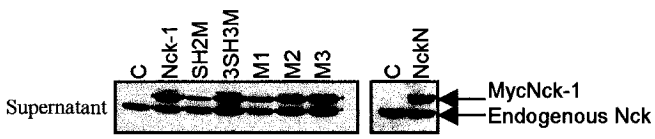
A


HEK293
HTC-IR
TCL S P S P
WB: eIF2β
WB: Nck
WB: L4
WB: CrkII
WB: Grb2

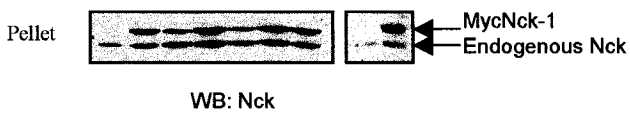
B


IP Nck
IP eIF2β
IP NRS
S P S P TCL S P
WB: eIF2 β
WB: Nck

C


C
Nck-1
SH2M
3SH3M
M1
M2
M3
C
NckN
Supernatant
MycNck-1
Endogenous Nck

D

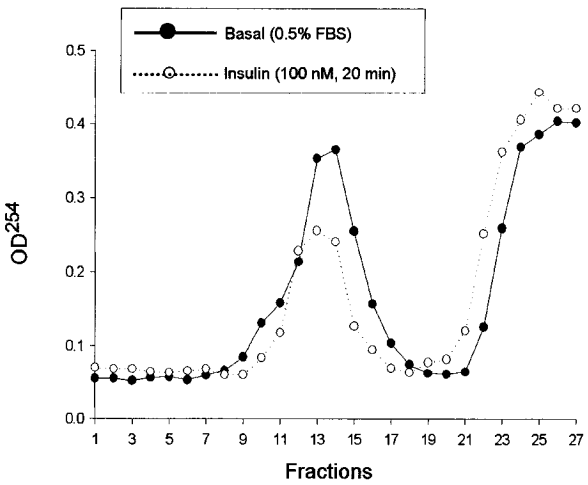

Pellet
MycNck-1
Endogenous Nck

WB: Nck

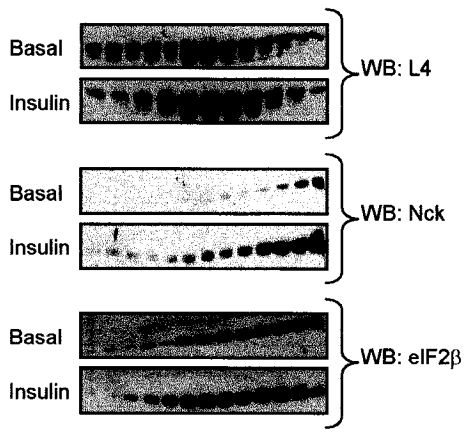
The presence of Nck in Purified Ribosomal Fractions is Enhanced Following Insulin Stimulation. To further characterize whether Nck localization to ribosomes is modulated by growth factor stimulation, sedimentation on linear sucrose gradients of lysates from serum-starved HEK293 cells treated or not with insulin were performed. The assignment of ribosomal enriched components were in fractions 8 to 18 as revealed by immunoblotting for the ribosomal protein L4 (Fig.3B, *Top*). These fractions were then immunoblotted for the presence of Nck (Fig.3B, *Middle*), eIF2β (Fig.3B, *Bottom*) and p85 of PI-3K (data not shown). As reported by others (19), the distribution of RNA content suggested that fractions 1-8 contain the polyribosomes while fractions 13-17 represent the ribosomal subunits (Fig.3A). Nck association with ribosomal subunits was importantly enhanced by insulin stimulation as compared with basal state (Fig.3B, *Middle*). eIF2β was detected in both conditions, but with a slight increase upon insulin treatment. p85 of PI-3K was not detected in the ribosomal fractions under both conditions (data not shown). Finally, the increase in the amount of Nck associated with ribosomal subunits upon insulin treatment did not result from a nonspecific effect of insulin on total Nck content (Fig.3C). Taken together, these results demonstrate that Nck is dynamically translocated into ribosomal compartments upon insulin stimulation.

Fig.3. Translocation of Nck to Ribosomal Fractions is Enhanced upon Insulin Stimulation. Equal amounts of proteins from insulin-stimulated and non-stimulated HEK293 cells were separated on 15-35% linear sucrose gradients. (A) **Ribosomal profile.** Optical density at 254nm representing the RNA content of each fractions. (B) **Nck and eIF2β detected in ribosomal fractions.** Immunoblot analysis of each fraction. Membranes were cut and the lower part probed for the ribosomal protein L4 whereas the upper part was probed first for Nck, then stripped and reprobed for eIF2β. (C) **Total Nck levels upon Insulin stimulation.** Nck immunoblot analysis. Results are representative of three independent experiments.

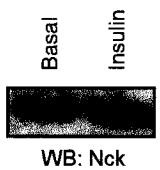
A


Basal (0.5% FBS)
Insulin (100 nM, 20 min)
OD254
0.0
0.1
0.2
0.3
0.4
0.5
1 3 5 7 9 11 13 15 17 19 21 23 25 27
Fractions

B


Basal
Insulin
WB: L4
Basal
Insulin
WB: Nck
Basal
Insulin
WB: eIF2β

C


Basal
Insulin
WB: Nck

Nck-1 Overexpression Increases Protein Translation in HEK293 Cells. We studied the effects of Nck-1 on protein translation. HEK293 cells were cotransfected with a reporter plasmid (Fig.4A) (20) and increasing amounts of Myc-tagged Nck-1. DNA transfected was kept constant at 1 μg by the addition of the empty vector. In this system, translation of RLUC is Cap-dependent whereas translation of the FLUC cistron, directed by the poliovirus internal ribosomal entry site (IRES) is Cap-independent (23). Expression of Nck-1 correlated with the amount of Nck-1 cDNA transfected (Fig.4B). Overexpression of Nck-1 enhanced the activity of both luciferases proportionally to its expression levels and reached a maximum of 1.5-fold increase at 0.5μg of cDNA transfected (Fig.4C). Higher concentrations of Nck-1 cDNA (1 and 2μg) were detrimental to the cells (data not shown).

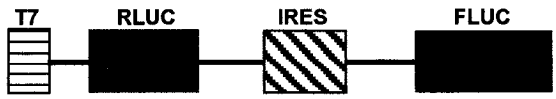
Nck-1 Effect on Translation Depends on the Integrity of Its First and Third SH3 Domains. We further characterized the Nck-1 effect by determining the domain(s) responsible for the enhancement of translation. Myc-tagged Nck-1 constructs cotransfected independently with the bicistronic luciferase reporter vector into HEK293 cells showed comparable expression levels (Fig.5A). The stimulatory effect of Nck-1 on translation was completely abolished by functional mutation of its first, third SH3 domain or all SH3 domains, whereas the Nck-1 mutated in its second SH3 domain resulted in a slight increase as compared with wild type Nck-1 (Fig.5B). Importantly, in the tranfected cells above, we found similar amounts of both luciferase mRNAs as demonstrated by quantitative PCR

and densitometric analysis (Fig.5C). These data confirm that the effect of Nck-1 is on translation only and depends on the functional integrity of its first and third SH3 domains.

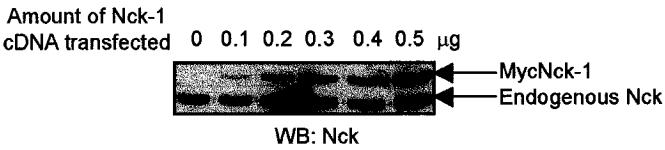
Nck-1 enhances protein translation *in vitro*. We assessed the *in vitro* translation of the luciferase mRNAs in presence of increasing amounts of recombinant GST-Nck-1. As shown in Fig.5D, GST-Nck-1 at 0.9 μg and 1.2 μg significantly enhanced overall translation of luciferase mRNAs by 1.5-2.0 fold. Results from *in vitro* translation strongly support a direct role for Nck-1 in the regulation of protein translation.

Fig.4. *In vivo*, Nck-1 Enhances both Cap dependent and –independent Protein Translation. (A) Schematic representation of the pcDNA3-RLUC-POLIRES-FLUC reporter vector. (B) Immunoblot analysis for the presence of endogenous Nck and Myc-Nck-1 in HEK293 cells. (C) Luciferase activity of the FLUC cistron (*Left*) and the RLUC cistron (*Right*) measured 36 h postransfection from HEK293 cells cotransfected with reporter vector and increasing amounts of MycNck-1 constructs. Experiments were carried out five times in triplicate, and the results represent the mean value ± S.E.M. *, At least $P<0.00,1$ as demonstrated by Student *t* test compared with control.

A


T7
RLUC
IRES
FLUC

B


Amount of Nck-1
cDNA transfected 0 0.1 0.2 0.3 0.4 0.5 µg
MycNck-1
Endogenous Nck
WB: Nck

C

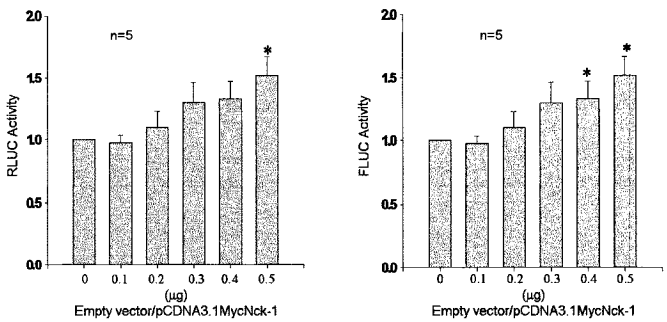
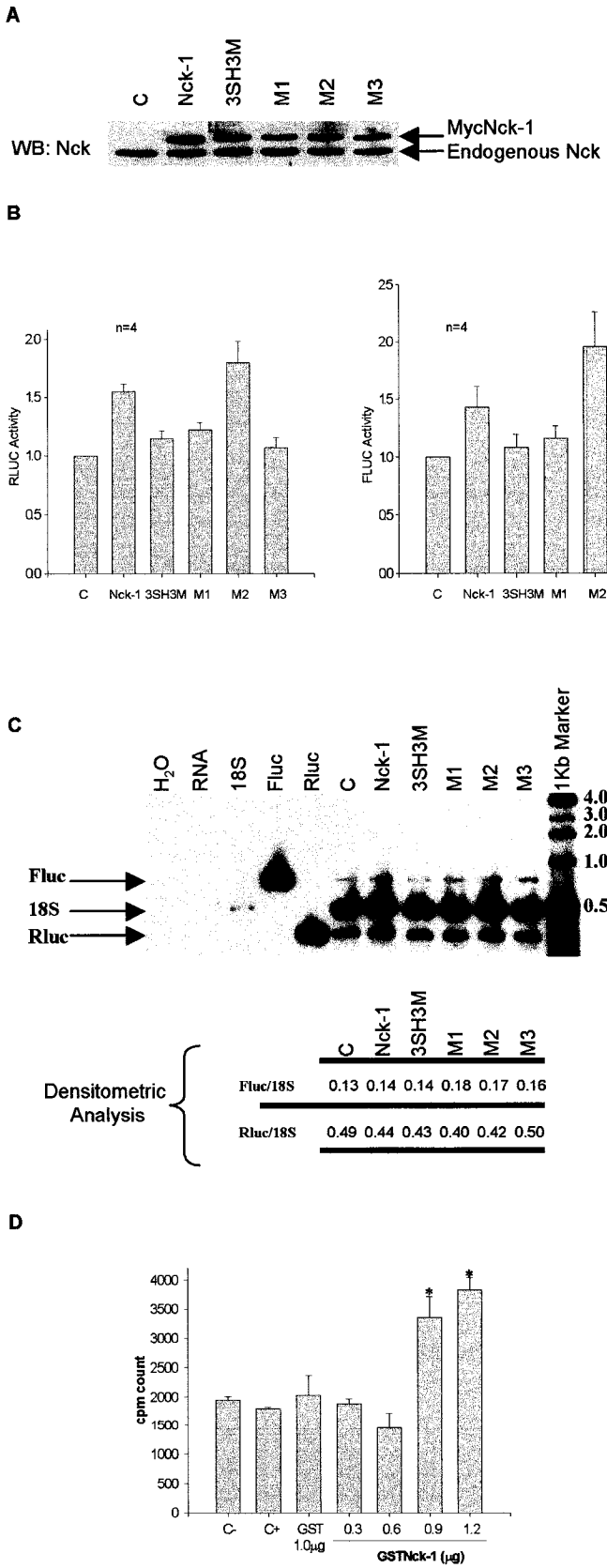

n=5
RLUC Activity
2.0
1.5
1.0
0.5
0.0
0 0.1 0.2 0.3 0.4 0.5
(µg)
Empty vector/pCDNA3.1MycNck-1
n=5
FLUC Activity
2.0
1.5
1.0
0.5
0.0
0 0.1 0.2 0.3 0.4 0.5
(µg)
Empty vector/pCDNA3.1MycNck-1

Fig.5. The Effect of Nck-1 on Protein Translation is Dependent on the Integrity of Its First and Third SH3 Domain. HEK293 cells were cotransfected with the bicistronic luciferase reporter vector (0.5 μg) and either empty vector or the indicated Myc-Nck-1 constructs (0.5 μg). (A) Immunoblot analysis for the expression of endogenous Nck and Myc-Nck-1 constructs. (B) Activity of the RLUC cistron (*Left*) and FLUC cistron (*Right*). Each experiment was performed four times in triplicate, and the results represent the mean ± S.E.M. (C) Quantitative reverse transcription PCR performed on total RNA prepared from above transfected HEK293. Water (H_2O) or control RNA (RNA) were used as controls. Amplified products were analyzed by densitometry for the calculation of the ratios of Fluc/18S and Rluc/18S. Results shown are representative of three experiments. (D) *In vitro* translation of luciferase mRNAs in wheat germ extract supplemented with increasing amounts of GST-Nck-1 and incorporation of [^{35}S] methionine was measured. C- and C+ denote samples without or with bicistronic luciferase vector, respectively. Results are representative of two independent experiments performed in triplicate. *, At least $P< 0.01$ as determined by Student *t* test.


A
C
Nck-1
3SH3M
M1
M2
M3
WB: Nck
MycNck-1
Endogenous Nck
B
n=4
RLUC Activity
FLUC Activity
C
Nck-1
3SH3M
M1
M2
M3
C
RNA
18S
Fluc
Rluc
1Kb Marker
4.0
3.0
2.0
1.0
0.5Kb
Fluc
18S
Rluc
Densitometric Analysis
Fluc/18S 0.13 0.14 0.14 0.18 0.17 0.16
Rluc/18S 0.49 0.44 0.43 0.40 0.42 0.50
D
cpm count
C-
C+
GST 1.0µg
0.3
0.6
0.9
1.2
GSTNck-1 (µg)

Discussion

This study reports an SH3 mediated interaction between the adapter Nck-1 and eIF2β, a component of the eIF2 complex responsible for one of the earliest steps in the initiation of protein synthesis. Here, we demonstrated that Nck and eIF2β not only interact but also colocalize in ribosomal subcellular compartments. Furthermore, we also provided evidence that in these compartments, their levels are regulated by insulin, a hormone known to stimulate protein synthesis. We observed that the ribosomal localization of Nck is independent of its Src homology domains, suggesting that a mechanism, at least independent of its interaction with eIF2β, is mediating its translocation to ribosomes. Given that Nck is reported to be phosphorylated on several residues following growth factor stimulation (24, 25), we hypothesize that such postranslational modifications may govern its translocation to ribosomal compartments.

Our study also shows that *in vivo*, Nck-1 enhances both Cap-dependent and – independent protein translation. In mammals most mRNAs are thought to be translated through a Cap-dependent mechanism involving ribosomal scanning. (reviewed in 26). However, a small population of mRNAs, coding for cell survival factors, cell cycle molecules, oncogenes and viral proteins are translated by a Cap-independent mechanism mediated by direct ribosome binding to IRES elements located in the 5' untranslated region (5'UTR) (reviewed in 27). Even though this mechanism is thought not to require any translation initiation factors, assembly of the 48S complexes on these IRES elements in an *in vitro*

reconstitution assay using purified eIF2/GTP/Met-tRNA$_i$ complex is sufficient for the 40S to lock onto the initiation codon (28). Therefore Nck-1 effect on both types of translation may suggest that Nck-1, by binding eIF2β, acts at the level of the eIF2/GTP/Met-tRNA$_i$ ternary complex upstream of both Cap-dependent and independent initiation of translation. This hypothesis is supported not only by the fact that the SH3 domains of Nck-1 important for its effect on protein translation are also responsible for its interaction with eIF2β, but also by the direct effect of Nck-1 on *in vitro* protein translation.

The involvement of Nck in insulin signaling has been suggested by the fact that Nck-1 and Nck-2 have been shown to bind substrates of the insulin receptor (29-32). Therefore, the insulin receptor could regulate the activity of effector molecules associated with the SH3 domains of Nck. Within this perspective, Sam68, an RNA binding protein (33), has been reported to interact with Nck (34) and to be a substrate of the insulin receptor (35). Moreover, Sam68 has been proposed to be a regulator of RNA metabolism and protein expression by modifying the mRNA stability and/or mRNA translation (36, 37). Therefore, Sam68 may contribute to the effect of Nck-1 on protein translation by directly targeting specific mRNA to ribosomes through the molecular complex mRNA-Sam68-Nck-1-eIF2β.

Nck-1 interacts with several protein kinases (24, 25). Among them, we have identified the isoform γ2 of the Casein Kinase I (CKI γ2) (12) and recently, several CKI substrates identified by an *in vitro* expression cloning screening, include RNA helicase, nucleolar protein hNOP56, hnRNP A1 and the ribosomal proteins L4, L8 and L13 (38), suggesting a possible involvement of CKI in RNA metabolism and protein translation.

In the past, the regulation of protein translation at the level of initiation by growth factors has been well studied (reviewed in 39). Our contribution clearly establish that Nck, an adapter protein known to mediate receptor tyrosine kinase signaling at the membrane level, also acts more downstream in the signaling pathways after its translocation to ribosomal compartments. Nck may constitute a target for tight regulation of protein translation at the initiation level through its interaction with eIF2β. This opens up a field of investigation, which will contribute to a better understanding of how growth factors regulate protein translation.

Acknowledgment

We thank the members of Dr. Andre Veillette's laboratories for their expert technical advice. We also thank Drs. Nahum Sonenberg and Francis Poulin for the kind gift of the bicistronic vector and their technical support. We are also grateful to Dr C. Nicchitta for the L4 antibody, Dr. W. Li for the HA-tagged Nck constructs, and to Ali Fazel, Pamela Cameron, Gerald Baquiran and Nathalie Bedard for their valuable help. This work was supported by a grant from the Canadian Diabetes Association in memory of Aurele Labelle.

References

1. Hershey, J. W. (1991) *Annu Rev Biochem* **60,** 717-55.

2. Pain, V. M. (1996) *Eur J Biochem* **236,** 747-71.

3. Chen, J. J. & London, I. M. (1995) *Trends Biochem Sci* **20,** 105-8.

4. Proud, C. G. (1995) *Trends Biochem Sci* **20,** 241-6.

5. Sood, R., Porter, A. C., Olsen, D. A., Cavener, D. R. & Wek, R. C. (2000) *Genetics* **154,** 787-801.

6. Harding, H. P., Zhang, Y. & Ron, D. (1999) *Nature* **397,** 271-4.

7. Rhoads, R. E. (1993) *J Biol Chem* **268,** 3017-20.

8. Cigan, A. M., Bushman, J. L., Boal, T. R. & Hinnebusch, A. G. (1993) *Proc Natl Acad Sci U S A* **90,** 5350-4.

9. Asano, K., Krishnamoorthy, T., Phan, L., Pavitt, G. D. & Hinnebusch, A. G. (1999) *Embo J* **18,** 1673-88.

10. Laurino, J. P., Thompson, G. M., Pacheco, E. & Castilho, B. A. (1999) *Mol Cell Biol* **19,** 173-81.

11. Gaspar, N. J., Kinzy, T. G., Scherer, B. J., Humbelin, M., Hershey, J. W. & Merrick, W. C. (1994) *J Biol Chem* **269,** 3415-22.

12. Lussier, G. & Larose, L. (1997) *J Biol Chem* **272,** 2688-94.

13. Anafi, M., Kiefer, F., Gish, G. D., Mbamalu, G., Iscove, N. N. & Pawson, T. (1997) *J Biol Chem* **272,** 27804-11.

14. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. & Pease, L. R. (1989) *Gene* **77,** 51-9.

15. Tanaka, M., Gupta, R. & Mayer, B. J. (1995) *Mol Cell Biol* **15,** 6829-37.

16. Mayer, B. J., Jackson, P. K., Van Etten, R. A. & Baltimore, D. (1992) *Mol Cell Biol* **12,** 609-18.

17. Chevet, E., Jakob, C. A., Thomas, D. Y. & Bergeron, J. J. (1999) *Semin Cell Dev Biol* **10,** 473-80.

18. Laemmli, U. K. (1970) *Nature* **227,** 680-5.

19. Siomi, M. C., Zhang, Y., Siomi, H. & Dreyfuss, G. (1996) *Mol Cell Biol* **16,** 3825-32.

20. Poulin, F., Gingras, A. C., Olsen, H., Chevalier, S. & Sonenberg, N. (1998) *J Biol Chem* **273,** 14002-7.

21. Li, W., Hu, P., Skolnik, E. Y., Ullrich, A. & Schlessinger, J. (1992) *Mol Cell Biol* **12,** 5824-33.

22. Park, D. & Rhee, S. G. (1992) *Mol Cell Biol* **12,** 5816-23.

23. Pelletier, J. & Sonenberg, N. (1988) *Nature* **334,** 320-5.

24. Buday, L. (1999) *Biochim Biophys Acta* **1422,** 187-204.

25. McCarty, J. H. (1998) *Bioessays* **20,** 913-21.

26. Pestova, T. V., Kolupaeva, V. G., Lomakin, I. B., Pilipenko, E. V., Shatsky, I. N., Agol, V. I. & Hellen, C. U. (2001) *Proc Natl Acad Sci U S A* **98,** 7029-36.

27. Kozak, M. (2001) *Mol Cell Biol* **21,** 1899-907.

28. Pestova, T. V. & Hellen, C. U. (1999) *Virology* **258,** 249-56.

29. Lee, C. H., Li, W., Nishimura, R., Zhou, M., Batzer, A. G., Myers, M. G., Jr., White, M. F., Schlessinger, J. & Skolnik, E. Y. (1993) *Proc Natl Acad Sci U S A* **90,** 11713-7.

30. Xu, P., Jacobs, A. R. & Taylor, S. I. (1999) *J Biol Chem* **274,** 15262-70.

31. Tu, Y., Liang, L., Frank, S. J. & Wu, C. (2001) *Biochem J* **354,** 315-22.

32. Tang, J., Feng, G. S. & Li, W. (1997) *Oncogene* **15,** 1823-32.

33. Huang, S. (2000) *J Struct Biol* **129,** 233-40.

34. Lawe, D. C., Hahn, C. & Wong, A. J. (1997) *Oncogene* **14,** 223-31.

35. Sanchez-Margalet, V. & Najib, S. (1999) *FEBS Lett* **455,** 307-10.

36. Morley, S. J. & Thomas, G. (1991) *Pharmacol Ther* **50,** 291-319.

37. Brawerman, G. (1989) *Cell* **57,** 9-10.

38. Gao, Z. H., Metherall, J. & Virshup, D. M. (2000) *Biochem Biophys Res Commun* **268,** 562-6.

39. Taha, C. & Klip, A. (1999) *J Membr Biol* **169**, 1-12.

CHAPTER III

Nck-1 antagonizes Endoplasmic Reticulum stress-induced inhibition of translation.

Reproduced with permission from Sem Kebache, Duc Thang Nguyên, Eric Chevet and Louise Larose. 2002. *Nck-1 antagonizes Endoplasmic Reticulum stress-induced inhibition of translation*. J. Biol. Chem. Submitted

Preface

In chapter II, I have shown that Nck-1 modulates positively protein translation through its interaction with eIF2β. It is established that eIF2 is a trimeric complex composed of the subunit α, β and γ which remains associated throughout the initiation cycle (1). Under certain conditions such as environmental stress, eIF2α is phosphorylated on Ser51 by specific kinases and this leads to a general inhibition of protein synthesis (2). Taken all together, these data convinced me to study:

1) Nck effects on protein translation under conditions known to inhibit it, especially during endoplasmic reticulum stress and

2) Whether Nck modulates eIF2α phosphorylation through its interaction with eIF2β.

Abbreviations

A.U : arbitrary unit

BiP : immunoglobulin heavy-chain binding protein

Cal : calyculinA

CHOP : CAAT enhancer binding homologous protein

DTT : dithiothreitol

EIF : eukaryotic initiation factor

ER : endoplasmic reticulum

GCN2 : general control non-repressed-2

GEF : guanine nucleotide exchange factor

IRES : internal ribosomal entry site

MEF : mouse embryonic fibroblast

PERK : PKR-like endoplasmic reticulum kinase

PKR : the interferon-inducible double-stranded RNA-activated kinase

SDS-PAGE : sodium dodecylsulfate polyacrylamide gel electrophoresis

SH : Src homology

Ser51 : serine 51

TCL : total cell lysate

Tg : thapsigargin

Tn : tunicamycin

UPR : unfolded protein response

WB : Western blotting

Abstract

Eukaryotic cells have developed specific mechanisms to overcome environmental stress. Here we show that Nck-1 prevents the unfolded protein response (UPR) normally induced by ER stress agents. Overexpression of Nck-1 enhances protein translation while it abrogates activation of PERK, eIF2α phosphorylation and inhibition of translation in response to tunicamycin or thapsigargin treatment. Nck-1 overexpression also attenuates induction of the ER chaperone BiP and impairs cell survival in response to thapsigargin. In these conditions, we provided evidence that the effects of Nck on the UPR involve its second SH3 domain and a phosphatase-dependent mechanism. Protein translation is reduced in embryonic fibroblasts lacking both Nck isoforms, however in these cells, translation is still responsive to thapsigargin treatment. This establishes that Nck is required for optimal protein translation and demonstrates a novel implication for this adapter in regulating ER stress-induced signaling.

Introduction

The endoplasmic reticulum (ER) is the major signal-transducing organelle continuously sensing intracellular changes triggered by adverse physiological stress states. These conditions include heat shock, hypoxia, glucose deprivation, genetic defects that alter protein structure and pharmacological compounds affecting protein folding. To overcome environmental stress, all eukaryotic cells have developed specific mechanisms in order to maintain ER functions and to promote cell survival. This is achieved by ER-resident molecular machines involved in the activation of at least two distinct signaling pathways that contribute to a proper folding environment in the ER (2,3). In particular, these pathways lead to transcriptional induction of genes encoding ER chaperones and disulfide exchange proteins (4), and to inhibition of translation initiation (5) through phosphorylation of eIF2α by the ER-resident kinase PERK (6). Together these events form what is defined as the Unfolded Protein Response (UPR).

Accumulation of unfolded proteins in the ER lumen induces transcription of a large set of genes, many of which encode proteins that function to increase the volume and capacity for either protein folding or the degradation of misfolded proteins (7). For instance, transcription of the ER chaperone KAR2/BiP/GRP78 is a classical marker for UPR activation in yeast and mammalian cells (2). BiP interacts transiently with exposed hydrophobic patches on protein folding intermediates. This is thought to prevent their aggregation while maintaining these proteins in a folding competent state to ensure that only properly folded

and assembled proteins exit the ER compartment (8). CHOP/GADD153, another UPR marker, is also activated at the transcriptional level. Its maximal induction occurs after several hours and closely parallels the time course of BiP induction (reviewed in 2). In addition, transcription of GADD34 is also activated under stress conditions (9,10), and its profile of induction is very similar to CHOP and BiP. GADD34 is a homologue of the Herpes Simplex Virus-encoded protein $\gamma_1$34.5 (11), which plays an important role in preventing PKR-mediated shutdown of host protein synthesis in virally infected cells. This activity of $\gamma_1$34.5 is dependent on its ability to associate with the catalytic subunit of the protein phosphatase 1c (PP1c), and correlates with an increase in cellular phosphatase activity that dephosphorylates eIF2α (12,13). The COOH-terminal fragment of GADD34 can substitute for the role of $\gamma_1$34.5 (10). Moreover, phosphorylated eIF2α levels were markedly diminished in GADD34-overexpressing cells despite normal activity of PERK and GCN2 (9).

In parallel, accumulation of unfolded proteins in the ER also leads to the inhibition of translation. This is due to the activation of the protein kinase PERK, which specifically phosphorylates the α subunit of eIF2 (eIF2α) on Ser51 (6). In order to efficiently initiate protein translation, the trimeric eIF2 complex, composed of the α, β and γ subunits, needs to recycle from eIF2-GDP (inactive form) to eIF2-GTP (active form) regulated by the GEF, eIF2B (14). Phosphorylated eIF2α has a higher affinity for eIF2B and forms with it a stable complex where the bound GDP cannot be exchanged for GTP (15). Since eIF2 is normally in excess of

eIF2B, phosphorylated eIF2α in the eIF2 complex essentially sequesters the cellular eIF2B activity and leads to a general inhibition of translation (15).

Recently, we have shown that the adapter molecule Nck-1 modulates protein synthesis through its interaction with the β subunit of eIF2 (eIF2β) (16). Since eIF2α is the site of regulation of protein translation and appears to remain associated throughout the translation initiation cycle with the β and γ subunits (1), we investigated the effect of Nck under ER stress conditions, where translation is inhibited through the phosphorylation of eIF2α. Here, we report that overexpression of Nck-1 prevents eIF2α phosphorylation and inhibition of translation in response to ER-stress. This correlates with the lack of PERK activation mediated by a phosphatase-dependent mechanism. Interestingly, protein translation is reduced in Nck deficient cells and translational resistance to ER stress is established by reexpression of Nck-1 in these cells. Overall, our results strongly support a direct role for Nck-1 in the regulation of the ER unfolded protein response.

Materials and Methods

Cell Culture. Human embryonic kidney 293 (HEK293) cells were grown in DMEM (Invitrogen) containing 10% FBS (Invitrogen). The mouse embryonic fibroblasts (MEF) wild-type, Nck1/Nck2 knockout and Nck1/Nck2 knockout rescued by reexpression of HA-Nck-1, were kindly provided by Dr. T. Pawson (University of Toronto, Canada) (17). The MEFs were grown in DMEM high glucose (Invitrogen) containing 10% FBS (Invitrogen).

Antibodies. Nck and eIF2β antisera were prepared as previously described (16,18). PERK antibody was purchased from Santa Cruz Technology. eIF2α and Se51-phosphorylated eIF2α antibodies were purchased from Cell Signal Technology and Biosource International, respectively.

Western Blotting. Transfected HEK293 cells were treated with 2.5 μg ml^{-1} tunicamycin, 1 μM thapsigargin, or 10 mM DTT for 30 min, washed in ice-cold PBS and lysed in 20 mM HEPES [pH 7.5], 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 100 mM NaF, 17.5 mM β-glycerophosphate, 1 mM phenylmethysulfonyl fluoride, 4 μg ml^{-1} aprotonin, and 2 μg ml^{-1} leupeptin. The lysates were cleared by centrifugation at 14,000 rpm. 50 μg of total cell lysates were resolved by SDS-PAGE. Samples were loaded on 7.5 % (PERK) or 10% (Nck, eIF2α and eIF2αP Ser51) acrylamide gels and after electrophoresis, transferred onto nitrocellulose membranes. After incubation with the selected

antibodies, signals were revealed by chemiluminescence (Santa Cruz Biotech) using appropriate HRP-conjugated secondary antibodies.

Nck-1 Constructs and Transfection. Human Nck-1 constructs described earlier (16) were transiently transfected in HEK293 cells by calcium phosphate precipitation. For western blot and RNA analysis, HEK293 cells were plated at a density of $1x10^6$ cells in 100mm plates and cotransfected with 10μg of either the reporter vector and 10 μg of empty pcDNA3.1MycHis or encoding various Myc-Nck-1 molecules. 24hrs post-transfection, each plate was trypsinized and divided into a number of plates needed for each treatment conditions. For the luciferase assay, HEK293 cells were plated at a density of $5x10^4$ cells/well in a 24 well plate and transiently transfected with 0.5 μg of the reporter vector and either 0.5 μg of empty pcDNA3.1MycHis or encoding various Myc-Nck-1 molecules. Each treatment condition was analyzed in triplicate.

Luciferase Assay. The bicistronic reporter plasmid pcDNA3-RLUC-POLIRES-FLUC was kindly provided by Dr. N. Sonenberg (McGill University, Montreal) (19). HEK293 cells transiently co-transfected were treated as indicated with 2.5 μg ml^{-1} tunicamycin (Sigma), 1 μM thapsigargin (Sigma) or 5 nM CalyculinA (Upstate Biotech) for 30 min or with 10 mM DTT (Calbiochem) for 15 min. *Renilla reniformis* luciferase (RLUC) and firefly luciferase (FLUC) activities were measured by using the dual-luciferase reporter assay system (Promega) and a luminometer (LUMAT).

Quantitative RNA Analysis. Transfected HEK293 cells were incubated with or without 1 μM thapsigargin for 30 min, lyzed in Trizol (Invitrogen) and total RNA isolated according to the manufacturer's recommendations. Quantitative RNA analysis was performed with BiP and GAPDH oligonucleotides as described (20). For the luciferase mRNAs levels, Trizol (Invitrogen)-prepared RNAs (5 μg) from HEK293 cells transiently cotransfected with the luciferase reporter vector and Myc-Nck-1 plasmid, and treated with either 2.5 μg ml^{-1} tunicamycin, 1 μM thapsigargin or 10 mM DTT were analyzed by semi-quantitative PCR, as previously described (16) .

Survival Assay. Survival assays were performed as described by others (21), with minor modifications. Briefly, HEK293 cells or MEFs were plated in their respective media at a density of 12000 cells/well in 6-well dishes and grown for 16 hrs. HEK293 cells were transiently transfected with either 0.5 μg of empty vector or plasmids encoding Nck-1 molecules. 36 hrs postransfection, cells were treated with 0.4 μM thapsigargin (Sigma) in the presence or absence of 20 μg ml^{-1} cycloheximide (Sigma) for the indicated period of treatment. Cells remaining on the plate were stained with crystal violet 5 days later.

Phosphatase Assay. Transfected HEK293 cells were treated with either 1 μM thapsigargin, or 1 μM thapsigargin combined with 5 nM calyculinA for 30 min, washed in ice-cold phosphate- and serum-free DMEM (Invitrogen) and lysed in 20 mM HEPES [pH 7.5], 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM

EDTA, 1 mM phenylmethysulfonyl fluoride, 4 µg ml^{-1} aprotonin, and 2 µg ml^{-1} leupeptin. 5µg of TCL were used according to the manufacturer's recommendations, in the Ser/Thr Phosphatase Assay Kit 1 (Upstate Biotech, Cat #17-127) to measure PP1 and/or PP2A activity.

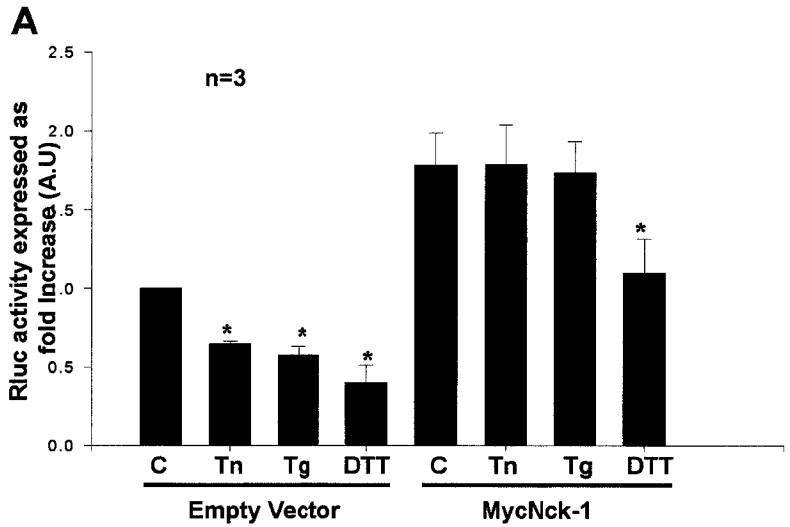
Results

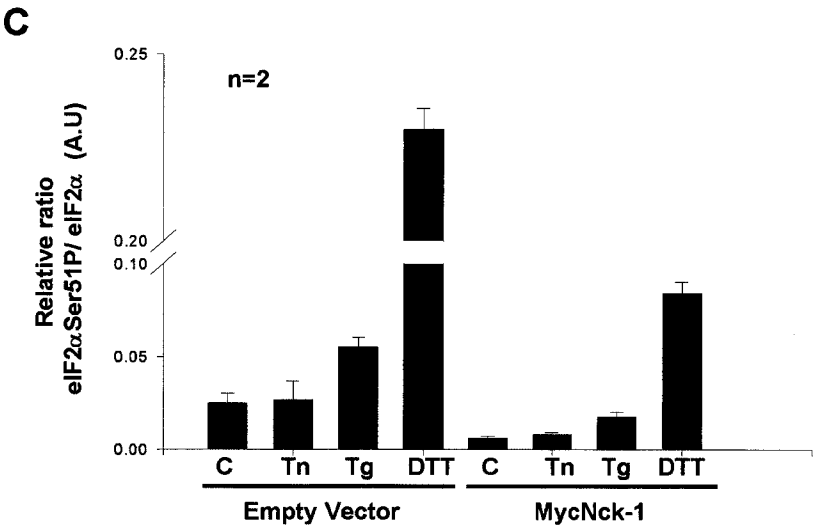
Overexpression of Nck-1 prevents ER stress-induced inhibition of protein translation and phosphorylation of eIF2α. HEK293 cells were cotransfected with the bicistronic luciferase reporter vector and either the empty pcDNA3.1MycHis vector or a plasmid containing Nck-1 cDNA. These transfected cells were treated with the ER-stress agents tunicamycin or thapsigargin for 30 min or with DTT for 15 min. In this system, translation of Rluc is Cap-dependent, whereas translation of the Fluc cistron directed by the poliovirus internal ribosomal entry site (IRES) is Cap-independent (19). In HEK293 cells transfected with the empty vector, Rluc activity is reduced by 25%, 30% and 50% when treated respectively with tunicamycin, thapsigargin or DTT, compared to similar untreated cells (Fig. 1A). As reported previously, in cells overexpressing Nck-1, Rluc activity was increased by 1.5 fold (16). However in these cells, translation was not affected by tunicamycin or thapsigargin treatment, and DTT induced only a 30% inhibition (Fig. 1A). Similar effects were observed when Fluc activity was monitored (data not shown). In all conditions, immunoblot analysis of total cell lysates (TLC) revealed that Nck was overexpressed and that the various cell treatments did not modify the levels of Nck expression. (Fig. 1B).

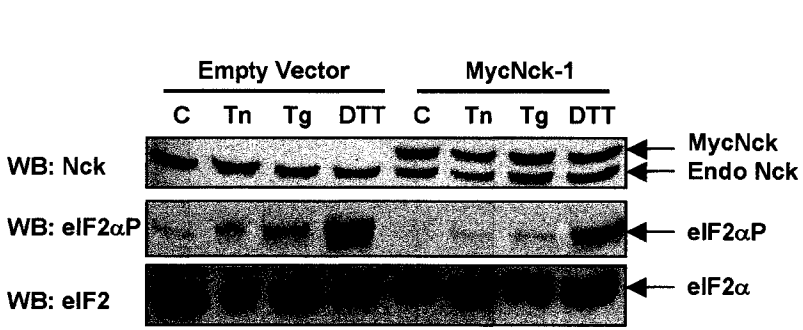
It is well recognized that increased eIF2α phosphorylation on Ser51 under ER stress conditions results in inhibition of translation. This prompted us to assess eIF2α phosphorylation in cells overexpressing Nck-1 upon similar drug treatments. eIF2α phosphorylation was increased in cells transfected with the

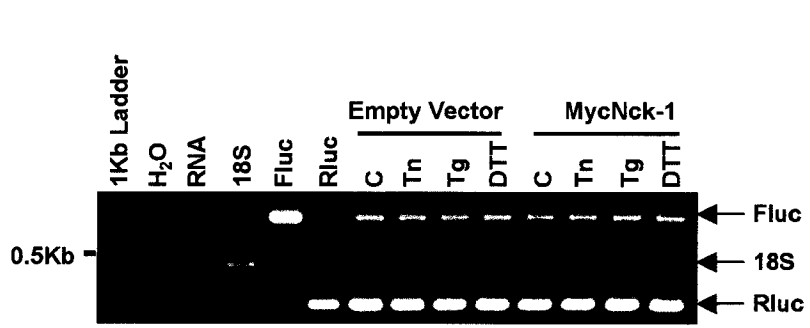
empty vector following all treatments, showing the greatest increase upon the DTT treatment (Fig. 1B, middle panel). In cells overexpressing Nck-1, eIF2α phosphorylation remained at the basal levels upon tunicamycin or thapsigargin treatment. Only Nck-1 overexpressing cells treated with DTT showed a clear increase in eIF2α phosphorylation but this level was reduced relative to the level detected in mock transfected cells under the same conditions (Fig. 1B, middle panel). Fig. 1B (lower panel) shows comparable levels of endogenous eIF2α in all conditions. These observations were confirmed by densitometric analysis and calculation of the ratio eIF2α Phospho Ser51/ eIF2α (Fig. 1C). Finally, semi-quantitative PCR (Fig. 1D) followed by densitometric analysis of the amplified products and calculation of the ratio Rluc/18S confirmed that treatments with ER stress pharmacological compounds did not affect luciferase mRNA levels (data not shown). This establishes that the variations in luciferase activity measured reflected translational rather than transcriptional changes. Overall, these data demonstrate that under ER stress conditions Nck-1 overexpression prevents eIF2α phosphorylation and inhibition of protein translation.

Fig. 1. Overexpression of Nck-1 prevents ER-stress-induced inhibition of protein translation and phosphorylation of eIF2α. A. Luciferase activity of RLUC cistron from HEK293 cells cotransfected with reporter vector and empty plasmid or containing MycNck-1, after 30 min treatment with tunicamycin (Tn), thapsigargin (Tg) or 15min treatment with DTT. Experiments were performed three times in triplicate, and the results represent the mean value +/- SEM. *At least $P<0.001$, as determined by Student's *t*-test compared with respective control (C). **B.** Western blot analysis of the corresponding HEK293 total cell lysates (50μg protein) performed with anti-Nck, anti phosphorylated Ser51 eIF2α, and anti-eIF2α antibodies. In each experiment, membranes containing the transferred proteins were cut at the proper molecular weight. The upper part was probed for Nck while the lower was first probed for phosphorylated Ser51 eIF2α, then stripped and reprobed for eIF2α. Results shown are typical of three independent experiments. **C.** Calculation of eIF2αPhospho Ser51/ eIF2α after densitometric analysis. Two experiments were quantified and the results represent the mean value +/- SEM. **D.** Quantitative reverse transcription-PCR performed on total RNAs prepared from above transfected HEK293. Water (H_2O) or RNA (RNA) were used as controls. Results shown are representative of two experiments.


A
n=3
Rluc activity expressed as fold Increase (A.U)
2.5
2.0
1.5
1.0
0.5
0.0
C Tn Tg DTT C Tn Tg DTT
Empty Vector
MycNck-1


C
n=2
Relative ratio eIF2αSer51P/ eIF2α (A.U)
0.25
0.20
0.10
0.05
0.00
C Tn Tg DTT C Tn Tg DTT
Empty Vector
MycNck-1


B
Empty Vector
MycNck-1
C Tn Tg DTT C Tn Tg DTT
WB: Nck
MycNck
Endo Nck
WB: eIF2αP
eIF2αP
WB: eIF2
eIF2α

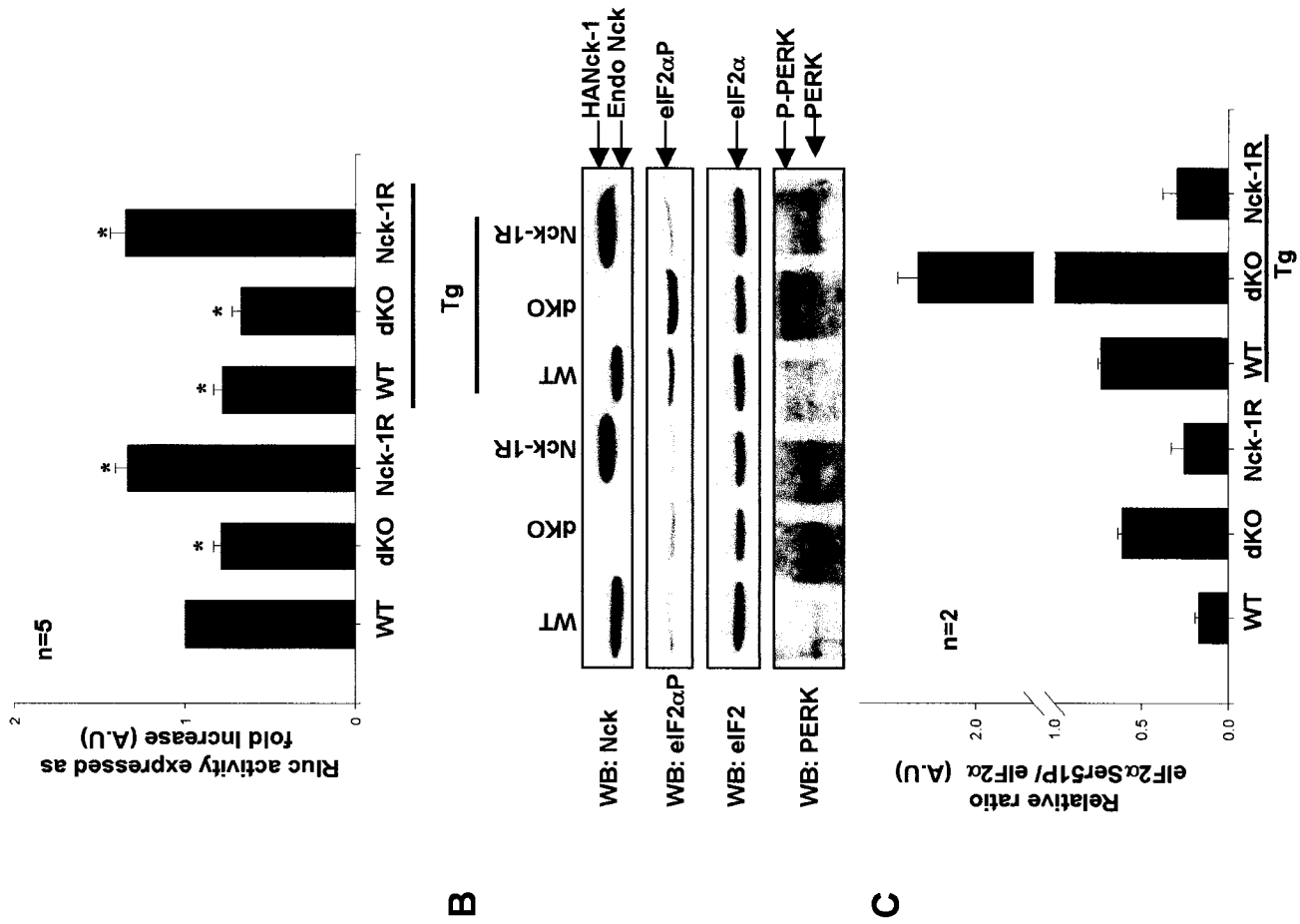

D
1Kb Ladder
H2O
RNA
18S
Fluc
Rluc
Empty Vector
MycNck-1
C Tn Tg DTT C Tn Tg DTT
0.5Kb
Fluc
18S
Rluc

Nck is required for optimal protein translation but its overexpression antagonizes the ER-induced UPR. To further establish a role of Nck in modulating translation and ER stress response, we used mouse embryonic fibroblasts (MEFs) in which Nck-1 and Nck-2 genes have been inactivated (dKO) (17). These MEFs were transfected with the bicistronic luciferase reporter vector; 36 hrs postransfection they were treated with thapsigargin and luciferase activity was measured. Compared to wild type MEFs (WT), protein translation is reduced by 20% in dKO MEFs and increased by 1.3 fold in dKO MEFs rescued with Nck-1 (Nck-1R) (Fig. 2A). Upon thapsigargin treatment, Rluc activity of the MEFs WT and dKO is reduced by 25% and 20% respectively compared to their reciprocal untreated cells, whereas in MEFs containing Nck-1, it remains unchanged (Fig. 2A). Similar data were observed when Fluc activity was monitored (data not shown). Western blot analysis showed the relative expression of Nck in the various MEFs lines (Fig. 2B, upper panel). Analysis of eIF2α phosphorylation on Ser51 revealed that in untreated dKO MEFs, the level of eIF2α phosphorylation is increased compared to untreated MEFs WT and Nck-1R (Fig. 2B, second panel and 2C). Treatment with thapsigargin further increases eIF2α phosphorylation in dKO MEFs compared to MEFs WT. However, it did not induce eIF2α phosphorylation in MEFs Nck-1R (Fig. 2B, second panel and 2C). Fig. 2B (third panel) showed comparable levels of total eIF2α in all conditions. Densitometric analysis and calculation of the ratio eIF2α Phospho Ser51 over total eIF2α showed that in absence of Nck (dKO), phosphorylation of eIF2α on Ser51 is increased by 3 fold independently of the treatment, while phosphorylation of

eIF2α is totally prevented by overexpressing Nck-1 (Nck-1R) in the same conditions (Fig. 2C). These results clearly demonstrate that Nck participates in the regulation of translation and eIF2α phosphorylation.

In response to ER-stress conditions, phosphorylation of eIF2α on Ser 51 is attributed to the activation of the protein kinase PERK. We assessed the activity of PERK in the MEFs expressing different levels of Nck. Activation of PERK analyzed by SDS-PAGE is characterized by slower migrating bands. As shown in Fig. 2B (lower panel), upon thapsigargin treatment, PERK is hyperactivated in MEFs dKO compared to MEFs WT, while remaining inactivated in MEFs Nck-1R. These observations revealed that PERK activation under ER stress conditions is influenced by the cellular content of Nck. In addition, we also observed that the level of eIF2α phosphorylation correlates well with the state of PERK activation.

Fig. 2. Nck-1 is required to sustain optimal levels of protein translation. A. Luciferase activity of RLUC cistron measured in MEFs transfected with luciferase reporter vector treated or not for 30 min treatment with thapsigargin (Tg). WT genotype is Nck-$1^{+/-}$/Nck-$2^{+/+}$, dKO: Nck-$1^{-/-}$/Nck-$2^{-/-}$ and Nck-1R denotes dKO rescued by stable expression of HA-Nck-1. Experiments were performed five times in triplicate, and the results represent the mean value +/- SEM. * At least $P<0.001$, as determined by Student's *t*-test compared with control. **B.** Western blot analysis of the above MEF cells performed with anti-Nck, anti phosphorylated Ser51 eIF2α, anti-eIF2α and PERK antibodies. 50μg of TCL proteins were resolved on 7.5% (for PERK) or 10% (for Nck, Ser51eIF2α and eIF2α) acrylamide gels by SDS-PAGE. In each experiment, membranes were cut at the proper molecular weight, and the upper parts was probed for Nck and PERK while the lower was first probed for Ser51 phosphorylated eIF2α, then stripped and reprobed for eIF2α. Results shown are typical of three independent experiments. **C.** eIF2α Phospho Ser51/ eIF2α ratio after densitometric analysis of two blots. The results represent the mean value +/- SEM.


A
n=5
Rluc activity expressed as fold Increase (A.U)
WT dKO Nck-1R WT dKO Nck-1R
Tg
B
WB: Nck
WB: eIF2αP
WB: eIF2
WB: PERK
WT dKO Nck-1R WT dKO Nck-1R
Tg
HANck-1
Endo Nck
eIF2αP
eIF2α
P-PERK
PERK
C
n=2
Relative ratio eIF2αSer51P/ eIF2α (A.U)
WT dKO Nck-1R WT dKO Nck-1R
Tg

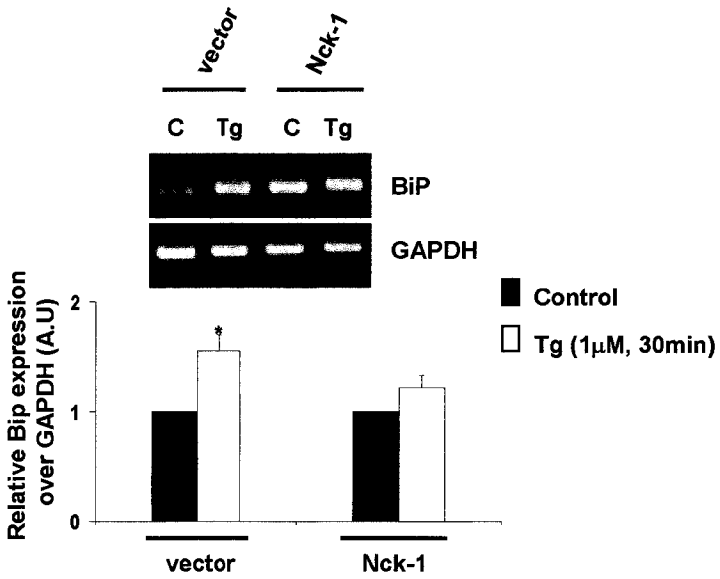
To further characterize the effect of Nck on the UPR, we analyzed the induction of BiP in HEK293 cells transfected with either empty vector or a vector encoding MycNck-1, and treated with or without thapsigargin for 30 min. Overexpression of Nck-1 showed comparable levels in all conditions (data not shown). Total RNAs were isolated and semi-quantitative RT-PCR experiments carried out to assess expression of BiP mRNAs. Thapsigargin treatment resulted in BiP induction by 1.5 fold in control HEK293 cells transfected with an empty vector, whereas overexpression of Nck-1 kept BiP transcript to almost basal level (Fig. 3A). These data clearly demonstrate that Nck-1 also prevents BiP induction in response to ER stress conditions.

It is well characterized that long exposure to ER stress compounds has a profound effect on cell survival, especially when the UPR is prevented (reviewed in 2). Therefore we exposed HEK293 cells overexpressing Nck-1 to thapsigargin for various times and assessed cell survival. For this purpose, cells were plated at low density and transfected with either empty vector or the vector containing MycNck-1 cDNA. At 36 hrs postransfection, cells were exposed to thapsigargin for 30 min, 1 or 2 hrs. After thapsigargin treatment, cells were allowed to resume growth in normal media in the absence of the drug. As shown in Fig. 3B, overexpression of Nck-1 has a dramatic effect on survival of the cells at all times compared to cells transfected with the empty vector. Interestingly, when combined with cycloheximide, a known inhibitor of protein translation, Nck-1 overexpressing cells survived the 30 min and 1 hour thapsigargin treatment, while treatment for 2 hours was still detrimental (Fig. 3B). Overall these data

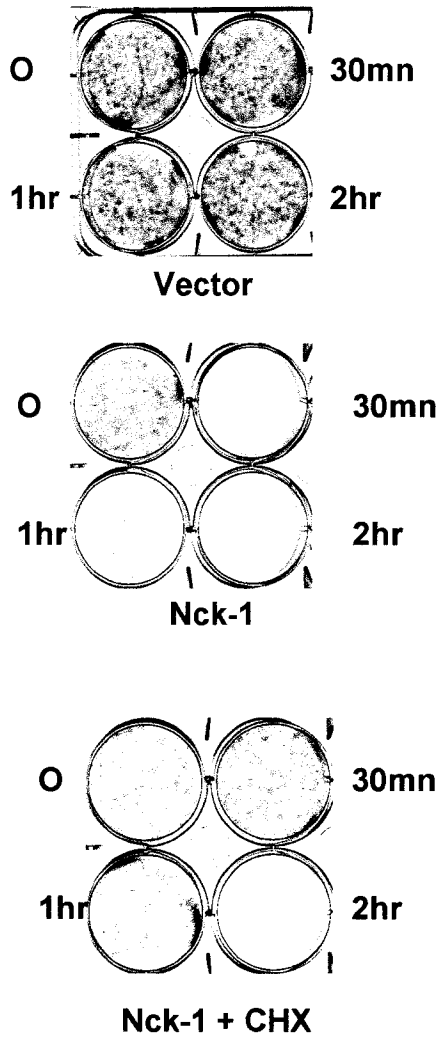
establish that Nck is required for optimal protein translation and for maintaining low levels of eIF2α phosphorylation. Also, our data determine that Nck participates in regulating the ER stress response (UPR). In fact, by preventing activation of PERK, overexpression of Nck-1 abolishes the UPR and leads to cell death.

Fig. 3. Nck-1 reduces BiP induction and cell survival in response to ER stress. A. HEK293 cells transfected either with an empty vector or a vector containing Nck-1 cDNA were treated for 30 min with 1 μM thapsigargin. Total mRNA was extracted and retrotranscribed into cDNA prior to PCR amplification with BiP and GAPDH specific oligonucleotides. Amplification products are shown on the gels (a representative experiment is shown; three independent experiments were performed in duplicate). Scanning densitometry analysis of the PCR products represents the mean +/-SD of three independent experiments. *, indicates significant changes ($P<0.01$) compared to control. **B.** Control and Nck-1 overexpressing cells were exposed for different times to thapsigargin (0.4 μM) in presence or not of cycloheximide (CHX, 20 μg ml^{-1}). Cells survival was determined 5 days later by crystal violet staining.

A

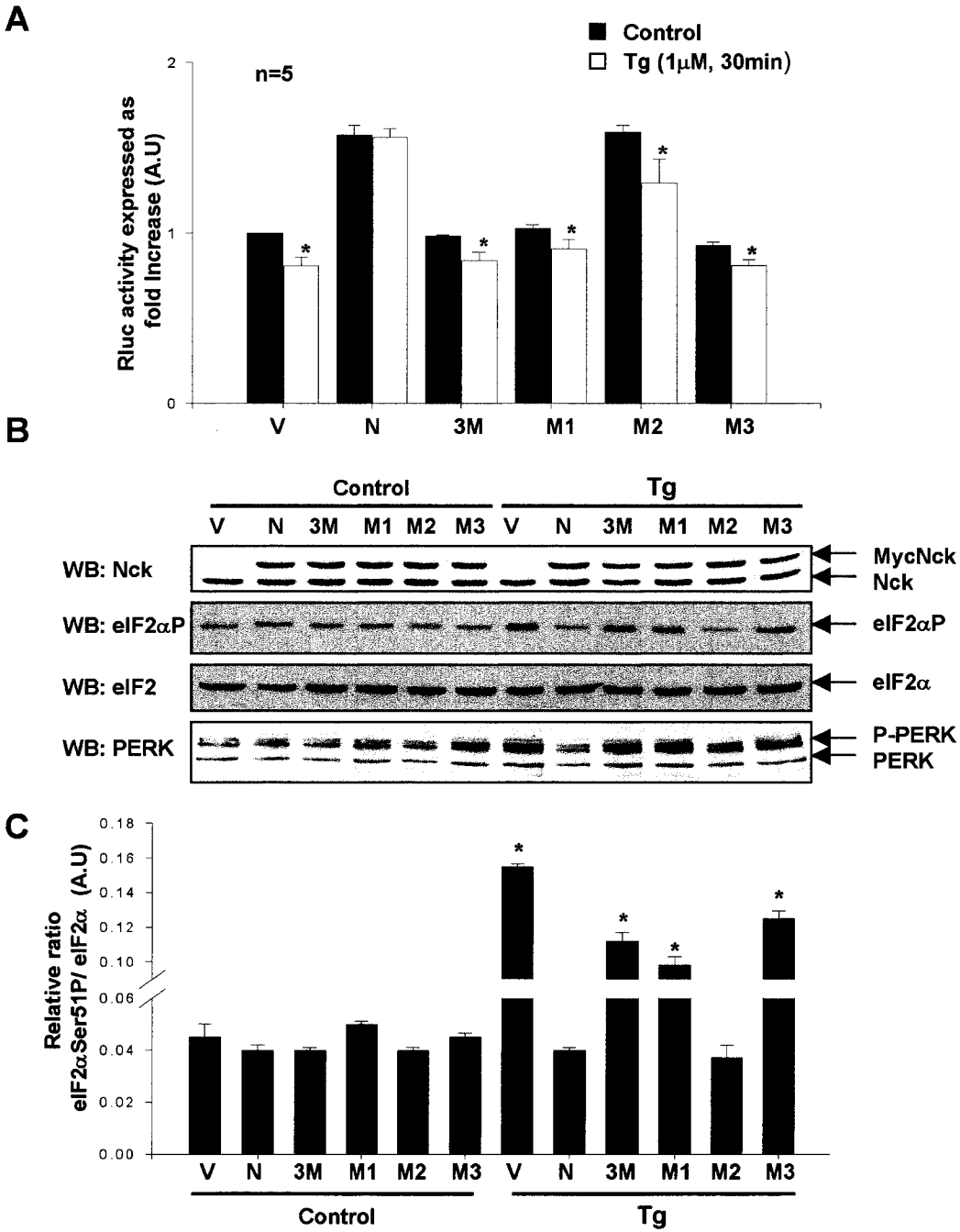

vector
Nck-1
C
Tg
C
Tg
BiP
GAPDH
Relative Bip expression over GAPDH (A.U)
2
1
0
*
Control
Tg (1μM, 30min)
vector
Nck-1

B


O
30mn
1hr
2hr
Vector
O
30mn
1hr
2hr
Nck-1
O
30mn
1hr
2hr
Nck-1 + CHX

Effect of Nck-1 on the UPR involves its second SH3 domain. We further characterized which Nck-1 SH domain(s) is required to prevent the ER stress-induced inhibition of translation. For this purpose, various Myc-tagged Nck-1 constructs were cotransfected with the bicistronic luciferase reporter vector into HEK293 cells. This results in comparable levels of Nck-1 expression (Fig. 4B, upper panel). As previously reported (16), the effect of Nck-1 on translation in serum growing conditions is abolished by mutation of its first (M1), third (M3) and all SH3 (3M) domains (Fig. 4A). Moreover, overexpression of these Nck-1 mutants did not prevent inhibition of translation achieved by thapsigargin treatment. However in these conditions, they allowed PERK activation and eIF2α phosphorylation on Ser51 (Fig. 4B and C). On the other hand, Nck-1 mutated on its second SH3 domain (M2), like Nck-1 wild type, enhanced protein translation. However in contrast to Nck-1, the M2 mutant permitted inhibition of translation in response to thapsigargin treatment (Fig. 4A) and surprisingly, still prevented PERK activation and eIF2α phosphorylation (Fig. 4B and C). Taken together, these data suggest that Nck-1 requires the functional integrity of its second SH3 domain in order to impair the translational component of the ER-induced UPR. Our data also suggest that in the absence of a functional second SH3 domain, Nck-1 allows inhibition of translation in response to ER stress but in a PERK/eIF2α phosphorylation independent manner.

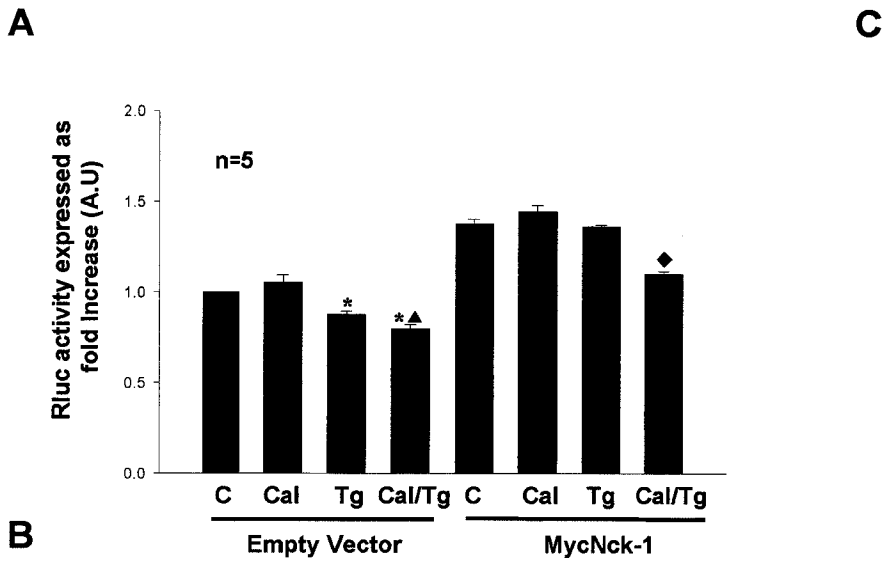
Fig. 4. Nck-1 effects on the translational component of the UPR require the integrity of its second SH3 domain. A. Luciferase activity of RLUC cistron from HEK293 cells cotransfected with the bicistronic luciferase reporter vector (0.5 μg) and either empty vector or the indicated Myc-Nck-1 constructs (0.5 μg). V denotes, empty vector, N: wild type Nck-1, 3M: Nck-1 mutated in its three SH3 domains, M1, M2 and M3: Nck-1 mutated in the first, second or third SH3 domain, respectively. Experiments were performed five times in triplicate, and the results represent the mean value +/- SEM. * At least $P<0.001$, as determined by Student's *t*-test compared with respective controls. **B.** Western blot analysis of the corresponding HEK293 cells was performed with anti-Nck, anti phosphorylated Ser51 eIF2α, anti-eIF2α and PERK antibodies. Total cell lysates (50 μg protein) were resolved on 7.5% (for PERK) or 10% (for Nck, Ser51eIF2α and eIF2α) acrylamide gels by SDS-PAGE. In each experiments, membranes were processed as described above. Results shown are typical of three independent experiments. **C.** Densitometric analysis of three independent experiments of eIF2α Phospho Ser51/ eIF2α ratio. Results represent the mean value +/- SEM. *At least $P<0.01$, as determined by Student's *t*-test compared with reciprocal untreated controls.

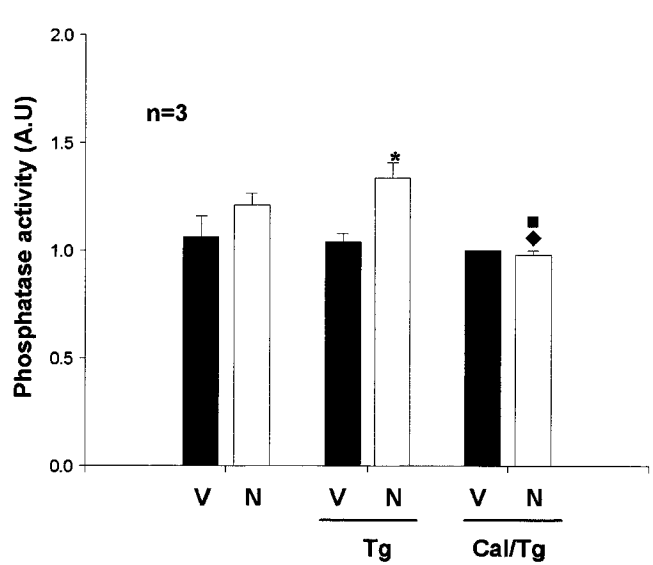

A
Control
Tg (1μM, 30min)
n=5
Rluc activity expressed as fold Increase (A.U)
0
1
2
V
N
3M
M1
M2
M3
B
Control
Tg
V N 3M M1 M2 M3 V N 3M M1 M2 M3
WB: Nck
MycNck
Nck
WB: eIF2αP
eIF2αP
WB: eIF2
eIF2α
WB: PERK
P-PERK
PERK
C
Relative ratio eIF2αSer51P/ eIF2α (A.U)
0.00
0.02
0.04
0.06
0.10
0.12
0.14
0.16
0.18
V N 3M M1 M2 M3 V N 3M M1 M2 M3
Control
Tg

The effect of Nck-1 under ER stress is phosphatase-dependent. As reported previously by others, PP1 is a phosphatase that dephosphorylates eIF2α to permit recovery from ER stress conditions (9). To determine whether the effects of Nck-1 on the UPR were mediated by a phosphatase dependent mechanism, we used calyculinA, a potent inhibitor of PP1 and PP2A phosphatases. HEK293 cells were cotransfected with the bicistronic luciferase reporter vector and either the empty pCDNA3.1MycHis vector or the vector containing the Nck-1 cDNA. These cells were treated for 30 min with either calyculinA, thapsigargin or both. As shown in Fig. 5A, calyculinA did not alter the effect of Nck-1 on protein translation in serum growing cells. However, when combined with thapsigargin, calyculinA allows recovery of the inhibition of translation in response to thapsigargin in Nck-1 overexpressing cells (Fig. 5A). Furthermore, western blot analysis revealed that in cells treated only with calyculinA, the level of eIF2α phosphorylation is increased in both control and Nck-1 overexpressing cells compared to the same cells untreated (Fig. 5B). In addition, when both calyculinA and thapsigargin are combined, eIF2α is phosphorylated in Nck-1 overexpressing cells to the same extent as detected in cells transfected with the empty vector. PERK activation followed a similar profile to eIF2α phosphorylation, showing no activation in cells overexpressing Nck-1 treated with thapsigargin alone and activation in cells overexpressing Nck-1 and treated with both calyculinA and thapsigargin (Fig. 5B). Fig. 5 shows comparable levels of Nck-1 overexpression and endogenous eIF2α in all conditions. These data strongly suggest that a phosphatase, by preventing PERK activation and thus phosphorylation of eIF2α,

mediates the effects of Nck-1 under ER stress conditions. In agreement with this hypothesis, in cells overexpressing Nck-1 and treated with thapsigargin, total cell lysates calyculin-sensitive Ser/Thr phoshatase activity is increased compared to thapsigargin treated cells transfected with an empty vector (Fig. 5C). This demonstrates that overexpression of Nck results in the activation of a calyculin sensitive phosphatase activity.

Fig. 5. The effect of Nck-1 under ER stress is phosphatase-dependent. A. Luciferase activity of RLUC cistron from HEK293 cells cotransfected with reporter vector and either empty vector or containing MycNck-1 cDNA and treated for 30 min with either 5 nM CalyculinA (Cal) or 1 μM thapsigargin (Tg) or both. (C) denotes untreated cells. Experiments were performed five times in triplicate, and the results represent the mean value +/- SEM. * At least $P<0.001$, as determined by Student's *t*-test when treated empty vector samples are compared to the untreated empty vector sample. ▲At least $P<0.01$ when compared to the Tg treated control. ◆ At least $P<0.01$ when compared to the Tg treated Nck-1. **B.** Corresponding HEK293 TCL (50 μg protein) were used for western blot analysis as described above. Results shown are typical of two independent experiments. **C.** Phosphatase Assay performed on HEK293 cells transfected with either empty vector (V) or containing MycNck-1 cDNA and treated for 30 min with either 1 μM Tg or 1 μM Tg combined with 5 nM Cal (Tg/Cal). Three experiments were performed in triplicate, and the results represent the mean value +/- SEM. * At least $P<0.01$, as determined by Student's *t*-test when Nck-1 samples are compared to their respective empty vector. ■At least $P<0.01$ when Nck-1 Tg/Cal is compared to the Nck-1 Tg. ◆ At least $P<0.01$ when Nck-1 Tg+Cal is compared to untreated Nck-1.


A
Rluc activity expressed as fold increase (A.U)
n=5
2.0
1.5
1.0
0.5
0.0
C Cal Tg Cal/Tg C Cal Tg Cal/Tg
Empty Vector
MycNck-1
B
Cal
Tg
Cal/Tg
V N V N V N V N
WB: Nck
WB: eIF2αP
WB: eIF2
WB: PERK
MycNck
Endo Nck
eIF2αP
eIF2α
P-PERK
PERK


C
Phosphatase activity (A.U)
n=3
2.0
1.5
1.0
0.5
0.0
V N V N V N
Tg
Cal/Tg

Discussion

Nck is involved in many cellular events such as mitogenesis, oncogenesis, cell proliferation, embryonic development and more predominantly cytoskeletal rearrangement (reviewed in 22-25). Recently, we reported a role for Nck in modulating protein translation through its interaction with eIF2β (16). Indeed, we demonstrated that the effect of Nck on translation is dependent on the integrity of its first and third SH3 domains that have been found originally to directly interact with eIF2β (16). Here, we establish that in the absence of expression of both isoforms of Nck, the levels of protein translation is decreased in serum growing mouse embryonic fibroblasts and this correlates with higher levels of eIF2α phosphorylation on Ser51. In this study, to further characterize the effect of Nck on translation, we investigated whether Nck could sustain translation under circumstances, such as ER stress, where translation is normally repressed. We demonstrate that Nck-1 prevents inhibition of translation in response to ER stress pharmacological treatments. Interestingly, we observe under these conditions that Nck-1 also abrogates activation of PERK, induction of BiP and eIF2α phosphorylation. This suggests that Nck-1 impairs the UPR by acting as a negative regulator of PERK. PERK is the major eIF2α kinase responsible for the ER-induced UPR. It is an ER resident transmembrane protein whose activity is repressed by the ER chaperone BiP. When unfolded proteins accumulate in the ER, BiP dissociates from PERK, resulting in its activation. Activated PERK in turn phosphorylates eIF2α (26) which inhibits translation in order to alleviate the load of unfolded protein in the ER (27,28) (Fig.6).

The inhibitory effect of Nck-1 on PERK activation in response to ER stress is also consistent with reduced BiP induction. Studies of BiP induction in yeast and mammals suggest that the signal for the UPR is not directly the accumulation of unfolded polypeptides, but rather the decrease in the ER concentration of free BiP that occurs when BiP is sequestered into complexes with unfolded proteins (reviewed in 29). The UPR is regulated at the transcription level (29-32) and the genes involved are responsive to low levels of free BiP in the ER. Recently, evidence was provided showing that BiP expression is tightly controlled at a posttranslational level (33). Indeed, cellular BiP mRNA does not lead to increased synthesis of BiP in unstressed cells and protein levels remain constant. Under ER stress conditions, this homeostatic restriction is lost (33). In our case, by interfering with the activation of PERK, Nck-1 may prevent dissociation of BiP from PERK, therefore inhibiting downstream events triggered by both BiP transcription and translation.

It has been shown previously that overexpression of IRE1, an ER stress protein kinase, induces cell death (34). In addition, a recent study revealed that PERK deficient cells have a reduced survival, which was partially recovered when translation is inhibited by cycloheximide (28). Interestingly, BiP overexpression increases cell survival under ER stress conditions (35), without increasing the maturation or secretion of proteins in the secretory pathway (36). Our results show that Nck-1 overexpression has a dramatic effect on cell survival. Knowing that the two major UPR pathways are mediated by PERK and IRE1 (reviewed in 2,3), and given that PERK activation is inhibited and BiP induction reduced in

Nck-1 overexpressing cells, this may favor the IRE1 UPR alternative pathway which interferes with cell survival. The IRE1 pathway has been coupled to the stress-activated protein kinases JNK (37). In an independent study, we report that Nck interacts directly with IRE1 (20). Moreover, immunodepletion of Nck in immuno-isolated ER membranes interfered with the SAPK/MAPK activation and this was rescued by the add-back of recombinant Nck-1 in the system. Overall these data suggest that Nck-1 promotes cross talk between ER stress signaling and SAPK/MAPK pathways, which could account for the reduced survival in cells overexpressing this adapter.

Recently, feedback inhibitory mechanisms for the regulation of the PERK-mediated UPR have been reported in the literature (9,38). For instance, eIF2α dephosphorylation mediated by the complex GADD34 and the subunit c of the phosphatase PP1, promotes recovery from the UPR (9). in addition, a second mechanism involving the P58^{IPK} Hsp40 family member, has been shown to inhibit activation of PERK by binding to PERK during ER stress (38). Our present data led us to hypothesize that Nck-1 could be involved in a feedback loop mechanism regulating PERK activation and protein translation under ER stress conditions. Indeed, we provided evidence for the existence of a such mechanism by demonstrating that calyculinA, a specific inhibitor of the Ser/Thr protein phosphatases PP1 and PP2A, efficiently reverses the effect of Nck-1 by allowing inhibition of translation under ER stress conditions. Therefore, several mechanisms may exist to recover from the UPR and the regulation of a Ser/Thr

phophatase by Nck-1 is one of them. However, it remains to be determined whether Nck interacts with a phosphatase *in vivo* and that the latter can dephosphorylate PERK.

In summary, from our findings the following mechanisms can be proposed. In ER stress conditions, overexpression of Nck-1 could favor a complex made of Nck-1 and either PP1 or PP2A phosphatase (Fig.6, mechanism 1). This complex contributes to keep PERK inactivated, thus preventing phosphorylation of eIF2α and enhancing protein translation. On the other hand, mechanism 2 presented in Fig. 6 implies that overexpression of Nck-1 could also favor an excess of Nck-1/eIF2 complex, which further increases protein translation. All together, these mechanisms lead to general cell death due to an overload of misfolded proteins in the ER.

Fig. 6. Schematic model of Nck-1 mediated effects in response to ER stress. Up arrows denote Nck-1 overexpression. Other arrows designate positive signaling. Crossed arrows indicate inhibition. 1) Under ER stress conditions, overexpression of Nck-1 favors the formation of Nck-1 complexed with a phosphatase. This prevents PERK activation, therefore keeps eIF2α in an unphosphorylated state and makes it available for initiation of translation. 2) Under the same conditions, overexpression of Nck-1 also favors the formation of Nck-1 and eIF2 trimer complex, which also leads to an increase of protein translation. Combined action of Nck-1 results in an overload of misfolded proteins in the ER protein leading to general cell death.

Endoplasmic Reticulum Stress
PERK
ER Lumen
SH3-1
SH3-2
SH3-3
SH2
Nck
1
P
P
P
P
1
P
Nck
SH3-2
SH2
SH3-1
SH3-3
γ
2
Translation initiation
2
Cycloheximide
Death
Survival

The physiological significance of our study could be linked to the translational control in the metabolism of glucose. It has been shown previously that PERK is highly expressed in pancreatic β cells. More interestingly, recent studies using PERK $^{-/-}$ mice (39) or eIF2α Ser51 mutant mice (40) showed imbalanced glucose homeostasis. PERK-deficient animals developed marked hyperglycemia at 4 weeks of age whereas Ser51 mutant mice were normal at birth but died of severe hypoglycemia few hours later. Both mutant mice had defects in pancreatic β cells. PERK deficient mutants showed a decrease in the β cell population due mainly to apoptosis concomitant with the hyperglycemic phenotype. In contrast, in the eIF2α mutant mice, hypoglycemia is a consequence of the loss of β cells function due to inability of eIF2α to mediate translational control. Whether a correlation exists between PERK function and Nck-1 expression in glucose homeostasis remains to be addressed. However, a possible role for Nck in insulin signaling and diabetes has been pointed out in several studies. One of the first accounts showed that Nck is capable of an insulin-dependent association with IRS-1 and IRS-3 (41-43), suggesting a role for Nck downstream of the insulin receptor. Moreover, in streptozotocin-treated rats (animal model for type-I diabetes), the levels of Nck in liver are reduced whereas they remain unchanged in adipose tissue (44). In obese insulin resistant diabetic KKA^{y} mice, an animal model of type-II diabetes, Nck levels remain unchanged in liver and dramatically increased in adipose tissue (45). Taken together, these observations suggest that modulation of Nck expression, with other key signaling molecules such PERK and eIF2, may account for altered glucose metabolism in insulin target tissues.

Acknowledgments

We thank Dr. Tony Pawson for its kind gift of the double Nck knock out MEFs. We also thank Drs. André Nantel and Malcom Whiteway for the critical review of the manuscript.

References

1. Pain, V. M. (1996) *Eur J Biochem* **236**, 747-771
2. Kaufman, R. J. (1999) *Genes Dev* **13**, 1211-1233.
3. Kaufman, R. J., Scheuner, D., Schroder, M., Shen, X., Lee, K., Liu, C. Y., and Arnold, S. M. (2002) *Nat Rev Mol Cell Biol* **3**, 411-421.
4. Gething, M. J., and Sambrook, J. (1992) *Nature* **355**, 33-45.
5. Brostrom, C. O., and Brostrom, M. A. (1998) *Prog Nucleic Acid Res Mol Biol* **58**, 79-125
6. Harding, H. P., Zhang, Y., and Ron, D. (1999) *Nature* **397**, 271-274.
7. Travers, K. J., Patil, C. K., Wodicka, L., Lockhart, D. J., Weissman, J. S., and Walter, P. (2000) *Cell* **101**, 249-258
8. Hurtley, S. M., and Helenius, A. (1989) *Annu Rev Cell Biol* **5**, 277-307
9. Novoa, I., Zeng, H., Harding, H. P., and Ron, D. (2001) *J Cell Biol* **153**, 1011-1022
10. He, B., Chou, J., Liebermann, D. A., Hoffman, B., and Roizman, B. (1996) *J Virol* **70**, 84-90
11. Chou, J., and Roizman, B. (1994) *Proc Natl Acad Sci U S A* **91**, 5247-5251
12. He, B., Gross, M., and Roizman, B. (1997) *Proc Natl Acad Sci U S A* **94**, 843-848
13. He, B., Gross, M., and Roizman, B. (1998) *J Biol Chem* **273**, 20737-20743
14. Merrick, W. C. (1992) *Microbiol Rev* **56**, 291-315
15. Kimball, S. R., Horetsky, R. L., and Jefferson, L. S. (1998) *J Biol Chem* **273**, 30945-30953
16. Kebache, S., Zuo, D., Chevet, E., and Larose, L. (2002) *Proc Natl Acad Sci U S A* **99**, 5406-5411
17. Gruenheid, S., DeVinney, R., Blat, F., Goosney, D., Gelkops, S., Gish, G. D., Pawson, T., and Finlay, B. B. (2001) *Nature Cell biology* **3**, 856-859
18. Lussier, G., and Larose, L. (1997) *J Biol Chem* **272**, 2688-2694

19. Poulin, F., Gingras, A. C., Olsen, H., Chevalier, S., and Sonenberg, N. (1998) *J Biol Chem* **273**, 14002-14007.

20. Nguyen, D. T., Jenna, S., Fazel, A., Kebache, S., Emadali, A., Wong, H. N., Larose, L., Bergeron, J. J., Kaufman, R. J., and Chevet, E. (2002) *(submitted)*

21. Harding, H. P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000) *Mol Cell* **6**, 1099-1108

22. Buday, L. (1999) *Biochim Biophys Acta* **1422**, 187-204.

23. Li, W., Fan, J., and Woodley, D. T. (2001) *Oncogene* **20**, 6403-6417

24. Li, W., and She, H. (2000) *Histol Histopathol* **15**, 947-955

25. McCarty, J. H. (1998) *Bioessays* **20**, 913-921.

26. Bertolotti, A., Zhang, Y., Hendershot, L. M., Harding, H. P., and Ron, D. (2000) *Nat Cell Biol* **2**, 326-332

27. Sood, R., Porter, A. C., Ma, K., Quilliam, L. A., and Wek, R. C. (2000) *Biochem J* **346 Pt 2**, 281-293

28. Harding, H. P., Zhang, Y., Bertolotti, A., Zeng, H., and Ron, D. (2000) *Mol Cell* **5**, 897-904

29. Kohno, K., Normington, K., Sambrook, J., Gething, M. J., and Mori, K. (1993) *Mol Cell Biol* **13**, 877-890

30. Normington, K., Kohno, K., Kozutsumi, Y., Gething, M. J., and Sambrook, J. (1989) *Cell* **57**, 1223-1236

31. Mori, K., Sant, A., Kohno, K., Normington, K., Gething, M. J., and Sambrook, J. F. (1992) *Embo J* **11**, 2583-2593

32. Kozutsumi, Y., Segal, M., Normington, K., Gething, M. J., and Sambrook, J. (1988) *Nature* **332**, 462-464.

33. Gulow, K., Bienert, D., and Haas, I. G. (2002) *J Cell Sci* **115**, 2443-2452

34. Wang, X. Z., Harding, H. P., Zhang, Y., Jolicoeur, E. M., Kuroda, M., and Ron, D. (1998) *Embo J* **17**, 5708-5717

35. Morris, J. A., Dorner, A. J., Edwards, C. A., Hendershot, L. M., and Kaufman, R. J. (1997) *J Biol Chem* **272**, 4327-4334

36. Dorner, A. J., Wasley, L. C., and Kaufman, R. J. (1992) *Embo J* **11**, 1563-1571

37. Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H. P., and Ron, D. (2000) *Science* **287**, 664-666

38. Yan, W., Frank, C. L., Korth, M. J., Sopher, B. L., Novoa, I., Ron, D., and Katze, M. G. (2002) *Proc Natl Acad Sci U S A* **22**, 22

39. Harding, H. P., Zeng, H., Zhang, Y., Jungries, R., Chung, P., Plesken, H., Sabatini, D. D., and Ron, D. (2001) *Mol Cell* **7**, 1153-1163

40. Scheuner, D., Song, B., McEwen, E., Liu, C., Laybutt, R., Gillespie, P., Saunders, T., Bonner-Weir, S., and Kaufman, R. J. (2001) *Mol Cell* **7**, 1165-1176

41. Lee, C. H., Li, W., Nishimura, R., Zhou, M., Batzer, A. G., Myers, M. G., Jr., White, M. F., Schlessinger, J., and Skolnik, E. Y. (1993) *Proc Natl Acad Sci U S A* **90**, 11713-11717

42. Tu, Y., Liang, L., Frank, S. J., and Wu, C. (2001) *Biochem J* **354**, 315-322.

43. Xu, P., Jacobs, A. R., and Taylor, S. I. (1999) *J Biol Chem* **274**, 15262-15270

44. Bonini, J. A., Colca, J., and Hofmann, C. (1995) *Biochem Biophys Res Commun* **212**, 933-938.

45. Bonini, J. A., Colca, J. R., Dailey, C., White, M., and Hofmann, C. (1995) *Am J Physiol* **269**, E759-765.

CHAPTER IV

General Discussion

Since its discovery, Nck by its SH3 domains has been described mainly as interacting with effector proteins that are key regulators of the actin cytoskeleton and thus Nck is believed to play a role in cytoskeleton rearrangement (reviewed in 1). In addition, a few studies have pointed out its oncogenic potential by showing that it induces cell transformation and tumor formation when overexpressed in fibroblasts. This is in agreement with the fact that Nck transduces signals of several proliferative mitogenic receptors such as the PDGFR and EGFR, known to play a critical role in cancer (reviewed in 2, 3). Even though several groups have isolated and characterized Nck binding partners and have attempted to find the meaning of these interactions, it still remains to determine by which mechanisms Nck modulates specific cellular functions. The work accomplished in this thesis reveals for the first time a novel role for Nck in regulating protein translation and ER-stress signaling.

In Drosophila, loss of function of Dock, the homologue of the human Nck, impaired axonal guidance and targeting required for eye and nervous system development (4, 5). This was the first evidence revealing a potential role for Nck in neuronal biology. Dock is ubiquitously expressed in the central nervous system, especially concentrated in neuronal growth cones (4, 5). In mammals, Nck is believed to play an important role by linking membrane receptors to regulators of the actin cytoskeleton (1, 6). Dock in Drosophila may exert similar functions by linking growth cone guidance receptors to regulators of the actin cytoskeleton implicated in axonal navigation and targeting. The role of Nck in axonal guidance and its effects on protein translation may be closely linked by

the fact that both dendrite outgrowth and axonal guidance appear to depend on local protein synthesis occurring in these neuronal structures. This is strengthened by the fact that specific mRNAs and components necessary for translation, post-translational modification and translocation of proteins have been detected locally in dendrites and axons (7). Clear evidence for active protein synthesis within dendrites was provided first (7-9) and recently proposed to couple synaptic plasticity and receptor repertoire in response to synaptic stimulation (10). Local axonal translation has been also demonstrated to mediate guidance cue effects of netrin-1 and Semaphorin-3A using isolated *Xenopus* retinal axons (11). Moreover, recently, Li et al. have shown in commissural neurons that Nck-1 binds the deleted colorectal cancer (DCC) receptor for netrin-1 and this interaction was dependent on the first and third SH3 domains of Nck (12). Although upstream signaling events regulating axonal protein translation and cytoskeleton rearrangements during guidance and targeting need to be elucidated, it is nevertheless tempting to propose that Nck could act as a crucial mediator of both of these processes. Confirming this hypothesis would really place Nck as an important key regulator of nervous system plasticity.

To date, several proteins of the translational apparatus have been shown to participate in the process of tumor formation. For example, eIF4E overexpression causes malignant transformation of immortalized cells. Elevated levels of eIF4E mRNA were found in a broad spectrum of transformed cell lines, solid tumors (13), and in breast (13, 14), head and neck carcinomas (15). High levels of eIF4AI mRNA are detected in human melanoma cells, whereas mRNAs

encoding other initiation factors such as eIF4AII, eIF2γ, eIF4B and eIF4E are not affected (16). Interestingly, increased levels of eIF2α are found in non-Hodgkin's lymphoma (17) and mouse mammary tumors (18), whereas eIF2B is elevated in some human breast cancer lines (19). It would be interesting to determine in tumors, whether Nck levels are also correlated with increased expression of these initiation factors, especially with eIF2α.

An important mechanism regulating global protein synthesis is the phosphorylation of eIF2α by eIF2α kinases (PKR, HEM, PERK). eIF2α phosphorylation on ser51 leads to lower levels of ternary complex (Met-tRNA-eIF2GTP) and reduced levels of initiation of translation. Expression of a dominant negative mutant form of PKR, causes malignant transformation of immortalized NIH 3T3 cells (20). This suggests that PKR could act as a tumor suppressor (21). Since PKR also phosphorylates other substrates than eIF2α, for example IκB, the inhibitor of the transcription factor NF-κB, its function in growth may not be strictly related to translation. A direct role for protein translation in transformation was later demonstrated when it was shown that overexpression of a eIF2α mutated on ser51 led to cell transformation while cells transfected with wild-type eIF2α did not (22). These results indicate that regulation of protein synthesis by phosphorylation of eIF2α is crucial for normal cell growth. According to our findings that Nck-1 modulates PERK activation, we suggest that indirectly Nck modulates eIF2α phosphorylation. In this situation, it is tempting to speculate that like PKR, PERK might have an anti-oncogenic

function and Nck by preventing PERK activation disrupts the equilibrium towards oncogenic transformation. Nevertheless, recent studies where PERK deficient mice and transgenic animals bearing ser51 mutation on eIF2α were generated, revealed an implication of PERK in the glucose metabolism rather than cell transformation (23, 24). Indeed, PERK-deficient mice developed marked hyperglycemia at 4 weeks of age, whereas eIF2α mutated on ser51 mice were normal at birth but died of severe hypoglycemia several hours later. Both mutant strains had defects in pancreatic β cells, especially in Ser51 mutant animals. The difference between the two types of animal models suggests that more than one type of eIF2α kinase may be operating in the insulin-producing pancreatic cells. The common feature of the two mice is the loss of pancreatic β cells, which undergo apoptosis, especially in PERK-deficient mice. Despite a defect in glucose metabolism and diabetes phenotypes, these animal models however did not demonstrate any tumor development.

Other cDNA clones, whose protein products interacted with the SH3 domains of Nck, were isolated from the yeast two-hybrid screen, where we initially detected eIF2β interaction with Nck. Among those, one coded for δ subunit of the protein phosphatase 2A (PP2A) (L. Larose, personal communication). Several observations support a role for PP2A in tumorigenesis. PP2A is required for the transforming activity of small T and middle T antigens (25, 26). Deregulation of PP2A leads to increased cell motility and invasiveness in endothelial cells through disorganization of actin and microtubules (27-29).

Especially, mutations in B56 subunits promote cell invasiveness and neoplastic progression (28). In this aspect, inappropriate regulation of the PP2A activity or its sequestration in an inadequate subcellular compartment may lead to tumorigenesis. Therefore, Nck-1 may promote oncogenesis effects by modulating PP2A activity and/or subcellular localization. Moreover, our results in chapter III showed a calyculinA sensitive phosphatase activity (PP1 and/or PP2A) associated with the Nck effect on PERK activation. Altogether my data demonstrate that the Nck expression level is critical for the welfare of the cell, especially under ER stress conditions. It is believed that apoptosis represents the main mechanism by which altered/deregulated cells are killed. In this aspect, Nck may play a role of a suicide gene when its expression bypasses normal physiological levels and interestingly, this could be tissue specific. Confirming this hypothesis would open up interesting strategies of cancer therapy.

In a collaborative study, we showed that Nck is in a complex with IRE1α and STAT-5 (30). IRE1α is an ER resident protein and STAT-5, a cytoplasmic transcription factor that mediates cellular responses to diverse cytokines and growth factors (reviewed in 31-33). Recent studies demonstrated that regulation of apoptotic pathways by STATs is largely due to transcriptional activation of genes that encode protein regulators of cell death process, such as Bcl-xL, caspases, Fas and TRAIL (reviewed in 34). Interestingly, STAT proteins may also regulate apoptosis through a non-transcriptional mechanism by inhibiting the anti-apoptotic protein NF-κB (reviewed in 34). In addition, ER stress conditions may

also activate JNKs (35). Indeed, lysates from rat pancreatic cells treated with thapsigargin, tunicamycin or DTT exhibited increased JNK activity (36). Overexpression of IRE1 leads to JNK activation, and IRE1$\alpha^{-/-}$ fibroblasts were impaired in JNK activation by ER stress. The cytoplasmic part of IRE1 binds TNF receptor-associated factor-2 (TRAF2), an adapter protein that couples plasma membrane receptors like TNF receptor to JNK activation (36). Interestingly, the study of Nguyen et al. demonstrated that, under ER stress conditions, Nck mediates the JNK activation pathway but not p38 (30). Consistent with these results we can hypothesize that under ER stress conditions the complex STAT-5/IRE1α/Nck, may inhibit the NF-κB-mediated anti-apoptotic signal, thus triggering programmed cell death.

Overall this work reveals novel mechanisms on how adapter proteins may promote late signaling events triggered by RTKs. My studies reported a role for the SH2/SH3 adapter protein Nck in regulating crucial cell functions, solely through protein interactions mediated by its SH3 domains, independently of its SH2 domain. These could lead to the establishment of new endeavors and discoveries in the fields of neurobiology, diabetes and cancer therapy.

Future Perspectives

For the first time, an SH2/SH3 domain containing protein involved in tumorigenesis, development, proliferation, differentiation and cytoskeleton rearrangement, is shown to play an important role in the modulation of protein translation by interacting with the translational key molecule eIF2β. Interestingly, Nck positive effects on protein synthesis are maintained under ER stress, a condition known to inhibit translation. Nck antagonizes ER stress-induced inhibition of translation, by preventing activation of the eIF2α kinase, PERK, thereby maintaining eIF2α in a non-phosphorylated state. These effects of Nck appear to be mediated by a CalyculinA-sensitive phosphatase. Therefore, It would be interesting to identify this phosphatase and to determine its relation with Nck, PERK and ER stress conditions.

It has been shown that mice lacking PERK or bearing an eIF2α mutated on Ser51 impair glucose metabolism, by developing a hyperglycemia and hypoglycemia, respectively (23, 24). In addition, these mice showed an elevated pancreatic β cell death. PERK is known to be highly expressed in pancreas. It would be interesting to study in this organ 1) whether Nck levels varies in certain conditions such as diabetes and 2) whether PERK activity is affected by Nck levels.

Finally, The role of Nck in axonal guidance and targeting as well as in protein synthesis, may suggest that these processes are related through Nck in neuronal cells. In order to demonstrate this hypothesis, mapping the site of interaction on eIF2β with Nck and preventing the association of these molecules *in vivo* would inform us whether Nck-dependent protein translation is required for actin cytoskeleton rearrangement observed in axonal guidance and targeting.

References

1. Li, W., Fan, J., and Woodley, D. T. (2001) Nck/Dock: an adapter between cell surface receptors and the actin cytoskeleton. *Oncogene* 20, 6403-6417

2. Li, W., and She, H. (2000) The SH2 and SH3 adapter Nck: a two-gene family and a linker between tyrosine kinases and multiple signaling networks [In Process Citation]. *Histol Histopathol* 15, 947-955

3. McCarty, J. H. (1998) The Nck SH2/SH3 adaptor protein: a regulator of multiple intracellular signal transduction events. *Bioessays* 20, 913-921.

4. Desai, C. J., Garrity, P. A., Keshishian, H., Zipursky, S. L., and Zinn, K. (1999) The Drosophila SH2-SH3 adapter protein Dock is expressed in embryonic axons and facilitates synapse formation by the RP3 motoneuron. *Development* 126, 1527-1535

5. Garrity, P. A., Rao, Y., Salecker, I., McGlade, J., Pawson, T., and Zipursky, S. L. (1996) Drosophila photoreceptor axon guidance and targeting requires the dreadlocks SH2/SH3 adapter protein. *Cell* 85, 639-650

6. Buday, L., Wunderlich, L., and Tamas, P. (2002) The Nck family of adapter proteins. Regulators of actin cytoskeleton. *Cell Signal* 14, 723-731

7. Crino, P. B., and Eberwine, J. (1996) Molecular characterization of the dendritic growth cone: regulated mRNA transport and local protein synthesis. *Neuron* 17, 1173-1187

8. Aakalu, G., Smith, W. B., Nguyen, N., Jiang, C., and Schuman, E. M. (2001) Dynamic visualization of local protein synthesis in hippocampal neurons. *Neuron* 30, 489-502

9. Torre, E. R., and Steward, O. (1996) Protein synthesis within dendrites: glycosylation of newly synthesized proteins in dendrites of hippocampal neurons in culture. *J Neurosci* 16, 5967-5978

10. Kacharmina, J. E., Job, C., Crino, P., and Eberwine, J. (2000) Stimulation of glutamate receptor protein synthesis and membrane insertion within isolated neuronal dendrites. *Proc Natl Acad Sci U S A* 97, 11545-11550

11. Campbell, D. S., and Holt, C. E. (2001) Chemotropic responses of retinal growth cones mediated by rapid local protein synthesis and degradation. *Neuron* 32, 1013-1026

12. Li, X., Meriane, M., Triki, I., Shekarabi, M., Kennedy, T. E., Larose, L., and Lamarche-Vane, N. (2002) The adaptor protein Nck-1 couples the netrin-1 receptor DCC (deleted in colorectal cancer) to the activation of the small GTPase Rac1 through an atypical mechanism. *J Biol Chem* 30, 30

13. Miyagi, Y., Sugiyama, A., Asai, A., Okazaki, T., Kuchino, Y., and Kerr, S. J. (1995) Elevated levels of eukaryotic translation initiation factor eIF-4E, mRNA in a broad spectrum of transformed cell lines. *Cancer Lett* 91, 247-252

14. Anthony, B., Carter, P., and De Benedetti, A. (1996) Overexpression of the proto-oncogene/translation factor 4E in breast-carcinoma cell lines. *Int J Cancer* 65, 858-863

15. Nathan, C. A., Liu, L., Li, B. D., Abreo, F. W., Nandy, I., and De Benedetti, A. (1997) Detection of the proto-oncogene eIF4E in surgical margins may predict recurrence in head and neck cancer. *Oncogene* 15, 579-584.

16. 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

17. Wang, S., Rosenwald, I. B., Hutzler, M. J., Pihan, G. A., Savas, L., Chen, J. J., and Woda, B. A. (1999) Expression of the eukaryotic translation initiation factors 4E and 2alpha in non-Hodgkin's lymphomas. *Am J Pathol* 155, 247-255

18. 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

19. Kim, S. H., Forman, A. P., Mathews, M. B., and Gunnery, S. (2000) Human breast cancer cells contain elevated levels and activity of the protein kinase, PKR. *Oncogene* 19, 3086-3094

20. Koromilas, A. E., Roy, S., Barber, G. N., Katze, M. G., and Sonenberg, N. (1992) Malignant transformation by a mutant of the IFN-inducible dsRNA-dependent protein kinase. *Science* 257, 1685-1689.

21. Meurs, E. F., Galabru, J., Barber, G. N., Katze, M. G., and Hovanessian, A. G. (1993) Tumor suppressor function of the interferon-induced double-stranded RNA-activated protein kinase. *Proc Natl Acad Sci U S A* 90, 232-236

22. Donze, O., Jagus, R., Koromilas, A. E., Hershey, J. W., and Sonenberg, N. (1995) Abrogation of translation initiation factor eIF-2 phosphorylation causes malignant transformation of NIH 3T3 cells. *Embo J* 14, 3828-3834

23. Harding, H. P., Zeng, H., Zhang, Y., Jungries, R., Chung, P., Plesken, H., Sabatini, D. D., and Ron, D. (2001) Diabetes mellitus and exocrine pancreatic dysfunction in perk-/- mice reveals a role for translational control in secretory cell survival. *Mol Cell* 7, 1153-1163

24. Scheuner, D., Song, B., McEwen, E., Liu, C., Laybutt, R., Gillespie, P., Saunders, T., Bonner-Weir, S., and Kaufman, R. J. (2001) Translational control is required for the unfolded protein response and in vivo glucose homeostasis. *Mol Cell* 7, 1165-1176

25. Mungre, S., Enderle, K., Turk, B., Porras, A., Wu, Y. Q., Mumby, M. C., and Rundell, K. (1994) Mutations which affect the inhibition of protein phosphatase 2A by simian virus 40 small-t antigen in vitro decrease viral transformation. *J Virol* 68, 1675-1681.

26. Campbell, K. S., Auger, K. R., Hemmings, B. A., Roberts, T. M., and Pallas, D. C. (1995) Identification of regions in polyomavirus middle T and small t antigens important for association with protein phosphatase 2A. *J Virol* 69, 3721-3728.

27. Gabel, S., Benefield, J., Meisinger, J., Petruzzelli, G. J., and Young, M. (1999) Protein phosphatases 1 and 2A maintain endothelial cells in a resting state, limiting the motility that is needed for the morphogenic process of angiogenesis. *Otolaryngol Head Neck Surg* 121, 463-468.

28. Ito, A., Kataoka, T. R., Watanabe, M., Nishiyama, K., Mazaki, Y., Sabe, H., Kitamura, Y., and Nojima, H. (2000) A truncated isoform of the PP2A B56 subunit promotes cell motility through paxillin phosphorylation. *Embo J* 19, 562-571.

29. Jackson, J., Meisinger, J., Patel, S., Lim, Z. C., Vellody, K., Metz, R., and Young, M. R. (1997) Protein phosphatase-2A associates with the cytoskeleton to maintain cell spreading and reduced motility of nonmetastatic Lewis lung carcinoma cells: the loss of this regulatory control in metastatic cells. *Invasion Metastasis* 17, 199-209

30. Nguyen, D. T., Jenna, S., Fazel, A., Kebache, S., Emadali, A., Wong, H. N., Larose, L., Bergeron, J. J., Kaufman, R. J., and Chevet, E. (2002) Mechanism of Regulation of Endoplasmic Reticulum Stress Response by STAT-5 and Nck downstream of IRE Alpha. *(submitted)*

31. Darnell, J. E., Jr. (1997) STATs and gene regulation. *Science* 277, 1630-1635.

32. Kaufman, R. J. (1999) Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev* 13, 1211-1233.

33. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H., and Schreiber, R. D. (1998) How cells respond to interferons. *Annu Rev Biochem* 67, 227-264

34. Battle, T. E., and Frank, D. A. (2002) The role of STATs in apoptosis. *Curr Mol Med* 2, 381-392.

35. Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J., and Woodgett, J. R. (1994) The stress-activated protein kinase subfamily of c-Jun kinases. *Nature* 369, 156-160.

36. Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H. P., and Ron, D. (2000) Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* 287, 664-666

CHAPTER V

Contributions to Original Research

1) In the work reported in Chapter Two, I characterized *in vivo* a novel interaction between the SH2/SH3 domains-containing adapter molecule Nck-1 and the eukaryotic initiation factor 2 β (eIF2β) by using pull down and coimmunoprecipitation experiments. In addition, by sucrose cushion sedimentation method, I showed that these two molecules colocalized in ribosomal enriched fractions. Moreover, using cell fractionation experiments, I demonstrated that this localization is enhanced upon growth factor stimulation. Interestingly, using a bicistronic luciferase reporter vector, I showed that overexpression of Nck enhances protein translation significantly, and this effect is mediated by the same Nck SH3 domains shown to interact with eIF2β.

2) In the work reported in Chapter Three, using the same bicistronic luciferase reporter vector used in Chapter Two, I demonstrated that Nck-1 effects on protein translation are maintained under ER stress conditions, where protein translation is normally inhibited. I also showed under these conditions by immunoblotting analysis that Nck-1 overexpression prevents activation of PERK and eIF2α phosphorylation. Morevover, under the same conditions, the use of a phosphatase inhibitor reversed the effects of Nck-1 overexpression on protein translation, PERK activation and eIF2α phosphorylation, thus demonstrating a phosphatase-dependent mechanism associated with Nck-1. Interestingly, I showed in Nck-deficient cells that Nck is required to sustain optimal protein translation levels.

APPENDIX I

Reprint

Modulation of protein translation by Nck-1

Sem Kebache*, Dongmei Zuo*, Eric Chevet†, and Louise Larose*‡

Departments of *Medicine and †Surgery, Polypeptide Laboratory, Division of Endocrinology, McGill University, Montreal, QC, Canada H3A 2B2

Edited by Lewis Clayton Cantley, Beth Israel Deaconess Medical Center, Boston, MA, and approved February 12, 2002 (received for review September 13, 2001)

In mammals, Nck represented by two genes, is a 47-kDa SH2/SH3 domain-containing protein lacking intrinsic enzymatic function. Here, we reported that the first and the third SH3 domains of Nck-1 interact with the C-terminal region of the β subunit of the eukaryotic initiation factor 2 (eIF2β). Binding of eIF2β was specific to the SH3 domains of Nck-1, and *in vivo*, the interaction Nck/eIF2β was demonstrated by reciprocal coimmunoprecipitations. In addition, Nck was detected in a molecular complex with eIF2β in an enriched ribosomal fraction, whereas no other SH2/SH3 domain-containing adapters were found. Cell fractionation studies demonstrated that the presence of Nck in purified ribosomal fractions was enhanced after insulin stimulation, suggesting that growth factors dynamically regulate translocation of Nck to ribosomes. In HEK293 cells, we observed that transient overexpression of Nck-1 significantly enhanced Cap-dependent and -independent protein translation. This effect of Nck-1 required the integrity of its first and third SH3 domains originally found to interact with eIF2β. Finally, *in vitro*, Nck-1 also increased protein translation, revealing a direct role for Nck-1 in this process. Our study demonstrates that in addition to mediate receptor tyrosine kinase signaling, Nck-1 modulates protein translation potentially through its direct interaction with an intrinsic component of the protein translation machinery.

Translation initiation is a complex process in which initiator tRNA (Met-tRNA$_i$) and the 40S and 60S ribosomal subunits of initiator tRNA (Met-tRNA$_i$) are assembled into 80S ribosomes at the initiation codon of mRNA by the coordinated action of the eukaryotic initiation factors (eIFs). Thus far, signaling-dependent events regulating eIFs have involved changes in their intrinsic activity or protein-interacting properties, resulting from their phosphorylation/dephosphorylation and/or sequestration into inactive complex. For example, eIF2 is a molecular complex of three subunits (α, β, and γ) responsible for one of the earliest steps in the initiation of protein synthesis (1). eIF2 forms a ternary complex with the Met-tRNA and GTP and, in collaboration with other initiation factors, binds the 40S ribosomal subunit to give rise to the preinitiation 43S complex (2). Inhibition of protein synthesis correlates with the phosphorylation of the eIF2α subunit (1) by the heme-regulated eIF2α kinase (HRI) (3), the IFN-inducible RNA-dependent protein kinase (PKR) (4), the serum starvation kinase (GCN2) (5), and the endoplasmic reticulum stress kinase (PERK) (6). Phosphorylation of eIF2α on Ser-51 by these eIF2α kinases inhibits the early steps of translation by blocking on eIF2 the exchange of GDP for GTP, a reaction under the control of the guanine exchange factor, eIF2B (7, 8). Thus, growth factors may enhance initiation of protein translation by preventing activation of eIF2α kinases or by activating specific phosphatases to maintain low levels of eIF2α phosphorylation. In contrast to eIF2α, the β and γ subunits of eIF2 known to interact with critical components of the translational initiation machinery such as eIF2B, eIF5, mRNA, GTP, and Met-tRNA (9–11), have not yet been reported to be regulated by signaling molecules.

To date, with the exception of the p85 adapter subunit of phosphatidylinositol 3′-kinase (PI-3K), no SH2/SH3 domain-containing adapters have been directly implicated in the regulation of the initiation of protein synthesis by growth factors. Here, we demonstrate a direct interaction between the adapter protein Nck-1 and the translation initiation factor subunit eIF2β in an enriched ribosomal fraction. Given these observations and the striking finding that the presence of Nck in ribosomes was increased upon insulin treatment, we investigated the role of Nck-1 in protein translation.

Materials and Methods

Antibodies. Nck antiserum was prepared as described (12). eIF2β antisera were obtained after rabbit immunization with a glutathione *S*-transferase (GST) chimera of the C-terminal region of mouse eIF2β (residues 133–333). L4 antibody was provided by C. Nicchitta (Duke University Medical Center, Durham, NC). p85, CrkII, Grb2, and hemagglutinin (HA) antibodies were purchased from Santa Cruz Biotechnology.

Cell Culture. Transformed rat hepatocytes overexpressing the human insulin receptor (HTC-IR) cells were grown in DMEM (Invitrogen) containing 10% FBS (Invitrogen) and Geneticin (G418) at 40 μg/ml (Invitrogen). Human embryonic kidney 293 (HEK293) cells were grown in DMEM containing 10% FBS.

Yeast Two-Hybrid Assays. Yeast two-hybrid screen was performed as described (13). pACTII vector encoding the transcriptional activation domain of Gal4 fused to mouse T cell cDNA library fragments were introduced into *Saccharomyces cerevisiae* strains already transfected with the pASI vector encoding a fusion between the DNA-binding domain of Gal4 and the three SH3 domains of Nck-1 (residues 1–251).

Immunoprecipitation. Mouse tissues were homogenized in 5 mM Tris, pH 7.4/1 mM $MgCl_2$/0.25 M sucrose/2 mM $NaVO_4$/1 mM PMSF/1 mM benzamidine/1 mM NaF and centrifuged at 200,000 × *g* for 30 min. The resulting supernatants were submitted to Nck or eIF2β immunoprecipitation. HTC-IR lysates were prepared as described (12). Clarified lysates were submitted to either Nck or eIF2β immunoprecipitation and analyzed by immunoblotting.

Nck-1 Constructs and Transfection. Human Nck-1 mutants were produced by overlapping PCR with specific primers containing appropriate mutated sites (14–16), subcloned into pcDNA 3.1Myc/His plasmid (Invitrogen) and sequenced. The HA-tagged Nck-1 constructs subcloned into pRK5 were provided by W. Li (Norris Cancer Center, University of Southern California, Los Angeles). Transient transfections of HEK293 cells were performed by using calcium phosphate precipitation.

GST Fusion Proteins. Wild-type Nck-1 and eIF2β cDNAs were subcloned into pGEX-2TK and GST fusion proteins were expressed, purified as recommended by the manufacturer (Amer-

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: eIF, eukaryotic initiation factor; PI-3K, phosphatidylinositol 3′-kinase; SH, Src homology domain; IRES, internal ribosomal entry site; NRS, normal rabbit serum; TCL, total cell lysate; IP, immunoprecipitation; WB, immunoblotting; GST, glutathione *S*-transferase; HA, hemagglutinin.

‡To whom reprint requests should be addressed. E-mail: louise.larose@mcgill.ca.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

www.pnas.org/cgi/doi/10.1073/pnas.082483399

sham Pharmacia), and used for binding assay experiments. GST and GST-Nck-1 were eluted from the glutathione beads before their use in overlay assays or in the *in vitro* translation system.

Overlay Assay. Recombinant GST and GST-Nck-1 proteins (5 μg) were spotted onto nitrocellulose and dried. Membranes were blocked in Tris-buffered saline containing 0.05% Tween-20 (TBST) and 10% nonfat dry milk and probed overnight at 4°C with ^{32}P-labeled GST-eIF2β. Membranes were washed in TBST and exposed for autoradiography.

Binding Assay. HEK293 cells transiently transfected with empty vector or plasmid encoding either HA-tagged Nck-1 wild type or mutated in its individual SH3 domains were lysed (10 mM Hepes, pH7.4/1% (vol/vol) Triton X-100/10 μg/ml aprotinin/10 μg/ml leupeptin/1 mM PMSF). After preclearing with glutathione immobilized on beads, lysates were mixed with 25 μg of GST or GST-eIF2β recombinant protein for 2 h at 4°C. Samples were analyzed by immunoblotting with Nck (Transduction Laboratories, Lexington, KY) or HA antibodies.

Sucrose Cushion Sedimentation. Sucrose cushion sedimentation on confluent HTC-IR and HEK293 cells were performed as described (17). Supernatants were trichloroacetic acid-precipitated and resuspended in Laemmli buffer (18) or subjected to immunoprecipitation with either Nck, eIF2β antibodies, or normal rabbit serum (NRS). Pellets were resuspended in Laemmli buffer or solubilized with 30 mM Tris·HCl, pH 7.5/150 mM NaCl/1% Triton-X-100 containing protease inhibitors and heated at 65°C for 10 min to facilitate protein resuspension before immunoprecipitation. Samples were analyzed by immunoblotting with either Nck, eIF2β, p85, Grb2, CrkII, or L4 antibodies.

Subcellular Fractionation. Insulin-stimulated and nonstimulated HEK293 cell lysates were prepared and layered onto a 15–35% linear sucrose gradient as described (19). Fractions were analyzed by immunoblotting with either Nck, eIF2β, p85, or L4 antibodies.

Luciferase Assay. The bicistronic reporter plasmid pcDNA3-RLUC-POLIRES-FLUC was kindly provided by N. Sonenberg (McGill University, Montreal) (20). HEK293 cells were transiently transfected with 0.5 μg of the reporter vector and either 0.5 μg of plasmids encoding various Myc-Nck-1 molecules (Fig. 1*A*). *Renilla reniformis* luciferase (RLUC) and firefly luciferase (FLUC) activities were measured by using a dual-luciferase reporter assay system (Promega) in a luminometer (LUMAT) 36 h after transfection.

***In Vitro* Transcription and Translation Assay.** Linearized pcDNA3-RLUC-POLIRES-FLUC vector was *in vitro* transcribed and translated in the TNT-coupled wheat germ Extract System (Promega) supplemented with [^{35}S]methionine and increasing amounts of recombinant GST-Nck-1. Samples were prepared for [^{35}S]methionine incorporation assay according to the manufacturer's instructions (Promega) and quantified by scintillation counting.

Quantitative RNA Analysis. Trizol (Invitrogen)-prepared RNAs (5 μg) from HEK293 cells transiently cotransfected with the luciferase reporter vector and other various Myc-Nck-1 plasmids (Fig. 1*A*) were converted into single-stranded cDNAs (SuperScript II kit, Invitrogen) followed by PCR reactions with the primers FLUC: 5′-TACAATTTGGACTTTCCGCC-3′and 5′-TTCTTCGCCAAAAGCACTCT-3′, RLUC: 5′-AACGCG-GCCTCTTCTTATTT-3′, and 5′-TATCAGGCCATTCATC-CCAT-3′ and classic 18S internal standard primers

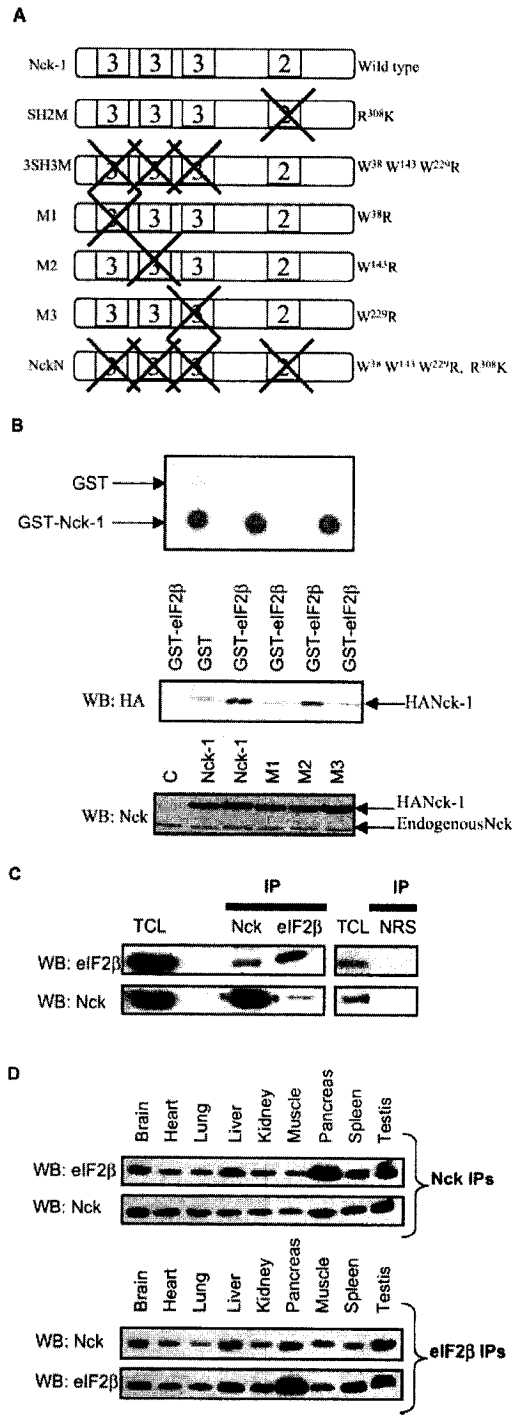


Fig. 1. Nck and eIF2β interaction. (*A*) Schematic representation of Nck-1 molecules. X represents functional mutation of the SH domain. (*B*) Overlay assay (*Top*). Autoradiography of GST or GST-Nck-1 (5 μg) triplicates immobilized on nitrocellulose and overlaid with ^{32}P-labeled GST-eIF2β. Binding assay (*Middle* and *Bottom*). *In vitro* binding by GST or GST-eIF2β (25 μg) of HA-Nck-1 molecules transiently expressed in HEK293 cells (*Middle*). Expression of endogenous and HA-tagged Nck-1 (*Bottom*). (*C*) HTC-IR cell lysates subjected to Nck, eIF2β, or NRS immunoprecipitation. (*D*) Tissue homogenates from adult male BalbC mice. Four milligrams of proteins used for coimmunoprecipitations and 1 mg for protein expression. Results are typical of two (tissues) and three (cultured cells) experiments.

Table 1. Yeast two-hybrid assay

Molecules	Interaction with eIF2β
Nck 3SH3	+
Nck SH3-1	+
Nck SH3-2	−
Nck SH3-3	+
Abl	−
Grb2 SH3-N	−
Grb2 SH3-C	−
CrkII SH3-C	−
PLCγ-1 SH3	−

Individual Nck SH3 domains and SH3 domains of Grb2, CrkII, p85 of PI-3K, Abl, and PLCγ-1 were used in yeast two-hybrid assays to characterize their interaction with eIF2β. The 3SH3 construct is the region of Nck comprising the three Src homology 3 domains (residues 1–251). The SH3-1 (residues 1–56), SH3-2 (residues 113–160) and SH3-3 (residues 197–251) constructs denote the first or second or third SH3 domain of Nck, respectively. SH3-C and SH3-N denote the C-terminal and N-terminal SH3 domain of Grb2, respectively.

(QuantumRNA, Ambion, Austin, TX). PCR reactions were supplemented with 10 μCi of 3000 Ci/nM of [α-^{32}P]dATP. Size markers were prepared by 5′ end-labeling of 1-kb ladder marker (Invitrogen). Samples were submitted to electrophoresis on agarose, transferred onto nylon membranes, and analyzed by autoradiography; reciprocal bands were quantified by densitometry.

Results

Proteins Interacting with the SH3 Domains of Nck-1. Two clones encoding a fusion protein containing the C-terminal region of the β subunit of the eukaryotic initiation factor 2 (eIF2β) were identified interacting with the SH3 domains of Nck-1 in the yeast two-hybrid system. Further characterization of this interaction in the same system revealed that the first and the third SH3 domains of Nck-1 were positive, but neither the second SH3 domain of Nck-1 nor the SH3 domains of Abl, Grb2, CrkII or PLCγ-1 interacted with eIF2β (Table 1). By overlay assays, we observed a direct interaction of GST-eIF2β with GST-Nck-1 (Fig. 1*B Top*). Furthermore, *in vitro* binding assays (Fig. 1*B Middle* and *Bottom*) also showed that Nck-1 interacts with eIF2β and that this interaction required the first and the third SH3 domains of Nck-1.

***In Vivo* Nck/eIF2β Interaction.** Reciprocal coimmunoprecipitations with lysates from cultured cells (Fig. 1*C*) and mouse tissue homogenates (Fig. 1*D*) were performed. As reported by others for Nck (21, 22) and expected for eIF2β, the two proteins are expressed in all tissues with high levels of expression in brain, pancreas, spleen, and testis (Fig. 1*D*). Furthermore, Nck and eIF2β coimmunoprecipitated in HTC-IR (Fig. 1*C*), HEK293 (data not shown) cell lysates, and tissue homogenates (Fig. 1*D*), demonstrating *in vivo* their interaction.

Nck and eIF2β Colocalize in a Ribosome-Enriched Fraction. By using the sucrose cushion sedimentation, a ribosome-enriched pellet (P) and supernatant containing soluble cytosolic components (S) were generated from HEK293 and HTC-IR cells. In both cell lines eIF2β, as expected, and Nck are detected in the enriched ribosomal fraction (Fig. 2*A*). Other SH2/SH3 domain-containing adapter molecules such as Grb2, CrkII (Fig. 2*A Lower*) or the p85 subunit of PI-3K (data not shown) were not detected in the ribosomal-enriched fraction. As control, immunoblot for the ribosomal protein L4 showed that the pellets were enriched in ribosomes (Fig. 2*A*).

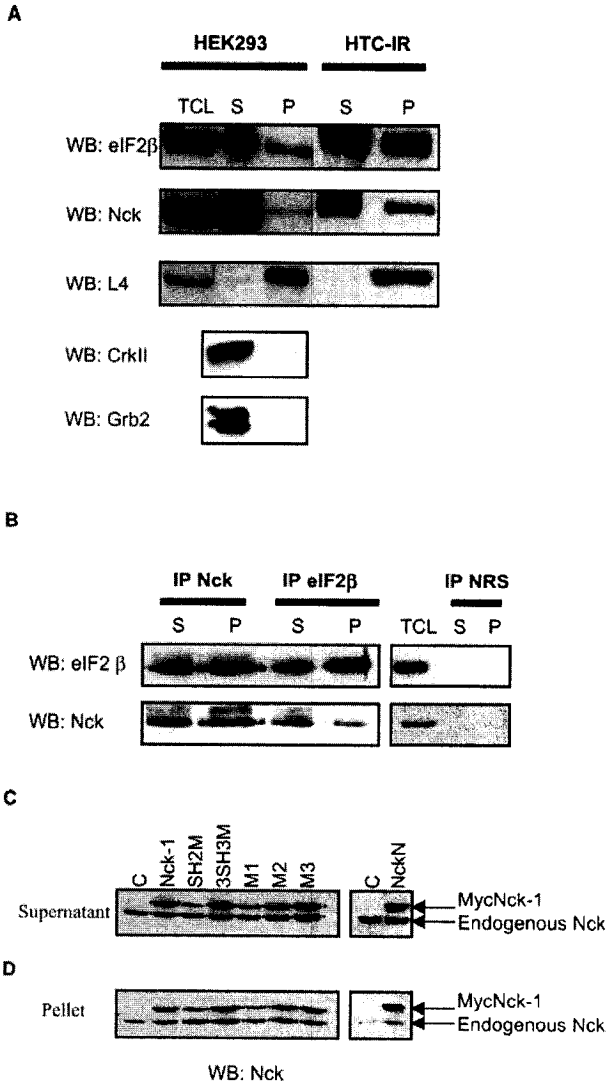


Fig. 2. Nck is detected and interacts with eIF2β in an enriched ribosomal fraction, and Nck ribosomal localization is independent of its functional SH domains. Sucrose cushion sedimentation. (*A*) Immunoblot analysis of an aliquot of the supernatant (S) and the entire pellet (P). (*B*) Sucrose cushion supernatants (S) and resuspended pellets (P) were immunoprecipitated in duplicate by using Nck, eIF2β antibodies, or NRS. One tenth of the immunoprecipitated supernatant and the entire immunoprecipitated pellet were used for immunoblotting. Results are representative of three independent experiments. (*C* and *D*) Immunoblot analysis of one tenth of the supernatants (*C*) and the total pellets (*D*) from HEK293 cells transiently transfected with various Myc-Nck-1 constructs. Results shown are typical of two independent experiments.

Nck and eIF2β Coimmunoprecipitate from the Ribosome-Enriched Fraction. To determine whether Nck and eIF2β interact in the ribosomal-enriched fraction, endogenous Nck and eIF2β were coimmunoprecipited by using supernatant and pellet fractions after sucrose cushion sedimentation. As shown in Fig. 2*B*, Nck was detected in eIF2β immunoprecipitates in pellet and supernatant fractions, and reciprocal results were also obtained for eIF2β in Nck immunoprecipitates. Taken together, these results demonstrate that Nck and eIF2β are part of the same molecular complex in this compartment.

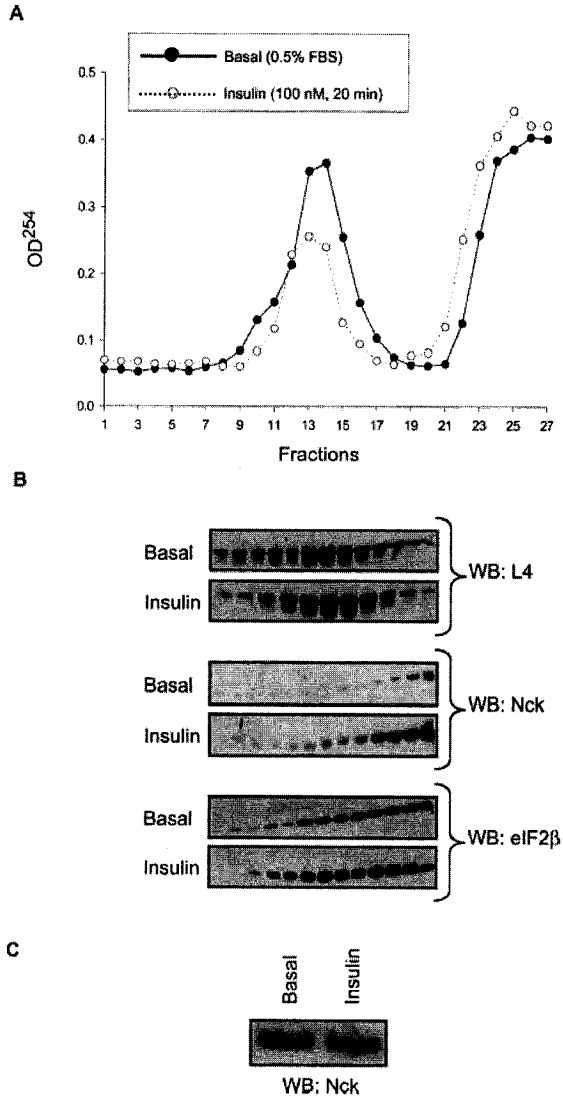


Fig. 3. Translocation of Nck to ribosomal fractions is enhanced upon insulin stimulation. Equal amounts of proteins from insulin-stimulated and nonstimulated HEK293 cells were separated 15–35% linear sucrose gradients. (*A*) Ribosomal profile. Optical density at 254nm representing the RNA content of each fractions. (*B*) Nck and eIF2β detected in ribosomal fractions. Immunoblot analysis of each fraction. Membranes were cut and the lower part probed for the ribosomal protein L4, whereas the upper part was probed first for Nck, then stripped and reprobed for eIF2β. (*C*) Total Nck levels upon insulin stimulation. Nck immunoblot analysis. Results are representative of three independent experiments.

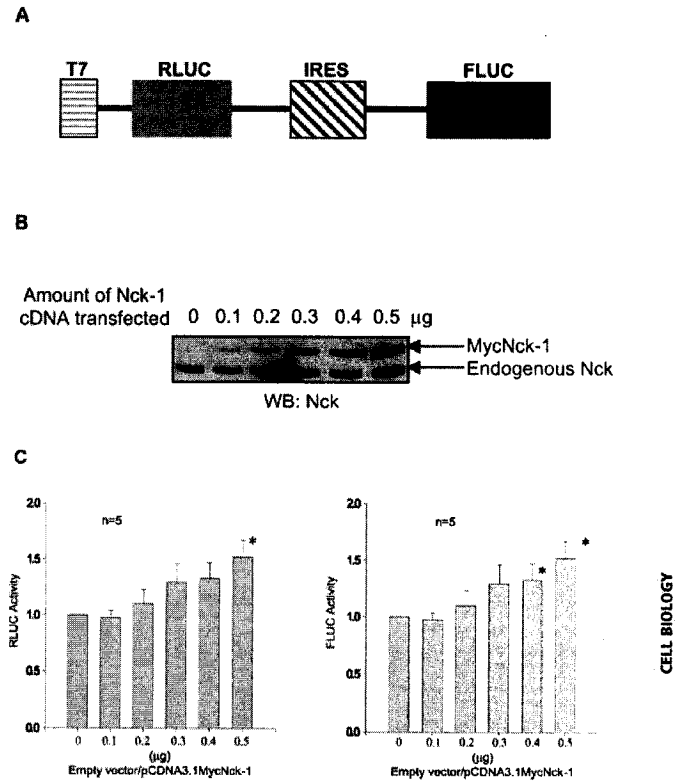


Fig. 4. *In vivo*, Nck-1 enhances both Cap-dependent and -independent protein translation. (*A*) Schematic representation of the pcDNA3-RLUC-POLIRES-FLUC reporter vector. (*B*) Immunoblot analysis for the presence of endogenous Nck and Myc-Nck-1 in HEK293 cells. (*C*) Luciferase activity of the FLUC cistron (*Left*) and the RLUC cistron (*Right*) measured 36 h postransfection from HEK293 cells cotransfected with reporter vector and increasing amounts of MycNck-1 constructs. Experiments were performed five times in triplicate, and the results represent the mean value ± SEM. *, At least $P < 0.001$, as determined by Student's *t* test compared with control.

Nck Translocation to Ribosomes Is Independent of the Functional Integrity of Its SH Domains. To determine whether the SH domains of Nck were involved in mediating its ribosomal localization, enriched ribosomal fractions were prepared from HEK293 cells transiently transfected with various Myc-Nck-1 constructs (Fig. 1*A*). All Nck-1 mutants were detected in the ribosomal-enriched fraction (Fig. 2*D*), demonstrating that the localization of Nck into ribosomes is modulated by a mechanism independent of its Src homology domains.

The Presence of Nck in Purified Ribosomal Fractions Is Enhanced After Insulin Stimulation. To further characterize whether Nck localization to ribosomes is modulated by growth factor stimulation, sedimentation on linear sucrose gradients of lysates from serum-starved HEK293 cells treated or not with insulin were performed. The assignment of ribosomal-enriched components were in fractions 8 to 18 as revealed by immunoblotting for the ribosomal protein L4 (Fig. 3*B Top*). These fractions were then immunoblotted for the presence of Nck (Fig. 3*B Middle*), eIF2β (Fig. 3*B Bottom*) and p85 of PI-3K (data not shown). As reported (19), the distribution of RNA content suggested that fractions 1–8 contain the polyribosomes, whereas fractions 13–17 represent the ribosomal subunits (Fig. 3*A*). Nck association with ribosomal subunits was importantly enhanced by insulin stimulation as compared with basal state (Fig. 3*B Middle*). eIF2β was detected in both conditions, but with a slight increase upon insulin treatment. p85 of PI-3K was not detected in the ribosomal fractions under both conditions (data not shown). Finally, the increase in the amount of Nck associated with ribosomal subunits upon insulin treatment did not result from a nonspecific effect of insulin on total Nck content (Fig. 3*C*). Taken together, these results demonstrate that Nck is dynamically translocated into ribosomal compartments upon insulin stimulation.

Nck-1 Overexpression Increases Protein Translation in HEK293 Cells. We studied the effects of Nck-1 on protein translation. HEK293 cells were cotransfected with a reporter plasmid (Fig. 4*A*) (20) and increasing amounts of Myc-tagged Nck-1. DNA transfected

was kept constant at 1 μg by the addition of the empty vector. In this system, translation of RLUC is Cap-dependent, whereas translation of the FLUC cistron, directed by the poliovirus internal ribosomal entry site (IRES), is Cap-independent (23). Expression of Nck-1 correlated with the amount of Nck-1 cDNA transfected (Fig. 4*B*). Overexpression of Nck-1 enhanced the activity of both luciferases proportionally to its expression levels and reached a maximum of 1.5-fold increase at 0.5 μg of cDNA transfected (Fig. 4*C*). Higher concentrations of Nck-1 cDNA (1 and 2 μg) were detrimental to the cells (data not shown).

Nck-1 Effect on Translation Depends on the Integrity of Its First and Third SH3 Domains. We further characterized the Nck-1 effect by determining the domain(s) responsible for the enhancement of translation. Myc-tagged Nck-1 constructs cotransfected independently with the bicistronic luciferase reporter vector into HEK293 cells showed comparable expression levels (Fig. 5*A*). The stimulatory effect of Nck-1 on translation was completely abolished by functional mutation of its first, third SH3, or all SH3 domains, whereas the Nck-1 mutated in its second SH3 domain resulted in a slight increase as compared with wild-type Nck-1 (Fig. 5*B*). In the transfected cells above, we found similar amounts of both luciferase mRNAs as demonstrated by quantitative PCR and densitometric analysis (Fig. 5*C*). These data confirm that the effect of Nck-1 is on translation only and depends on the functional integrity of its first and third SH3 domains.

Nck-1 Enhances Protein Translation *in Vitro*. We assessed the *in vitro* translation of the luciferase mRNAs in the presence of increasing amounts of recombinant GST-Nck-1. As shown in Fig. 5*D*, GST-Nck-1 at 0.9 μg and 1.2 μg significantly enhanced overall translation of luciferase mRNAs by 1.5- to 2.0-fold. Results from *in vitro* translation strongly support a direct role for Nck-1 in the regulation of protein translation.

Discussion

This study reports an SH3-mediated interaction between the adapter Nck-1 and eIF2β, a component of the eIF2 complex responsible for one of the earliest steps in the initiation of protein synthesis. Here, we demonstrated that Nck and eIF2β not only interact but also colocalize in ribosomal subcellular compartments. Furthermore, we also provided evidence that in these compartments, their levels are regulated by insulin, a hormone known to stimulate protein synthesis. We observed that the ribosomal localization of Nck is independent of its Src homology domains, suggesting that a mechanism, at least independent of its interaction with eIF2β, is mediating its translocation to ribosomes. Given that Nck is reported to be phosphorylated on several residues after growth factor stimulation (24, 25), we hypothesize that such postranslational modifications may govern its translocation to ribosomal compartments.

Our study also shows that *in vivo*, Nck-1 enhances both Cap-dependent and –independent protein translation. In mammals most mRNAs are thought to be translated through a Cap-dependent mechanism involving ribosomal scanning (reviewed in ref. 26). However, a small population of mRNAs, coding for cell survival factors, cell cycle molecules, oncogenes, and viral proteins are translated by a Cap-independent mechanism mediated by direct ribosome binding to IRES elements located in the 5′-untranslated region (5′UTR) (reviewed in ref. 27). Even though this mechanism is thought not to require any translation initiation factors, assembly of the 48S complexes on these IRES elements in an *in vitro* reconstitution assay by using purified eIF2/GTP/Met-tRNA$_i$ complex is sufficient for the 40S to lock onto the initiation codon (28). Therefore Nck-1 effect on both types of translation may suggest that Nck-1, by binding eIF2β, acts at the level of the eIF2/GTP/Met-tRNA$_i$ ternary

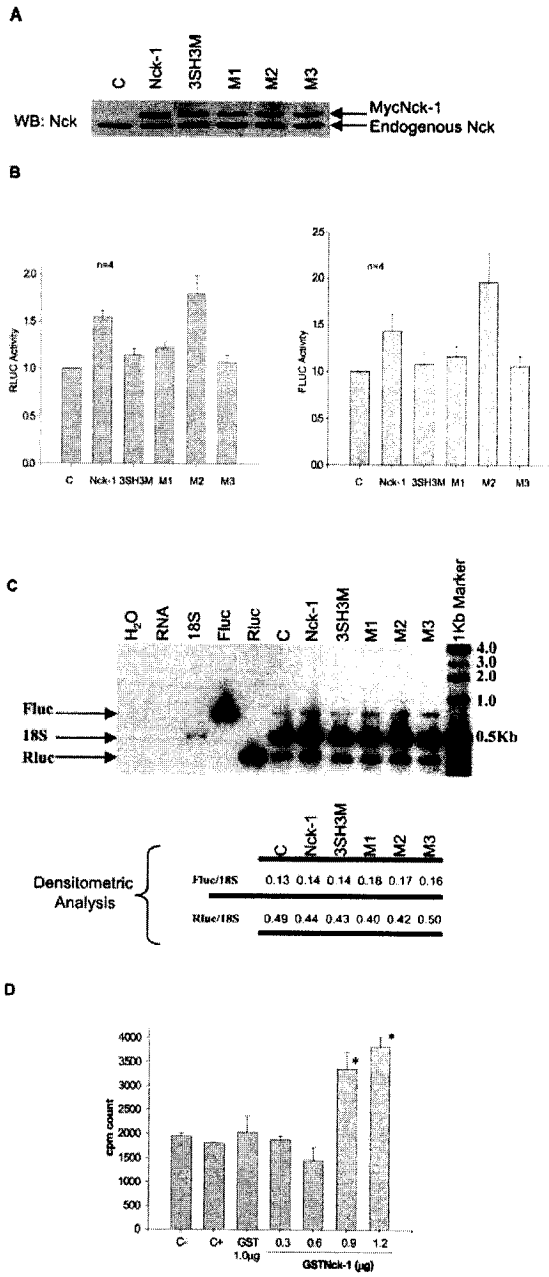


Fig. 5. The effect of Nck-1 on protein translation is dependent on the integrity of its first and third SH3 domains. HEK293 cells were cotransfected with the bicistronic luciferase reporter vector (0.5 μg) and either empty vector or the indicated Myc-Nck-1 constructs (0.5 μg). (*A*) Immunoblot analysis for the expression of endogenous Nck and Myc-Nck-1 constructs. (*B*) Activity of the RLUC cistron (*Left*) and FLUC cistron (*Right*). Each experiment was performed four times in triplicate, and the results represent the mean + SEM. (*C*) Quantitative reverse transcription–PCR performed on total RNA prepared from above transfected HEK293. Water (H_2O) or control RNA (RNA) were used as controls. Amplified products were analyzed by densitometry for the calculation of the ratios of Fluc/18S and Rluc/18S. Results shown are representative of three experiments. (*D*) *In vitro* translation of luciferase mRNAs in wheat germ extract supplemented with increasing amounts of GST-Nck-1 and incorporation of [^{35}S]methionine was measured. C− and C+ denote samples without or with bicistronic luciferase vector, respectively. Results are representative of two independent experiments performed in triplicate. *, At least $P <$ 0.01 as determined by Student's *t* test.

complex upstream of both Cap-dependent and independent initiation of translation. This hypothesis is supported not only by the fact that the SH3 domains of Nck-1 important for its effect on protein translation are also responsible for its interaction with eIF2β, but also by the direct effect on Nck-1 on *in vitro* protein translation.

The involvement of Nck in insulin signaling has been suggested by the fact that Nck-1 and Nck-2 have been shown to bind substrates of the insulin receptor (29–32). Therefore, the insulin receptor could regulate the activity of effector molecules associated with the SH3 domains of Nck. Within this perspective, Sam68, an RNA-binding protein (33), has been reported to interact with Nck (34) and to be a substrate of the insulin receptor (35). Moreover, Sam68 has been proposed to be a regulator of RNA metabolism and protein expression by modifying the mRNA stability and/or mRNA translation (36, 37). Therefore, Sam68 may contribute to the effect of Nck-1 on protein translation by directly targeting specific mRNA to ribosomes through the molecular complex mRNA-Sam68-Nck-1-eIF2β.

Nck-1 interacts with several protein kinases (24, 25). Among them, we have identified the isoform γ2 of the Casein Kinase I (CKI γ2) (12) and recently, several CKI substrates identified by an *in vitro* expression-cloning screening include RNA helicase, nucleolar protein hNOP56, hnRNP A1, and the ribosomal proteins L4, L8, and L13 (38), suggesting a possible involvement of CKI in RNA metabolism and protein translation.

In the past, the regulation of protein translation at the level of initiation by growth factors has been well studied (reviewed in ref. 39). Our contribution clearly establishes that Nck, an adapter protein known to mediate receptor tyrosine kinase signaling at the membrane level, also acts more downstream in the signaling pathways after its translocation to ribosomal compartments. Nck may constitute a target for tight regulation of protein translation at the initiation level through its interaction with eIF2β. This opens up a field of investigation, which will contribute to a better understanding of how growth factors regulate protein translation.

We thank the members of Dr. Andre Veillette's laboratories for expert technical advice. We also thank Drs. Nahum Sonenberg and Francis Poulin for the kind gift of the bicistronic vector and for technical support. We are also grateful to Dr. C. Nicchitta for the L4 antibody, Dr. W. Li for the HA-tagged Nck constructs, and Ali Fazel, Pamela Cameron, Gerald Baquiran, and Nathalie Bedard for valuable help. This work was supported by a grant from the Canadian Diabetes Association in memory of Aurele Labelle.

1. Hershey, J. W. (1991) *Annu. Rev. Biochem.* **60,** 717–755.
2. Pain, V. M. (1996) *Eur. J. Biochem.* **236,** 747–771.
3. Chen, J. J. & London, I. M. (1995) *Trends Biochem. Sci.* **20,** 105–108.
4. Proud, C. G. (1995) *Trends Biochem. Sci.* **20,** 241–246.
5. Sood, R., Porter, A. C., Olsen, D. A., Cavener, D. R. & Wek, R. C. (2000) *Genetics* **154,** 787–801.
6. Harding, H. P., Zhang, Y. & Ron, D. (1999) *Nature (London)* **397,** 271–274.
7. Rhoads, R. E. (1993) *J. Biol. Chem.* **268,** 3017–3020.
8. Cigan, A. M., Bushman, J. L., Boal, T. R. & Hinnebusch, A. G. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 5350–5354.
9. Asano, K., Krishnamoorthy, T., Phan, L., Pavitt, G. D. & Hinnebusch, A. G. (1999) *EMBO J.* **18,** 1673–1688.
10. Laurino, J. P., Thompson, G. M., Pacheco, E. & Castilho, B. A. (1999) *Mol. Cell. Biol.* **19,** 173–181.
11. Gaspar, N. J., Kinzy, T. G., Scherer, B. J., Humbelin, M., Hershey, J. W. & Merrick, W. C. (1994) *J. Biol. Chem.* **269,** 3415–3422.
12. Lussier, G. & Larose, L. (1997) *J. Biol. Chem.* **272,** 2688–2694.
13. Anafi, M., Kiefer, F., Gish, G. D., Mbamalu, G., Iscove, N. N. & Pawson, T. (1997) *J. Biol. Chem.* **272,** 27804–27811.
14. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. & Pease, L. R. (1989) *Gene* **77,** 51–59.
15. Tanaka, M., Gupta, R. & Mayer, B. J. (1995) *Mol. Cell. Biol.* **15,** 6829–6837.
16. Mayer, B. J., Jackson, P. K., Van Etten, R. A. & Baltimore, D. (1992) *Mol. Cell. Biol.* **12,** 609–618.
17. Chevet, E., Wong, H. N., Gerber, D., Cochet, C., Fazel, A., Cameron, P. H., Gushue, J. N., Thomas, D. Y. & Bergeron, J. J. (1999) *EMBO J.* **18,** 3655–3666.
18. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
19. Siomi, M. C., Zhang, Y., Siomi, H. & Dreyfuss, G. (1996) *Mol. Cell. Biol.* **16,** 3825–3832.
20. Poulin, F., Gingras, A. C., Olsen, H., Chevalier, S. & Sonenberg, N. (1998) *J. Biol. Chem.* **273,** 14002–14007.
21. Li, W., Hu, P., Skolnik, E. Y., Ullrich, A. & Schlessinger, J. (1992) *Mol. Cell. Biol.* **12,** 5824–5833.
22. Park, D. & Rhee, S. G. (1992) *Mol. Cell. Biol.* **12,** 5816–5823.
23. Pelletier, J. & Sonenberg, N. (1988) *Nature (London)* **334,** 320–325.
24. Buday, L. (1999) *Biochim. Biophys. Acta* **1422,** 187–204.
25. McCarty, J. H. (1998) *BioEssays* **20,** 913–921.
26. Pestova, T. V., Kolupaeva, V. G., Lomakin, I. B., Pilipenko, E. V., Shatsky, I. N., Agol, V. I. & Hellen, C. U. (2001) *Proc. Natl. Acad. Sci. USA* **98,** 7029–7036.
27. Kozak, M. (2001) *Mol. Cell. Biol.* **21,** 1899–1907.
28. Pestova, T. V. & Hellen, C. U. (1999) *Virology* **258,** 249–256.
29. Lee, C. H., Li, W., Nishimura, R., Zhou, M., Batzer, A. G., Myers, M. G., Jr., White, M. F., Schlessinger, J. & Skolnik, E. Y. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 11713–11717.
30. Xu, P., Jacobs, A. R. & Taylor, S. I. (1999) *J. Biol. Chem.* **274,** 15262–15270.
31. Tu, Y., Liang, L., Frank, S. J. & Wu, C. (2001) *Biochem. J.* **354,** 315–322.
32. Tang, J., Feng, G. S. & Li, W. (1997) *Oncogene* **15,** 1823–1832.
33. Huang, S. (2000) *J. Struct. Biol.* **129,** 233–240.
34. Lawe, D. C., Hahn, C. & Wong, A. J. (1997) *Oncogene* **14,** 223–231.
35. Sanchez-Margalet, V. & Najib, S. (1999) *FEBS Lett.* **455,** 307–310.
36. Morley, S. J. & Thomas, G. (1991) *Pharmacol. Ther.* **50,** 291–319.
37. Brawerman, G. (1989) *Cell* **57,** 9–10.
38. Gao, Z. H., Metherall, J. & Virshup, D. M. (2000) *Biochem. Biophys. Res. Commun.* **268,** 562–566.
39. Taha, C. & Klip, A. (1999) *J. Membr. Biol.* **169,** 1–12.

APPENDIX II

Ethics and Certificates

Nck-1antagonizes Endoplasmic Reticulum Stress-induced inhibition of translation

Sem Kebache[†], Duc Thang Nguyen[‡], Eric Chevet[‡] and Louise Larose[†]

[†]Polypeptide Hormone Laboratory, Division of Endocrinology, Department of Medicine,
[‡]Department of Surgery, McGill University, Montreal, Quebec, H3A 2B2, Canada.

PNAS Copyright Assignment and Documentation Report

Please complete both sections of this form and submit it with your manuscript.

Copyright Assignment

In order that the Proceedings of the National Academy of Sciences USA (PNAS) may disseminate the WORK to the fullest extent, the author(s) hereby irrevocably grants, assigns, conveys, and transfers exclusively to the National Academy of Sciences (USA) the copyright in the WORK identified herein (including abstracts or summaries submitted with the WORK), under all laws, treaties, and conventions throughout the world in all forms, languages, and media now or hereafter known or developed without limitation in consideration of the publication of the WORK in the PNAS.

The author(s) warrants that the WORK is original, that it has not been published, that the copyright interest has not been assigned, licensed, or otherwise transferred to another party, and that permissions have been obtained and are attached for any portions of the WORK subject to another copyright. Each author identified on the manuscript must sign this form. If any author's signature does not appear below, the signing author(s) represents and warrants that he or she signs this assignment as authorized agent(s) for and on behalf of all the authors and that this assignment is made on behalf of all the authors. The authors shall indemnify the Academy and/or its successors and assigns for any and all claims, costs and expenses, including attorney's fees, arising out of any breach of this warranty or other representations contained herein. This assignment is subject to the following conditions:

The author(s) reserves all proprietary rights, other than copyright, such as patent rights.

The author(s) reserves the right, after publication of the work in PNAS, to use all or part of the WORK in compilations or other publications of the author's own works, and to make copies of all or a part of the WORK for the author's use for lectures, classroom instruction, or similar uses.

If the WORK has been prepared by an employee within the scope of his or her employment, the employer reserves the right to make copies of the WORK for its own internal use.

Title of WORK

Author's Signature 11/11/02 Date

Print Name

Author's Signature Date

Print Name

Author's Signature 11/10/02 Date

Print Name

Author's Signature 11/15/02 Date

Print Name 12/6/02

Failure to complete and return **both** the Copyright Assignment and the Documentation Report will delay or prevent publication of the WORK.

Documentation Report

If any of the following items are pertinent to your paper, we must receive the appropriate documentation or statements ***before*** we can publish your paper.

Please check the appropriate boxes and sign below:

☒ **None of the 3 items below applies to this paper.**

☐ **Structural Coordinate Deposition and Release.** Authors of papers reporting new or revised structures must deposit their coordinates in the Protein Data Bank or an equivalent public archive, and the accession numbers must be supplied for publication. **You agree, by signing below, that the coordinates will be released *when the article is published.*** A footnote reporting the accession numbers will be added to your paper. You must provide the accession numbers on the page proof.

☐ **Sequence Database Deposition.** Authors of papers reporting new sequences must submit these data to an appropriate database, and the accession numbers must be supplied for publication. A footnote reporting the accession numbers will be added to your paper. You must provide the accession numbers on the page proof.

☐ **Permission to Print Previously Published Material.** If your paper includes material (e.g., figures or tables) that were published previously, whether or not you are an author of the earlier publication, you must provide the previous publisher's permission to republish this material ***in print and online.*** We acknowledge that copyright is held by the original publisher. Please state which items were published previously and where:

Author's Signature Date

Print Name

If WORK is a Work Made for Hire and the employer of the author(s) owns the copyright, this assignment must be signed by an authorized representative of the employer.

Name of Employer

Authorized Signature Date

Title

Name(s) of Employee(s)

Authorized Signature Date

Title

For U.S. Government Employees Only. I certify that the WORK was written as part of my official duties as an employee of the U.S. government. For coauthors who are not U.S. government employees, I, as authorized agent, hereby transfer the copyright interests of my co-authors to the National Academy of Sciences.

Author's Signature Date

Print Name

Author's Signature Date

Print Name

FORMS

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: Louise Larose

ADDRESS: Polypeptide Lab, Strathcona Anatomy Bldg. TELEPHONE

FAX NUMBER:

DEPARTMENT: Medicine

PROJECT TITLE: Function of Nck in insulin signalling.

---

2. FUNDING SOURCE: MRC ☐ NSERC ☐ NIH ☐ FCAR ☐ FRSQ ☐

INTERNAL ☐ OTHER ☐ (specify) Can.Diabetes Assoc

Grant No.: Beginning date July 1,1999 End date June 30, 2001

---

3. Indicate if this is

☐ Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
Approval End Date

☐ New funding source: project previously reviewed and approved under an application to another agency.

Agency Medical Research Council #3329 Approval End Date June 30,2000.

☐ New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".

Containment Level (circle 1): 1 (2) 3 4

Principal Investigator or course director: date: 30 (day) 7 (month) 99 (year)

Chairperson, Biohazards Committee: date: 9 (day) Aug (month) 99 (year)

SIGNATURE

Approved period: beginning 01 (day) 07 (month) 99 (year) ending 30 (day) 06 (month) 2001 (year)

* as defined in the "McGill Laboratory Biosafety manual"

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: Louise Larose

ADDRESS: Polypeptide Lab, Strathcona Anatomy Bldg. TELEPHONE:

FAX NUMBER:

DEPARTMENT: Medicine

PROJECT TITLE: A role for the adaptor protein Nck in insulin signalling and insulin resistance.

---

2. FUNDING SOURCE: MRC ☐ NSERC ☐ NIH ☐ FCAR ☐ FRSQ ☐

INTERNAL ☐ OTHER ☐ (specify) Can.Diabetes Assoc

Grant No.: Beginning date July 1, 2001 End date June 30, 2003

---

3. Indicate if this is

☒ Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
Approval End Date June 30, 2001.

☐ New funding source: project previously reviewed and approved under an application to another agency.

Agency Approval End Date

☐ New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual". Containment Level (circle 1): 1 (2) 3

Principal Investigator or course director SIGNATURE date: 5 (day) 06 (month) 01 (year)

DR. CARLOS J. BRIED'S
Dept. of Microbiology & Immunology
McGill University
3775 University Street
Montreal, Quebec H3A 2B4
Canada

Chairperson, Biohazards Committee SIGNATURE date: 07-06-01 (day-month-year)

Approved period beginning 01-07-01 (day-month-year) ending 30-06-2003 (day-month-year)

* as defined in the "McGill Laboratory Biosafety manual"

2nd REVISION, JANUARY 1996

4. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Check appropriate classification	Fellow

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: Louise Larose

ADDRESS: Polypeptide Lab, Strathcona Anatomy Bldg. TELEPHONE

FAX NUMBER:

DEPARTMENT: Medicine

PROJECT TITLE: Function of Nck in insulin signalling.

---

2. FUNDING SOURCE: MRC ☐ NSERC ☐ NIH ☐ FCAR ☐ FRSQ ☐

INTERNAL ☐ OTHER ☐ (specify) Can.Diabetes Assoc

Grant No.: Beginning date July 1,1999 End date June 30, 2001

---

3. Indicate if this is

☐ Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
Approval End Date

☐ New funding source: project previously reviewed and approved under an application to another agency.

Agency Medical Research Council #3329 Approval End Date June 30,2000.

☐ New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".

Containment Level (circle 1): 1 (2) 3 4

Principal Investigator or course director: date: 30 (day) 7 (month) 99 (year)

Chairperson, Biohazards Committee: SIGNATURE date: 9 (day) Aug (month) 99 (year)

Approved period: beginning 01 (day) 07 (month) 99 (year) ending 30 (day) 06 (month) 2001 (year)

* as defined in the "McGill Laboratory Biosafety manual"

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: Louise Larose

ADDRESS: Polypeptide Lab, Strathcona Anatomy Bldg. TELEPHONE:

FAX NUMBER:

DEPARTMENT: Medicine

PROJECT TITLE: A role for the adaptor protein Nck in insulin signalling and insulin resistance.

---

2. FUNDING SOURCE: MRC ☐ NSERC ☐ NIH ☐ FCAR ☐ FRSQ ☐

INTERNAL ☐ OTHER ☐ (specify) Can.Diabetes Assoc

Grant No.: Beginning date July 1, 2001 End date June 30, 2003

---

3. Indicate if this is

☒ Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
Approval End Date June 30, 2001.

☐ New funding source: project previously reviewed and approved under an application to another agency.

Agency Approval End Date

☐ New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".

(circle 1): 1 (2) 3 Containment Level

Principal Investigator (course director) SIGNATURE date: 5 (day) 06 (month) 01 (year)

DR. ORLUS J. BRED'G
Dept. of Microbiology & Immunology
McGill University
3775 University Street
Montreal, Quebec H3A 2B4
Canada

Chairperson, Biohazards Committee SIGNATURE date: 07-06-01 (day-month-year)

Approved period: beginning 01-07-01 (day-month-year) ending 30-06-2003 (day-month-year)

* as defined in the "McGill Laboratory Biosafety manual"

2nd REVISION, JANUARY 1996

4. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Check appropriate classification	Fellow

FOR OFFICE USE ONLY

Project #:	3329
Investigator #:	SSA 845
Approval End Date:	Aug 31, 1997
Facility Committee:	

McGill University
University Animal Care Committee

ACTION	✓	DATE
P.I.	✓	15/11/96
FACC		5
RGO		C
REM		
INVEST		
PROTO		

APPLICATION TO USE ANIMALS FOR RESEARCH OR TEACHING

No project should be commenced without prior approval of an application to use animals for research or teaching. Submit application to appropriate facility committee one month before starting new projects or expiry of previously approved application. Animal use applications must be renewed annually.

1.

Principal Investigator: Louise Larose

Department: Medicine

Address: McGill University,
Polypeptide Hormone Lab.,

Telephone:

Fax:

Animal Use: Teaching: ____ Specify Course No.: ____ Research: X

Project Title: Function of Nck, an adaptor protein in mitogenesis and transformation.

2.

FUNDING SOURCE: MRC X NSERC ____ NIH ____ FCAR ____ FRSQ X

INTERNAL* ____ OTHER* X (specify) Canadian Diabetes Association

* Submit evidence of peer review for scientific merit

Beginning Date: January 1995 End Date: September 1998

3.

INDICATE IF THIS IS

[X] Renewal animal use application: procedures involving animals have been previously approved and no alterations have been made to the protocol.

Previous Project #: 3329 Approval End Date: Aug 31, 1996

[X] New funding source: project previously reviewed and approved under an application to another agency.

Previous Project #: 3329 Agency: MRC, CDA Approval End Date: Aug, 31, 1996

[ ] New project: project not previously reviewed or procedures and/or species altered from previously approved application.

CERTIFICATION STATEMENT: The Facility Animal Care Committee, having examined the proposal for the above named project on matters relating to the animal care and use, approves the experimental procedures proposed and certifies with the applicant that the care and treatment of animals used will be in accordance with the principles outlined in the "Guide to the Care and Use of Experimental Animals" prepared by the Canadian Council on Animal Care.

Principal Investigator/Course Director:		Date:	OCTOBER 24, 96
Chairperson, Facility Animal Care Committee:		Date:	Oct. 25, 96
University Animal Care Officer:		Date:	96/11/14
Approved period for animal use:	Beginning: 01-09-96	Ending:	31-08-97

OCT 28 1996

McGill University
Animal Use Protocol

ACTION	✓	DATE
P.I.	✓	28/8/97
ACC		
RGO		
REM		
INVEST		
PROTO		

copy to RVP1

FOR OFFICE USE ONLY

Project #	3329
Investigator #	DSA 845
Approval End Date	June 30, 1998
Facility Committee	Dossier / ~~Health~~

☐ New Application

☒ Renewal of Project # 3329

1. Investigator Information

Principal Investigator: Dr. Louise Larose Telephone:

Department: Polypeptide Laboratory Fax:

Address:

E-mail:

Animal Use: Research ☒ Teaching ☐ Specify Course number:

Project Title: Function of Nck, an adaptor protein in mitogenesis and transformation.

2. Funding Source

External ☒ Internal ☐

Source(s): Canadian Diabetes Association

Peer Reviewed source: Yes ☒ No ☐ *If no, see instructions - section 2

Awarded ☒ Pending ☐

Funding Period: From: July 1, 1997 To: June 30, 1999.

Proposed Start Date of Research: (Day/Month/Year) 1/7/97

Expected Date of Completion: (Day/Month/Year) 30/6/99

3. Emergency: Person(s) designated to handle emergencies (2 emergency telephone numbers must be indicated)

Name: Louise Larose Phone #: Work: Alternative #:

Name: Geneviève Lussier Phone #: Work: Alternative #:

Certification:

The information in this application is exact and complete. I agree to follow the policies and procedures set forth by the Facility Animal Care Committees and McGill University, as well as those described in the "Guide to the Care and Use of Experimental Animals" prepared by the Canadian Council on Animal Care. I shall request the Animal Care Committee's approval prior to any deviations from the procedures described within.

Principal Investigator/Course Director Date July 31, 1997

Approval:

Chairperson, Facility Animal Care Committee		Date	8/8/97
University Animal Care Officer		Date	97/08/28
Approved period for animal use	Beginning 1/7/97	Ending	30/06/98

☒ NOTE REVIEWER'S MODIFICATION(S) ON PAGE 2

Revised 02/97

AUG 12 1997

D Level

McGill University
Animal Use Protocol

ACTION	✓	DATE
P.I.		13/7/98
FACC		
RCO		
REM		
INVEST		
PROTO		

FOR OFFICE USE ONLY

Project #	3329
Investigator #	DSA 845
Approval End Date	June 30, 1999
Facility Committee	Medicine

☐ New Application

☒ Renewal of Project # 3329

Copy to RVH

1. Investigator Information

Principal Investigator: Dr. Louise Larose Telephone:

Department: Polypeptide Laboratory Fax:

Address:

E-mail:

Animal Use: Research ☒ Teaching ☐ Specify Course number:

Project Title: Function of Nck, an adaptor protein in mitogenesis and transformation.

2. Funding Source

External ☒ Internal ☐

Source(s): Canadian Diabetes Association

Peer Reviewed source: Yes ☒ No ☐ *If no, see instructions - section 2

Awarded ☒ Pending ☐

Funding Period: From: July 1, 1997 99 To: June 30, 1999. 2002

Proposed Start Date of Research: (Day/Month/Year) 1/7/97

Expected Date of Completion: (Day/Month/Year) 30/6/99

3. Emergency: Person(s) designated to handle emergencies (2 emergency telephone numbers must be indicated)

Name: Louise Larose Phone #: Work: Alternative #:

Name: DONGMEI ZUO Phone #: Work: Alternative #:

Certification:

The information in this application is exact and complete. I agree to follow the policies and procedures set forth by the Facility Animal Care Committees and McGill University, as well as those described in the "Guide to the Care and Use of Experimental Animals" prepared by the Canadian Council on Animal Care. I shall request the Animal Care Committee's approval prior to any deviations from the procedures described within.

Principal Investigator/Course Director		Date	June 16, 1998

Approval:

Chairperson, Facility Animal Care Committee		Date	July 6, 98
University Animal Care Officer		Date	98/06/13
Approved period for animal use	Beginning 1/7/98	Ending	30/06/99

☐ NOTE REVIEWER'S MODIFICATION(S) ON PAGE 2

Revised 02/97

JUL 08 1998
Aug 13 1998

McGill University Animal Use Protocol

ACTION	✓	DATE
P.I.	✓	18/08/99
FACC	✓	4
RGO	✓	11
VET	✓	11
INVEST		
PROTO		

FOR OFFICE USE ONLY D level

Project #	4159
Investigator #	845
Approval End Date	June 30, 2000
Facility Committee	MED

[x] New Application – NB same protocol as #3329 for MRC

[ ] Renewal of Project # _____

1. Investigator Information

Principal Investigator: Dr. Louise Larose Telephone:

Department: Polypeptide Laboratory Fax:

Address:

E-mail: mdll@musica.mcgill.ca

Animal Use: Research [X] Teaching [ ] Specify Course number:

Function of Nck in insulin signalling.

2. Funding Source

External [X] Internal [ ]

Source(s): Canadian Diabetes Association

Peer Reviewed source: Yes [X] No [ ] *If no, see instructions - section 2

Awarded [X] Pending [ ]

Funding Period: From: July 1, 1999 To: June 30, 2001.

Proposed Start Date of Research: (Day/Month/Year) 1/7/99

Expected Date of Completion: (Day/Month/Year) 30/6/01

3. Emergency: Person(s) designated to handle emergencies (2 emergency telephone numbers must be indicated)

Name: Louise Larose Phone #: Work: Alternative #:

Name: Dongmei Zuo Phone #: Work: Alternative #:

Certification:

The information in this application is exact and complete. I agree to follow the policies and procedures set forth by the Facility Animal Care Committees and McGill University, as well as those described in the "Guide to the Care and Use of Experimental Animals" prepared by the Canadian Council on Animal Care. I shall request the Animal Care Committee's approval prior to any deviations from the procedures described within.

Principal Investigator/Course Director		Date	July 30, 1999

Approval:

Chairperson, Facility Animal Care Committee			Date	9 Aug 99
University Animal Care Officer			Date	99/08/11
Approved period for animal use	Beginning	1/7/99	Ending	30/06/2000

[ ] NOTE REVIEWER'S MODIFICATION(S) ON PAGE 2

Revised 02/97

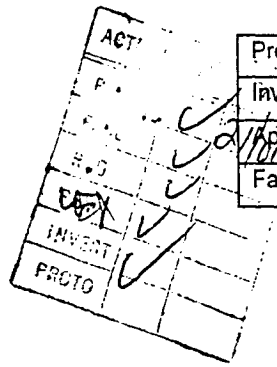
AUG 18 1999

Dlevel CCFA

McGill University
Animal Use Protocol

FOR OFFICE USE ONLY

Project #	4159
Investigator #	845
Approval End Date	June 30, 2001
Facility Committee	MED



☐ New Application – NB same protocol as #3329 for MRC

☑ Renewal of Project # 4159

1. Investigator Information

Principal Investigator: Dr. Louise Larose Telephone:

Department: Polypeptide Laboratory Fax:

Address:

E-mail:

Animal Use: Research [X] Teaching [ ] Specify Course number:

Function of Nck in insulin signalling.

2. Funding Source

External [X] Internal [ ]

Source(s): Canadian Diabetes Association

Peer Reviewed source: Yes [X] No [ ] *If no, see instructions - section 2

Awarded [X] Pending [ ]

Funding Period: From: July 1, 1999 To: June 30, 2001

Proposed Start Date of Research: (Day/Month/Year) 1/7/00

Expected Date of Completion: (Day/Month/Year) 30/6/01

3. Emergency: Person(s) designated to handle emergencies (2 emergency telephone numbers must be indicated)

Name: Louise Larose Phone #: Work: Alternative #:

Name: Dongmei Zuo Phone #: Work: Alternative #:

Certification:

The information in this application is exact and complete. I agree to follow the policies and procedures set forth by the Facility Animal Care Committees and McGill University, as well as those described in the "Guide to the Care and Use of Experimental Animals" prepared by the Canadian Council on Animal Care. I shall request the Animal Care Committee's approval prior to any deviations from the procedures described within.

Principal Investigator/Course Director		Date	May 31, 2000

Approval:

Chairperson, Facility Animal Care Committee		Date	June 14th 2000
University Animal Care Officer		Date	June 20, 2000
Approved period for animal use	Beginning	Ending	June 30, 2001

☐ NOTE REVIEWER'S MODIFICATION(S) ~~ON BACK~~

Revised 02/97

JUN 16 2000

McGill University
Animal Use Protocol – Research

Guidelines for completing the form are available at www.mcgill.ca/fgsr/rgo/animal/

Protocol #: 4159
Investigator #: 845
Approval End Date: June 30, 2002
Facility Committee: MED

☐ Pilot ☐ New Application ☒ Renewal of Protocol # 4159-010630

Title (must match the title of the funding source application): A role for the adaptor protein Nck in insulin signaling and insulin resistance.

1. Investigator Data:

Principal Investigator: Dr. Louise Larose Office #:
Department: Polypeptide Laboratory Fax#:
Address: Email:

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: Dr. Louise Larose Work #: Emergency #:
Name: Dongmei Zuo Work #: Emergency #:

ACTION	✓	DATE
P.I.	C	July 30 01
FACC	C	4
RGO	C	4
VET	C	4
DB		
Approved		

3. Funding Source:

External ☒
Source (s): Can.Diabetes Assoc.
Peer Reviewed: ☒ YES ☐ NO**
Status : ☒ Awarded ☐ Pending
Funding period: July 1, 2001 - June 30, 2002

Internal ☐
Source (s):
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period:

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed . e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y): or ongoing ☒

Expected Date of Completion of Animal Use (d/m/y): June 30, 2002. or ongoing ☐

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator: Date: July 4, 01

Approval Signatures.

Chair, Facility Animal Care Committee: Date: 5/7/01
University Veterinarian: Date: 7/09/01
Chair, Ethics Subcommittee(as per UACC policy): Date: 7/26/01
Approved Period for Animal Use Beginning: July 1, 2001 Ending: June 30, 2002
☐ This protocol has been approved with the modifications noted in Section 13.

JUL 11 2001

McGill University
Animal Use Protocol – Research

Guidelines for completing the form are available at
www.mcgill.ca/fgsr/rgo/animal/

Protocol #: 4159
Investigator #: 345
Approval End Date: June 30, 2003
Facility Committee: MED

☐ Pilot ☐ New Application ☒ Renewal of Protocol # 4159

Title (must match the title of the funding source application): A role for the adaptor protein Nck in insulin signaling and insulin resistance.

1. Investigator Data:

Principal Investigator: Dr. Louise Larose Office #:

Department: Polypeptide Laboratory Fax#:

Address: Email:

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: Dr. Louise Larose Work #: Emergency #:

Name: Mr. Eric Cardin Work #: Emergency #:

3. Funding Source:

External ☒
Source (s): Can.Diabetes Assoc.
Peer Reviewed: ☒ YES ☐ NO**
Status : ☒ Awarded ☐ Pending
Funding period: July 1, 2002 - June 30, 2003

Internal ☐
Source (s):
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period:

ACTION	✓	DATE
CCC	✓	June 17
DE	✓	"
APPROVED		

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed . e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y): or ongoing ☒

Expected Date of Completion of Animal Use (d/m/y): . or ongoing ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator: Date: June 13, 2002

Approval Signatures:

Chair, Facility Animal Care Committee:		Date: 14/6/2002
University Veterinarian:		Date: June 18 2002
Chair, Ethics Subcommittee(as per UACC policy):		Date: 6/12/02
Approved Period for Animal Use	Beginning: July 1, 2002	Ending: June 30, 2003

☐ This protocol has been approved with the modifications noted in Section 13.

JUN 19 2002