NOTE TO USERS

The adenovirus E4orf4 protein induces G2 / M arrest and cell death by inhibiting PP2A phosphatase activity regulated by the B alpha subunit

By

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B.Sc.H.

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Abstract

The human adenovirus E4orf4 protein is toxic in human tumor cells and yeast. In both cases, interaction of E4orf4 with the Bα/Cdc55 regulatory subunit of protein phosphatase 2A (PP2A) appears to be critical for cell killing; however, the effect of E4orf4 binding on PP2A is not known. Bα is one of several B subunits that form holoenzymes with A and C subunits and regulate PP2A substrate specificity. In the present studies the effects of E4orf4 on PP2A activity was examined, as well as the role of PP2A in E4orf4-mediated cell death. Previously it was shown that binding of E4orf4 had little effect on the dephosphorylation of a universal peptide substrate by purified PP2A, however this study demonstrates that E4orf4 reduced PP2A activity to varying degrees against two substrates, phosphorylase *a* and histone H1, the latter known to represent a class of substrate against which Bα-containing PP2A complexes are highly active. Expression of E4orf4 was also found to result in the accumulation of hyperphosphorylated forms of two PP2A substrates, 4EBP-1 and $p70^{S6K}$ (Bα specific) *in vivo*, indicating a significant inhibition of PP2A activity against these substrates. The importance of such inhibition in cell killing by E4orf4 was supported by the fact that low levels of okadaic acid (OA) or expression of the heat stable I_1^{PP2A} inhibitor, both of which inhibit PP2A activity globally, enhanced E4orf4-induced cell death. Furthermore, E4orf4 killing was reduced by overexpression of a Y307F mutant form of the PP2A catalytic Cα subunit, which is insenstitive to downregulation by tyrosine phosphorylation. E4orf4 was found to induce a significant G2 / M arrest, an effect that was enhanced both by treatment with OA, which alone at high levels has similar effects, and by expression of simian virus 40 small T antigen (SV40-ST), which binds to PP2A holoenzymes and preferentially replaces the Bα subunit. These results suggest that E4orf4 induces cell death by binding to the Bα subunit and inhibiting its ability to direct PP2A activity against Bα-specific PP2A substrates, some of which may be involved in mitotic progression.

Resume

La protéine adénovirale E4orf4 cause de la toxicité dans les cellules cancéreuses humaines ainsi que dans les levures. Dans les deux cas, la toxicité cellulaire induite par E4orf4 nécessite l'intéraction de cette dernière avec la protéine Bα/Cdc55. Bα fait parti de la famille des sous-unités de type B qui, avec les unites A et C, forment la phosphatase PP2A. De plus, le rôle principale de la familles des unites B est de contrôller la spécificité des substrats de PP2A. La présente étude avait pour but de déterminer l'effet de E4orf4 sur l'activité enzymatique de PP2A, ainsi que le rôle de cette phosphatase dans la mort cellulaire induite par E4orf4. Des expériences antérieures ont démontré que E4orf4 avait aucun effet sur l'activité de PP2A envers un peptide universel utilisé comme pseudo-substrat. Par contre, les résultats présentés dans cette études démontrent que E4orf4 diminue l'activité enzymatique de PP2A envers la phosphorylase *a* et histone H1, deuz protéines spécifique aux complexes de PP2A contenant l'unité Bα. De plus, l'expression de E4orf4 est associée à une augmentation du niveau de phosphorylation des protéines 4EBP-1 et $p70^{S6K}$, deux substrats de PP2A. Ces résultats suggèrent que E4orf4 diminue l'activité de PP2A envers certain substrats et que cette inhibition est liée à la mort cellulaire cause par l'expression de E4orf4. La mort induite par E4orf4 est augmenté en présence d'une faible concentration d'acide okadaique ainsi que la protéine I_1^{PP2A}, tous deux étant des inhibiteurs global de PP2A. La co-expression d'un mutant dans l'unité catalytique Cα de PP2A Y307F, insensible à l'inactivation par la phosphorylation, réduit la toxicité cellulaire associée à E4orf4. Nos résultats démontrent que E4orf4 cause un arrêt dans la phase G2/M du cycle cellulaire et cet effet peut être augmenté dans la présence d'inhibiteur de PP2A, notamment l'acide okadaique et l'antigène du virus simian small T (SV40-ST). En conclusion, nos résultats indiquent que E4orf4 se lie à Bα pour bloquer l'activité enzymatique de PP2A contre des substrats spécifique à la protéine Bα. Cette interaction est importante pour la toxicité cellulaire induite par E4orf4 et certaines protéines affectées par cette inhibition pourraient être impliquées dans la progression mitotique du cycle cellulaire.

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Contribution of Co-authors

All of the figures in this thesis were done by myself, except for the *in vitro* assays in Figure 3-1 which were performed by Monica McQuoid of Dr. D.C. Pallas' laboratory.

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List of Abbreviations

243R - 243 residue E1A protein product of 12S mRNA
289R - 289 residue E1A protein product of 13S mRNA
aa - amino acids
AA - arachadonic acid
Ad - human adenovirus
ATF - activating transcription factor
C-terminal - carboxy terminal
cAMP - cyclic adenosine monophosphate
CAR - coxsackievirus and adenovirus receptor
CBP - CREB-binding protein
CDKs - cyclin dependent kinases
CMV - cytomegalovirus
CR1 - conserved region 1
CR2 - conserved region 2
CR3 - conserved region 3
CREB - cAMP responsive element binding protein
DAPI - 4'-6-diamidino-2-phenylindole-dihydrochloride
DBP - DNA binding protein
DNA - deoxyribonucleic acid
E1A - early region 1A
E1B - early region 1B
E2 - early region 2
E3 - early region 3
E4 - early region 4
ECL - enhanced chemiluminescence
eIF-2α - eukaryotic initiation factor-2α
eIF4E - eukaryotic translation initiation factor 4E
ER - endoplasmic reticulum
FACS - fluorescence-activated cell sorting
G1 phase - gap phase before S phase
G2 phase - gap phase before M phase
GFP - green fluorescent protein
HA- hemagglutinin
h.p.t. - hours post infection
h.p.i. - hours post transfection
kD - kilodalton
M phase - cell division
MAPK - mitogen-activated protein kinase
Mdm2 - murine double minute 2
MHC - major histocompatibility complex
MLC - myosin light chain
moi - multiplicity of infection

MPF - mitosis promoting factor
mRNA - messenger RNA
mTOR - mammalian target of rapamycin
N-terminal - amino terminal
OA - okadaic acid
orf - open reading frame
PARP - poly ADP ribose polymerase
PCR - polymerase chain reaction
pfu - plaque-forming units
PKR - protein kinase R
PML - promyelocytic leukemia protein
POD - PML oncogenic domains
PP1 - protein phosphatase 1
PP2A - protein phosphatase 2A
pRb - retinoblastoma tumor suppressor protein
pTP - precursor terminal protein
ROS - reactive oxygen species
RNA - ribonucleic acid
RGD - arginine, glycine, aspartic acid
rtTA - reverse tetracycline transactivator
S phase - DNA replication
SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis
SV40-ST - simian virus 40 small t antigen
TBP- TATA binding protein
tetO - tetracycline operator
TFIID - transcription factor IID
TNF - tumor necrosis factor
TOR - target of rapamycin
TP - terminal protein
TRAIL - TNF-related apoptosis inducing ligand
VA - virus associated
YY1 - yin yang protein 1

Chapter 1: Introduction

1.1 Adenoviruses

1.1.1 History

Adenoviruses were first identified by researchers studying the etiologic agents of acute respiratory infections. In 1953, Rowe and colleagues observed the spontaneous degeneration of primary cell cultures derived from human adenoids. The degeneration of cells was caused by replication of previously unidentified viruses found in the adenoid tissue (Rowe, Huebner et al. 1953). A year later while studying respiratory disease in military personnel, Hilleman and Werner isolated similar viral agents that induced cytopathic changes in human cell cultures (Hilleman and Werner 1954). These viruses were proven to be related (Huebner, Rowe et al. 1954) and in 1956 they were termed *adenoviruses* after the adenoid tissue.

Studies have confirmed that adenoviruses were the cause of acute febrile respiratory disease (Ginsberg, Gold et al. 1955; Dingle and Langmuir 1968), but not the cause of the common cold. Adenoviruses are also associated with acute hemorrhagic cystitis, hepatitis, gastroenteritis and myocarditis (Jones and Shenk 1979; Fowlkes and Shenk 1980; Cepko and Sharp 1982; Lee, Kao et al. 1991; Carvalho, Seeler et al. 1995).

In 1962, Trentin and colleagues reported that human adenovirus type 12 (Ad12) induced malignant tumors following injection into newborn hamsters (Trentin, Yabe et al. 1962). This study revealed for the first time that human virus had oncogenic potential and it became apparent that adenoviruses could be used as an experimental tool to analyze normal and malignant cell growth.

1.1.2 Classification

Adenoviruses are classified as members of the *Adenoviridae* family of viruses. This family is separated into two genera, *Mastadenovirus* and *Aviadenovirus* (Norrby, Bartha et al. 1976). The *Aviadenovirus* genus consists solely of bird viruses, whereas the

Mastadenovirus genus contains human, murine, simian, porcine, canine, bovine, equine, ovine, and opossum viruses.

Forty-nine human adenovirus serotypes were identified based on their resistance to neutralization by antisera to other known adenovirus serotypes (Norrby 1969; Crawford-Miksza and Schnurr 1996). These serotypes were then subdivided into six groups based on their ability to agglutinate red blood cells (A-F) (Table 1-1) (Rosen 1960). This type of classification is standard as the results are comparable to other methods of categorization including oncogenity in rodents (Green 1970), relatedness of tumour antigens (McAllister, Nicolson et al. 1969), percentage of G-C content (Pina and Green 1965), cross-hybridization (Garon, Berry et al. 1973), electrophoretic mobility of virion proteins (Wadell 1979) or digestion with restriction endonucleases (Wadell, Hammarskjold et al. 1980).

Subgroup		Hemagglutination groups	Serotypes	Oncogenic potential		Percentage of G–C in DNA
				Tumors in animals	Transformation in tissue culture	
A	IV	(little or no agglutination)	12,18,31	High	+	48–49
B	I	(complete agglutination of monkey erythrocytes)	3,7,11,14,16, 21,34,35	Moderate	+	50–52
C	III	(partial agglutination of rat erythrocytes)	1,2,5,6	Low or none	+	57–59
D	II	(complete agglutination of rat erythrocytes)	8,9,10,13, 15,17,19,20,22–30, 32,33,36–39,42–47	Low or none (mammary tumors)	+	57–61
E	III		4	Low or none	+	57–59
F	III		40,41	Unknown		

Table 1-1: Adenovirus classification. [Adapted from (Shenk 2001)]

1.1.3 Virion Structure

Adenoviruses are icosahedral particles (70 - 100 nm in diameter) containing 20 triangular surfaces and 12 vertices. The particles (virions) consist of a DNA-containing

core (13% mass) surrounded by a protein capsid (87% mass) (Furtado, Subramanian et al. 1989; Hasson, Ornelles et al. 1992). The capsid is composed of 240 hexons and 12 pentons (Ginsberg, Pereira et al. 1966). Each penton contains a base and a projecting fiber. The fibers interact with a cellular receptor protein and their length varies depending on the serotype (Figure 1-1) (Norrby 1966).

Structural studies of Ad2 and Ad5 have identified 11 polypeptides in the virion core and capsid (Maizel, White et al. 1968; Ishibashi and Maizel 1974; van Oostrum and Burnett 1985). The virion core consists of the viral genome and four known proteins: protein VII, V, mu and the terminal protein. Viral DNA is wrapped around clusters of the most predominant core polypeptide, protein VII (Mirza and Weber 1982; Chatterjee, Vayda et al. 1986). Protein V mediates the interaction of viral DNA with a penton base (Everitt, Lutter et al. 1975) and it might bridge the virion core and capsid. The mu protein associates with viral DNA (Chatterjee, Vayda et al. 1986; Anderson, Young et al. 1989); however, its function is unknown (Hosokawa and Sung 1976). The terminal protein is covalently attached to the 5' ends of the viral DNA (Rekosh, Russell et al. 1977). This protein facilitates attachment of the viral genome to the nuclear matrix (Bodnar, Hanson et al. 1989; Schaack, Ho et al. 1990; Fredman and Engler 1993) and is involved in DNA replication (Challberg, Desiderio et al. 1980; Lichy, Horwitz et al. 1981; Challberg, Ostrove et al. 1982).

The virion capsid contains seven identified proteins. The hexon consists of three molecules of protein II known as the hexon capsomere (Horwitz, Maizel et al. 1970). Protein VI, VIII and IX associate with the hexon (Everitt, Sundquist et al. 1973) and appear to stabilize the hexon capsomere. Protein VI and VIII seem to connect the virion capsid and core. Protein IIIa is associated with hexon units that surround the penton (Everitt, Lutter et al. 1975). The penton base consists of five molecules of protein III and protein IV forms the projecting fiber (van Oostrum and Burnett 1985). Together the penton base and fiber are known as the penton capsomere.

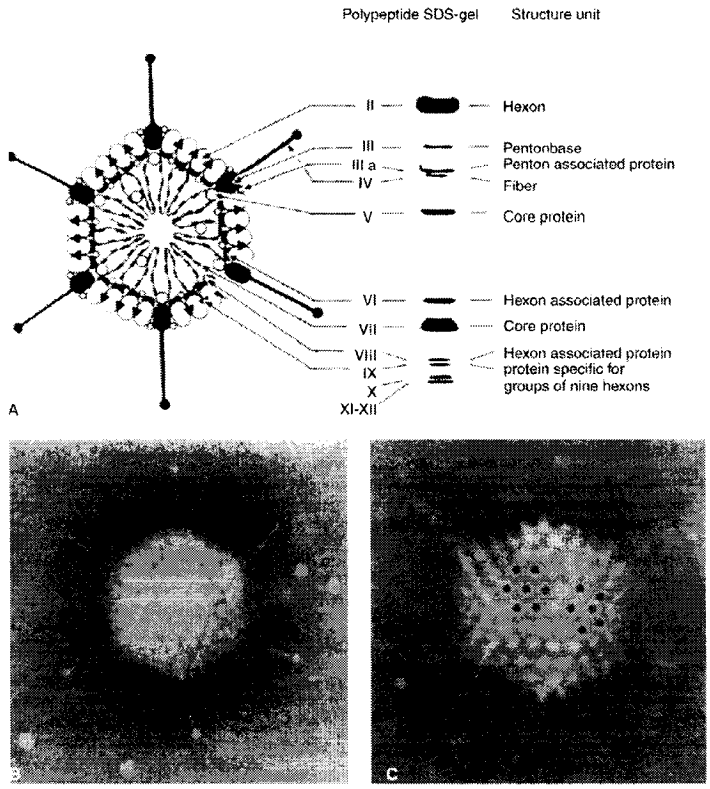


Figure1-1: Virion structure. [Adapted from (Horwitz 2004)]

1.1.4 Genome Organization

Adenovirus genomes consist of linear, double stranded DNA. Organization of the genome is similar amongst adenovirus serotypes. The genome consists of two terminal repeats each containing an origin for DNA replication. To facilitate an interaction between the viral DNA and its encapsidating protein (Hearing, Samulski et al. 1987) a *cis*-acting packaging sequence is located near an end of the chromosome (Hammarskjold and Winberg 1980; Hearing and Shenk 1983; Hearing and Shenk 1986; Grable and Hearing 1992).

The viral chromosome carries numerous transcription units transcribed by RNA polymerase II. These units are classified as early and late based on their order of expression before and after DNA replication. There are five early transcription units (E1A, E1B, E2, E3 and E4), two delayed early units (IX and IVa2) and one major late unit which produces five families of late mRNAs (L1 to L5) (Pettersson and Roberts

1986). Also, depending on the serotype, the viral chromosome contains one or two virus-associated (VA) genes transcribed by RNA polymerase III. The viral DNA is transcribed with the rightward reading strand coding for the E1A, E1B, IX, major late, VA, RNA and E3 units and the leftward reading strand coding for the E4, E2, and IVa2 units (Figure 1-2) (Shenk 2001).

Adenoviral proteins have diverse functions. The early transcription unit encodes polypeptides involved in transcriptional regulation, apoptosis, DNA replication and mRNA transport; whereas the late transcription unit encodes structural proteins. Furthermore, many individual transcription units encode several polypeptides with similar functions, for example E1B encodes two proteins (19 kD and 55 kD) involved in preventing apoptosis (Shenk 2001).

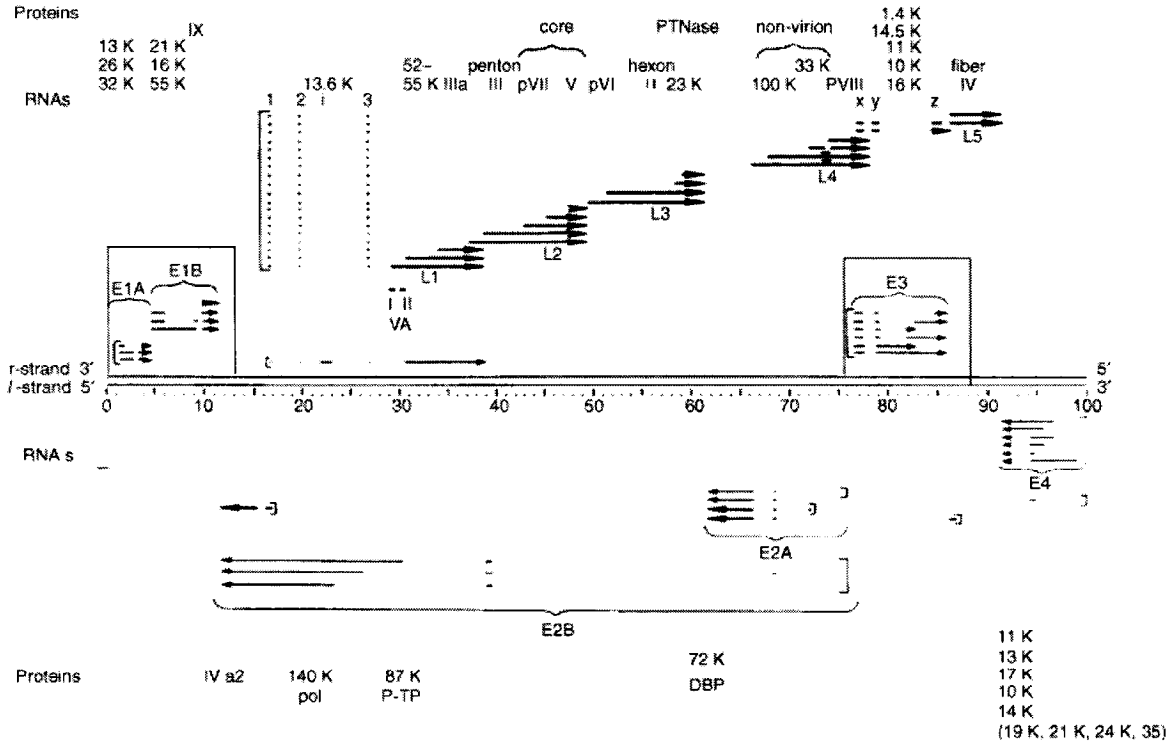


Figure 1-2: Adenovirus transcription map. [Adpated from (Horwitz 2004)]

1.1.5 Infectious Cycle

1.1.5.1 Overview

Most studies of the human adenovirus replicative cycle have involved infecting HeLa or KB cells with Ad2 and Ad5 at multiplicities of infection (moi) of more than 10-plaque forming units (pfu) per cell to ensure that the cells are infected synchronously.

The replicative cycle is divided into the early and late phase which are separated by the onset of viral DNA replication. The early phase involves adsorption, penetration, localization of the viral DNA to the nucleus and expression of early genes which facilitate DNA replication, cell cycle progression, prevent apoptosis and antagonize host antiviral measures. These early events begin when the virus interacts with the host cell and end after 5 to 6 hours. At this point viral DNA replication commences and with its onset the late phase begins. Late events include the expression of late viral genes and assembly of viral progeny. The replicative cycle takes approximately 20 to 24 hours to complete and about 10^4 progeny virus particles per cell are produced.

1.1.5.2 Adenoviral Adsorption and Entry

Attachment of the virus to the host cell is facilitated by its fiber protein (Lonberg-Holm and Philipson 1969) whose carboxy-terminal knob binds to the cellular receptor (Devaux, Caillet-Boudin et al. 1987). A common receptor for coxsackie B viruses, Ad2 and Ad5 is the CAR (coxsackievirus and adenovirus receptor) protein which belongs to the immunoglobin superfamily. The class I major histocompatibility complex (MHC) $\alpha 2$ domain is another receptor recognized by Ad 5 (Hong, Karayan et al. 1997).

Once the virus is attached to the host cell, a second protein-protein interaction is required for the virus to enter the cell (Silver and Anderson 1988). This event occurs between the penton base protein and members of the integrin family of heterodimeric cell surface receptors (Wickham, Mathias et al. 1993). The interaction requires binding of an arg-gly-asp (RGD) sequence present in the penton base to the $\alpha_v\beta_3$- and $\alpha_v\beta_5$- vitronectin-

binding integrins. This second interaction is required for internalization of the virus by receptor-mediated endocytosis (Wickham, Mathias et al. 1993).

The internalization process is very efficient (Greber, Willetts et al. 1993). Approximately 80-85% of virus that interacts with a host cell is successfully internalized and penetration occurs quickly, with half of the adsorbed virus localizing to endosomes within 10 minutes. About 90% of the virus within endosomes localizes to the cytosol (Seth, Fitzgerald et al. 1984; Svensson 1985). This movement occurs rapidly, with a half time of about 5 minutes, suggesting that the virus escapes from the endosome before formation of a lysosome occurs (Bai, Harfe et al. 1993). The released viral particles are transported by microtubules through the cytoplasm to the nucleus (Dales and Chardonnet 1973; Suomalainen, Nakano et al. 1999).

During the internalization process there is sequential dismantling of the virion (Greber, Willetts et al. 1993) by both proteolytic degradation and dissociation of viral constituents. The purpose of this disassembly is to eliminate structural proteins so that viral DNA can be transported to the nucleus. Once the viral DNA reaches the nucleus, its terminal protein enables it to interact with the nuclear matrix (Bodnar, Hanson et al. 1989; Schaack, Ho et al. 1990; Fredman and Engler 1993). This association is necessary for transcription of viral genes (Schaack, Ho et al. 1990).

1.1.6 Early Gene Expression

1.1.6.1 Overview

Early gene expression is essential to viral replication for three main reasons. The first is that it promotes the host cell to enter the S phase of the cell cycle, so that the replication machinery is available to the virus. The second is that it protects the infected cell against antiviral defenses of the host organism. The third is that it produces viral gene products necessary for viral DNA replication.

1.1.6.2. E1A Region

1.1.6.2.1 E1A Proteins

E1A is the first transcription unit expressed after the viral chromosome reaches the nucleus in infected cells (Boulanger and Blair 1991). During the early phase of infection E1A encodes two mRNAs (12S and 13S) that generate protein products of 243 (243R) and 289 (289R) amino acids, respectively. During the late phase of infection three other E1A mRNA species accumulate (11S, 10S and 9), but no specific function has been designated for their products (Stephens and Harlow 1987).

Studies regarding E1A proteins have focused mainly on 243R and 289R. These proteins localize in the nucleus (Douglas and Quinlan 1996), have a half-life of 20-30 minutes (Spindler and Berk 1984; Branton and Rowe 1985) and have a linear structure (Howe, Mymryk et al. 1990; Barbeau, Charbonneau et al. 1994).

Comparison of the amino acid sequences of 243R and 289R amongst various adenovirus serotypes indicates three conserved regions termed CR1, CR2, and CR3 (van Ormondt, Maat et al. 1980; Moran and Mathews 1987; Nevins 1992) were identified. Two regions common to both proteins consist of amino acids 41-80 (CR1) and 121-139 (CR2), which are separated by less conserved domains (van Ormondt, Maat et al. 1980; Moran and Mathews 1987; Nevins 1992). The CR3 (aas 140-188) region includes the unique 46 aa sequence found in the 289R protein. All of the conserved regions facilitate interactions with other proteins that are necessary to activate transcription (van Ormondt, Maat et al. 1980).

1.1.6.2.2 E1A Activated Transcription

E1A activates transcription of other early viral genes by associating with transcription factors and recruiting them to viral promoters (Ferguson, Krippl et al. 1985). This interaction is mostly mediated by the CR3 domain of 289R (Berk and Sharp 1977) which contains an N-terminal transactivation domain and a C-terminal promoter targeting region (Webster and Ricciardi 1991). The CR3 transactivation domain enables E1A to interact

with the TATA-binding protein (TBP) (Horikoshi, Maguire et al. 1991; Lee, Kao et al. 1991). TBP is the DNA-binding subunit of the transcription factor IID (TFIID) which interacts with TATA-containing promoters. Thus, binding of E1A to TBP stimulates activation of TATA box promoters (Green, Treisman et al. 1983; Wu, Rosser et al. 1987). The second part of this mechanism involves the cellular tumor suppressor protein, p53. p53 represses transcription by binding to TFIID at sites that overlap those that interact with E1A (Seto, Usheva et al. 1992; Liu, Miller et al. 1993). Therefore, E1A mediated transcriptional activation also involves CR3 binding to TBP to relieve p53-induced repression (Horikoshi, Usheva et al. 1995). 243R can also activate transcription through the TATA motif (Kraus, Moran et al. 1992). This E1A protein can bind to and inactivate Dr1 (Kraus, Inostroza et al. 1994), a factor that associates with TBP and inhibits transcription (Inostroza, Mermelstein et al. 1992). This interaction results in the stimulation of transcription, but it is not as efficient as 289R.

The C-terminal promoter targeting region of CR3 stimulates transcription by interacting with cellular DNA-binding transcription factors, including ATF-2. Other than E1B, all of the adenovirus promoters contain binding sites for the ATF (also known as *CREB*) family of transcription factors. E1A is able to simultaneously interact with the DNA binding domain of ATF-2 (Lillie and Green 1989; Liu and Green 1990; Abdel-Hafiz, Chen et al. 1993) and TBP through separate subdomains of CR3. Thus, it has been suggested that E1A functions as a bridge between ATF-2 and TBP to promote the formation of the transcription initiation complex (Lee, Kao et al. 1991). CR3 interacts with another transcription factor termed AP-1. AP-1 is a dimeric molecule made up of *c-fos* and *c-jun* family members; interestingly, members of the ATF family can also heterodimerize with *jun* family members (Benbrook and Jones 1990).

E1A proteins also stimulate transcription by enhancing the amount of AP-1 in collaboration with cyclic adenosine monophosphate (cAMP). These proteins increase the AP-1 level by activating the transcription of *c-fos* and *junB* genes through their TATA motifs (Kleinberger and Shenk 1993). By increasing the level of AP-1, the transcription of viral genes is activated (Müller, Roberts et al. 1989).

E1A also activates transcription through interacting with the transcription factor YY1 (Shi, Seto et al. 1991). YY1 binds upstream of a transcriptional initiation site and represses transcription. Binding of E1A through an amino terminal sequence to YY1 is correlated with the relief of YY1-mediated repression (Lewis, Tullis et al. 1995).

E1A proteins can also stimulate transcription through the binding sites of factors that bind upstream of the basal promoter, including E2F and E4F. E2F is a transcription factor that binds to sites in the E2 promoter and induces the expression of cellular proteins involved in cell cycle progression. E2F also associates with the cellular retinoblastoma tumor suppressor protein, pRb which inhibits transcriptional activation by E2F. Proteins encoded by the 13S and 12S mRNAs can bind to pRb (Whyte, Buchkovich et al. 1988) through their CR1 and CR2 domains (Dyson, Guida et al. 1992; Ikeda and Nevins 1993) and displace pRb from E2F (Bagchi, Raychaudhuri et al. 1990; Bagchi, Weinmann et al. 1991; Bandara and La Thangue 1991). By dissociating this complex, the transcription of both viral and cellular genes is activated. E4F is another transcription factor associated with E1A. E4F binds to sites in the E4 promoter and activates transcription. Although the mechanism has not yet been characterized, it is known that E1A enhances the phosphorylation of E4F and can interact with the transcription factor. These two events stimulate the binding of E4F to the E4 promoter, thus activating transcription (Müller, Roberts et al. 1989).

1.1.6.2.3 E1A-mediated DNA Synthesis

Adenovirus infection promotes quiescent cells to enter S phase of the cell cycle to produce an environment suitable for viral replication. E1A proteins are primarily responsible for the progression of cells from G1 to S phase. These proteins stimulate S-phase entry through mechanisms involving their CR1 and CR2 domains (Lillie, Loewenstein et al. 1987; Zerler, Roberts et al. 1987; Howe, Mymryk et al. 1990). Insight into these mechanisms came with the observation that a number of cellular proteins could be co-immunoprecipitated with the E1A polypeptides (Yee and Branton 1985; Harlow, Whyte et al. 1986) and had approximate molecular weights of 33, 60, 80, 90, 105, 107, 130, 300 and 400 kD.

The first protein to be identified was the retinoblastoma tumor suppressor protein, pRb (Whyte, Buchkovich et al. 1988). pRb, as mentioned in the previous section, regulates the ability of E2F to activate transcription. Growth arrest is correlated with the formation of the pRb / E2F complex which prevents E2F-induced transcription. It has been suggested that pRb masks the E2F activation domain and recruits histone deacetylases to inhibit transcription by promoting condensation of chromatin. This growth arrest is reversed when pRb is phosphorylated by cyclin-dependent kinases (CDKs). The hyperphosphorylated form of pRb does not interact with E2F, therefore E2F-induced transcription can occur and stimulate a series of genes important for S-phase entry and DNA synthesis (i.e. DNA polymerase α, *cdc2*, cyclin E, *c-myc*, etc.) (Hinds, Mittnacht et al. 1992; Nevins 1992).

E1A proteins drive S-phase entry by promoting E2F-mediated transcription. E1A proteins interact with pRb primarily through their CR2 domain and this association is stabilized by the amino terminal region of CR1 (Dyson, Guida et al. 1992; Ikeda and Nevins 1993). The E1A binding domain on pRb is often referred to as the pocket. This region contains two important sequences, termed the A and B boxes, which are separated by a spacer region (Hu, Dyson et al. 1990; Kaelin, Ewen et al. 1990). E2F proteins interact with pRb through the same region as E1A proteins. Therefore E2F and E1A proteins compete to bind pRb. When E1A polypeptides bind to pRb, E2F is free to activate cellular genes that facilitate cell cycle progression (Bagchi, Raychaudhuri et al. 1990; Bagchi, Weinmann et al. 1991; Bandara and La Thangue 1991; Dyson, Guida et al. 1992; Ikeda and Nevins 1993).

E1A proteins also activate E2F-mediated transcription by stimulating the activity of CDKs to hyperphosphorylate pRb. CDKs are regulated through various mechanisms including association with cyclin-dependent inhibitory proteins, such as $p27_{kip1}$. E1A antagonizes the inhibitory activity of $p27_{kip1}$, allowing CDKs to phosphorylate pRb. The hyperphosphorylated form of pRb will not bind to E2F, therefore E2F-mediated transcription is induced and the cells are pushed into S-phase of the cell cycle (Mal, Poon et al. 1996; Alevizopoulos, Catarin et al. 1998).

Two other proteins that bind E1A have been identified as p107 and p130. These proteins are cellular pRb family members and like pRb they can also interact with E2F and prevent E2F-mediated transcription. E1A binds to these proteins through its CR2 region and this interaction is stabilized by CR1 (Wang, Rikitake et al. 1993). When E1A proteins bind to p107 and p130, E2F is displaced enabling promotion of S-phase entry by activating genes involved in cell cycle progression.

E1A proteins are involved in a second mechanism that drives cells into the S phase of the cell cycle. This mechanism involves 243R and 289R binding through their CR1 domain to the CBP (CREB-binding proteins) and p300 proteins (Wang, Rikitake et al. 1993). p300 / CBP are transcriptional coactivators of genes involved in cell cycle arrest (Arany, Sellers et al. 1994). p300 / CBP are also histone acetyltransferases and they interact with a factor termed P / CAF which is also a histone acetyltransferase. Acetylation of histones appears to weaken interactions with DNA, creating a chromatin structure that is more conducive to transcription. E1A proteins inhibit the histone acetyltransferase activity of p300 / CBP by binding to these proteins and simultaneously displacing P / CAF (Yang, Ogryzko et al. 1996; Chakravarti, Ogryzko et al. 1999; Hamamori, Sartorelli et al. 1999; Perissi, Dasen et al. 1999). This interaction prevents the transcription of genes involved in cell cycle arrest.

S-phase entry is mediated by the ability of E1A to inhibit p300 / CBP activity. p53 is a tumor suppressor protein and high levels of p53 promotes cell cycle arrest or apoptosis. p300/CBP acts as a coactivator for p53, therefore when E1A interacts with p300/CBP it inhibits p53 function (Lill, Grossman et al. 1997; Somasundaram and El-Deiry 1997) relieving a block in cell cycle progression. This relief alone will not stimulate S-phase entry; however, it is believed that there are several unidentified genes whose expression is modified by the E1A-p300 / CBP interaction and these genes promote the cells to synthesize DNA.

Taken together, the CR1 and CR2 binding domains of E1A proteins mediate an interaction with pRb and p300 / CBP to stimulate S-phase entry in quiescent cells. However, these two binding regions are also essential for the cells to pass through the G2 to M phase checkpoint and progress to mitosis (Zerler, Roberts et al. 1987; Howe,

Mymryk et al. 1990; Wang, Draetta et al. 1991; Howe and Bayley 1992). This movement is critical to the virus because it permits viral progeny to divide, continue cycling and return to the replication phase of the cell cycle.

1.1.6.2.4 E1A and Apoptosis

Apoptosis, or programmed cell death, is a genetically regulated process that leads cells to commit suicide in response to a variety of stimuli. Apoptosis is stimulated during viral infection to prevent viral growth and inhibit its spread within the infected organism. Apoptosis is a non-inflammatory response characterized by certain features including some DNA fragmentation, membrane blebbing, cytoplasmic vascuolization and shrinkage, as well as chromatin condensation (Lavoie, Nguyen et al. 1998; Lavoie, Champagne et al. 2000). Apoptosis is induced by both p53-dependent and -independent cell death pathways.

The activity and level of p53 is stimulated in response to numerous stresses, including the presence of E1A proteins (Debbas and White 1993; Lowe and Ruley 1993; Samuelson and Lowe 1997). E1A proteins drive quiescent cells into the S phase of the cell cycle to promote viral replication. This unscheduled cell proliferation induces high levels of p53 resulting in apoptosis (Braithwaite, Nelson et al. 1990; Debbas and White 1993). E1A stimulates p53-dependent apoptosis through a pathway involving $p19^{ARF}$, the tumor suppressor protein (de Stanchina, McCurrach et al. 1998). As mentioned previously, E1A binds to pRb relieving E2F, thus enabling this transcription factor to transactivate genes such as $p19^{ARF}$ (de Stanchina, McCurrach et al. 1998). $p19^{ARF}$ binds to and inhibits Mdm2, preventing Mdm2-mediated proteolysis of p53 (Honda and Yasuda 1999). Therefore, expression of E1A proteins stabilizes the level of p53.

E1A proteins also induce p53-independent apoptosis by enhancing susceptibility to signaling by death receptor ligands, specifically TNF (tumor necrosis factor), Fas and TRAIL (TNF-related apoptosis inducing ligand) (Chen, Holskin et al. 1987; Cook, May et al. 1989; Routes, Ryan et al. 2000). Death receptors are transmembrane proteins found on the cell surface. These receptors induce a signaling cascade upon ligand binding which results in the activation of caspases and apoptosis. Although the mechanism has

not yet been characterized, the sensitivity of TNF-α has been mapped to the CR1 region of E1A proteins (Duerksen-Hughes, Hermiston et al. 1991; Shisler, Duerksen-Hughes et al. 1996).

Although E1A proteins can induce both p53-dependent and -independent cell death, the virus needs to block this apoptotic response in order to replicate. Therefore, E1A also has the ability to prevent programmed cell death. E1A proteins inhibit the transcriptional activation function of p53 by interacting with and sequestering p300 / CBP, an important p53 coactivator (as discussed in section 1.1.6.2.3). Also, E1A proteins induce the expression of polypeptides (E4orf6, E3, and E1B) that are the major viral defense against apoptosis (Sarnow, Ho et al. 1982; Rao, Debbas et al. 1992).

1.1.6.3 E1B Region

The early region 1B (E1B) gene is adjacent to E1A and generates four mRNA species. The two major mRNA species, 22S and 13S, are derived from a common mRNA precursor by alternative splicing. Both 22S and 13S have the same 5' and 3' termini. Translation of the 22S mRNA encodes two polypeptides of 175 residues, E1B 19 kD, and 495 residues, EIB 55 kD (Anderson, Schmitt et al. 1984; Takayesu, Teodoro et al. 1994). The 13S mRNA also produces the 19 kD protein and an 85 aa polypeptide consisting of 75 residues of the 55 kD protein. The two minor mRNA species 14.5S and 14S also encode the 19 kD protein common to the two major species, as well as two 55 kD related species, one consisting of 92 aa (14.5S) and the other 155 aa (14S) (Virtanen and Pettersson 1985).

E1B proteins contribute to cellular transformation by counteracting E1A-mediated growth arrest and apoptosis. The functions of the E1B 55 kD and 19 kD proteins have been the most well characterized. The E1B 55 kD protein prevents p53-dependent apoptosis by acting as a transcriptional repressor (Yew and Berk 1992) targeted to p53-responsive promoters (Yew, Liu et al. 1994) through binding to p53 (Sarnow, Ho et al. 1982). E1B 55 kD interacts with p53 by associating with the amino-terminal, acidic transcriptional activation domain of this protein (Kao, Yew et al. 1990). This association

may antagonize p53-induced apoptosis (Sabbatini, Lin et al. 1995; Teodoro and Branton 1997) and/or cell cycle arrest (Shepherd, Howe et al. 1993; Hutton, Turnell et al. 2000).

As mentioned, E1B 19 kD also prevents apoptosis. E1B 19 kD is capable of inhibiting both p53-dependent and independent (induced by TNF-α, Fas ligand, and TRAIL) apoptosis through mechanisms analogous to the Bcl-2 family of apoptosis regulators (White 2001). E1B 19 kD seems to block apoptosis by preventing most of the mitochondrial signaling events. These activities involve inhibiting cytochrome c and Smac / DIABLO release from mitochondria (Henry, Thomas et al. 2002) by binding to and modulating the proapoptotic proteins Bax, Bak and possibly related BH3-containing members of the Bcl-2 family (Boyd, Malstrom et al. 1994; Farrow, White et al. 1995; Han, Sabbatini et al. 1996), as well as suppressing the activation of caspase-9 and -3 downstream of mitochondria (Henry, Thomas et al. 2002).

1.1.6.4 E2 Region

Early region 2 (E2) is essential for the completion of a productive infection. E2 encodes proteins critical for viral DNA replication. The E2 unit produces two mRNA species, E2A and E2B as a consequence of alternative splicing and poly (A) sites (Berk, Lee et al. 1979). E2A encodes the DNA-binding protein and E2B encodes both the precursor terminal protein (pTP) and the adenovirus DNA polymerase.

The E2A DNA-binding protein is a 59 kD phosphoprotein that migrates in SDS-polyacrylamide gels with an apparent molecular weight of 72 kD. This protein binds in a sequence independent fashion to single-stranded DNA (van der Vliet and Levine 1973). The DNA-binding protein is essential for the elongation stage of DNA replication (Van Der Vliet, Levine et al. 1975).

The E2B precursor terminal protein is synthesized as an 80 kD polypeptide covalently attached to the 5' ends of the viral chromosome and active in initiation of DNA replication (Challberg, Desiderio et al. 1980; Stillman, Lewis et al. 1981). During the assembly of the virions it is processed by proteolysis to generate a 55 kD fragment termed the terminal protein (TP). The TP is covalently attached to the genome (Challberg and Kelly 1981), but the entire protein with cleaved peptide bonds remains associated with

the genomic termini (Schaack, Ho et al. 1990) and plays a critical role in DNA replication (Lichy, Horwitz et al. 1981; Tamanoi and Stillman 1982).

The E2B encoded DNA polymerase protein is biochemically distinct from other known polymerases (Enomoto, Lichy et al. 1981; Field, Gronostajski et al. 1984). This 140 kD protein contains both 5' to 3' polymerase activity for DNA replication and 3' to 5' exonuclease activity for proofreading (Field, Gronostajski et al. 1984). In solution the pTP and polymerase form a heterodimeric complex (Enomoto, Lichy et al. 1981; Lichy, Field et al. 1982; Stillman, Tamanoi et al. 1982; Temperley and Hay 1992), therefore it is expected that they bind together to the origin of replication.

1.1.6.5 E3 Region

The E3 transcription unit encodes at least seven polypeptides and four of these proteins, E3 19 kD, 14.7 kD, 10.4 kD, 14.5 kD, are known to prevent death of infected cells induced by the host immune system (Wold and Tollefson 1998). These E3 encoded proteins inhibit cytolysis of infected cells induced by both CTLs (cytotoxic T lymphocytes) and TNF-α.

CTLs recognize and lyse virus infected cells when a viral peptide antigen is displayed in complex with a class I MHC antigen on the surface of the infected cell. The E3 encoded 19 kD glycoprotein prevents the action of CTLs by binding to the peptide-binding domain of class I MHC antigens and inhibiting the transport of class I MHC antigens from the endoplasmic reticulum (ER) to the plasma membrane (Crystal 1990; Dai, Schwarz et al. 1995). The E3 19 kD protein is able to retain the class I antigen in the ER because this protein contains an ER retention signal that has been mapped to its C-terminal domain (Paabo, Bhat et al. 1987). E3 19 kD also interacts with TAP (Bennett, Bennink et al. 1999) and prevents it from transferring peptides processed in the cytosol to class I antigen retained in the ER. Through inhibiting TAP-mediated loading of class I antigen and diminishing the level of class I antigen on the cell surface, the E3 19 kD protein is able to protect the infected cell against premature lysis by CTLs.

CTLs also induce apoptosis when the Fas ligand, located on their cell surface, interacts with the Fas receptor on a target cell. This type of apoptosis is inhibited by three of the

E3 proteins. The membrane bound E3 14.5 kD and 10.4 kD protein complex can prevent Fas-mediated apoptosis by internalizing and degrading the Fas receptor causing a reduction in the levels of this receptor on the surface of infected cells (Carlin, Tollefson et al. 1989; Tollefson, Hermiston et al. 1998). The E3 14.7 kD protein associates with FLICE, a caspase acting downstream of the Fas receptor, and blocks Fas-ligand induced apoptosis (Chen, Tian et al. 1998).

TNF-α can also induce apoptosis and the subsequent lysis of cells infected with virus. TNF-α is a cytokine that is secreted by activated lymphocytes and macrophages. The E3 14.7 kD polypeptide (Gooding, Elmore et al. 1988) and the E3 14.5 kD/10.4 kD protein complex can independently inhibit TNF-α induced cytolysis (Gooding, Ranheim et al. 1991). Both of these proteins block the activation of cytosolic phospholipase A2, which acts downstream of the TNF-α receptor (Zilli, Voelkel-Johnson et al. 1992; Krajcsi, Dimitrov et al. 1996; Dimitrov, Krajcsi et al. 1997) to stimulate the release of arachadonic acid (AA). AA is associated with the onset of apoptosis in infected cells.

The E3 region also encodes a protein with pro-apoptotic properties. The E3 11.6 kD protein, also termed the adenovirus death protein, is not synthesized from an early promoter like the other E3 proteins (DeJong, Valderrama et al. 1983). Instead, the E3 11.6 kD protein is expressed late. Virus lacking E3 11.6 kD accumulate in infected cells suggesting that it is involved in cell death and the release of the virus once replication is complete.

1.1.6.6 E4 Region

The E4 region is situated at the right end of the virus genome and is transcribed in a leftward direction. The E4 transcription unit encodes at least seven different polypeptides identified according to the order and arrangement of their open reading frames (Rigolet and Galibert 1984). The E4 proteins are termed E4orf1, E4orf2, E4orf3, E4orf3/4, E4orf4, E4orf6 and E4orf6/7 and these polypeptides encompass a diverse set of functions required for several viral processes.

E4orf1 is a 14 kD protein that localizes primarily in the cytoplasm (Weiss, McArthur et al. 1996). This polypeptide induces cell transformation in various rodent cell lines,

including CREF (Javier 1994; Weiss, McArthur et al. 1996), as well as in the mammalian cell line TE85 (Weiss, Lee et al. 1997). Cell transformation by E4orf1 is dependent on its PDZ-binding motif located in the protein's carboxy terminus. This region mediates interactions between E4orf1 and PDZ domain-containing proteins, such as DLG, the mammalian orthologue of *Drosophila* disc large (dlg) tumor suppressor protein (Lee, Weiss et al. 1997; Weiss and Javier 1997; Glaunsinger, Lee et al. 2000). Although the mechanism of E4orf1-induced transformation has not yet been characterized, it has been suggested that PDZ-containing proteins function to suppress abnormal cell growth and E4orf1 targets these proteins for inactivation (Glaunsinger, Weiss et al. 2001).

E4orf2 is a 14.6 kD protein that localizes in the cytoplasm (Dix and Leppard 1993). The functional relevance of this protein has not yet been characterized.

E4orf3 is an 11 kD protein that localizes in the nucleus (Sarnow, Ho et al. 1982). This polypeptide associates with E1B 55 kD resulting in an accumulation of 55 kD in the nucleus and targeting of this protein to various virus-induced nuclear structures (Konig, Roth et al. 1999; Leppard and Everett 1999). E4orf3 also affects the distribution of important transcription and replication factors in the nucleus, termed PML oncogenic domains (PODs). E4orf3 stimulates a reorganization of PODs, with their protein components appearing first in the tracks and then relocalizing to peripheral regions of the virus replication zones (Carvalho, Seeler et al. 1995; Doucas, Ishov et al. 1996). This reorganization enhances the expression of viral genes.

E4orf3/4 is 7.1 kD fusion protein composed of residues from the amino terminus of E4orf3 and the carboxy terminus of E4orf4. The functional importance of this protein has not yet been characterized.

E4orf4 is a 14 kD protein that localizes primarily in the nucleus and induces cell death via a novel pathway. This protein will be discussed in more detail in section 1.2.

E4orf6 is a 34 kD protein that interacts with E1B 55 kD and carries out two essential functions. Firstly, E4orf6, like E4orf3, increases the level of 55 kD in the nucleus and sequesters 55 kD to various virus induced structures (Ornelles and Shenk 1991; Goodrum, Shenk et al. 1996; Lethbridge, Scott et al. 2003). Secondly, the interaction between E4orf6 and E1B 55 kD counteracts the E1A-mediated stabilization of p53 by

enhancing p53 proteolytic degradation through the 26S proteasome (Grand, Grant et al. 1994; Moore, Horikoshi et al. 1996; Nevels, Rubenwolf et al. 1997; Querido, Marcellus et al. 1997). E4orf6 is part of a multiprotein complex that consists of a Cullin-containing E3 ubiquitin ligase, a Cullin family member Cul5, as well as Elongins B and C and the RING-H2 finger protein Rbx1 (Querido, Blanchette et al. 2001). This complex is able to promote p53 ubiquitination *in vitro* in the presence of E1/E2 ubiquitin stimulating and conjugating enzymes (Querido, Blanchette et al. 2001).

E4orf6/7 is a 19.5 kD fusion protein containing residues from E4orf6 and E4orf7. E4orf6/7 complements the ability of E1A proteins to enhance the expression of E2 and cellular genes induced by E2F. E4orf6/7 interacts with free E2F causing E2F to dimerize (Obert, O'Connor et al. 1994) and bind tightly to the two-inverted E2F-binding sites in the E2 early promoter which stimulates transcription (Hardy, Engel et al. 1989; Hardy and Shenk 1989; Huang and Hearing 1989; Raychaudhuri, Bagchi et al. 1989; Marton, Baim et al. 1990).

1.1.7 Adenoviral DNA Replication

Adenoviral DNA replication commences approximately 5 to 8 hours post infection and continues until the host cell dies. *In vivo* studies have shown that the replication process occurs in two stages (Lechner and Kelly 1977). In the first stage, synthesis begins at either terminus of the DNA and continues to the other end of the genome. The products include a duplex consisting of a daughter and parental strand, as well as a single strand of DNA. In the second stage, a compliment to the single stand is produced. The single strand circularizes through annealing of its self-complimentary termini. The circularized DNA has the same structure as the termini of the duplex viral genome. Therefore, it is recognized by the same replication machinery used in the first stage and a second duplex is generated.

Initiation of viral replication occurs when the pre-initiation complex binds to the origin of replication on the viral DNA. The viral genome consists of two terminal repeats, each containing an origin for DNA replication. The origin consists of the terminal 51 residues of the repeats and contains three functional regions: domain A, B

and C. Domain A functions as a minimal origin of replication and within its 18 residues there is a 9 base conserved sequence amongst serotypes that acts a binding sequence for the pTP protein and DNA polymerase complex (discussed in more detail in section 1.1.6.4) (Chen, Mermod et al. 1990; Mul and Van der Vliet 1992; Temperley and Hay 1992). Initiation commences when the first nucleotide to be incorporated into the nascent strand, dCMP, is covalently bound to the pTP protein (Lichy, Horwitz et al. 1981). This pTP-dCMP complex has a free 3'-OH that acts as a primer for replication. The pTP-dCMP association occurs once the pTP-polymerase complex is bound to domain A (Lichy, Horwitz et al. 1981; Tamanoi and Stillman 1982). Domain B and C are not essential for DNA replication, but they enhance the efficiency of the initiation reaction. Domain B consists of residues 19 to 39 and the cellular factor NFI (nuclear factor I) interacts with this region. NFI binds to the DNA polymerase and stabilizes the pTP-polymerase complex at the origin (Bosher, Robinson et al. 1990; Chen, Mermod et al. 1990; Mul, Verrijzer et al. 1990). Domain C consists of residues 40-51 and the cellular factor NFIII (nuclear factor III) binds to this region. NFIII interacts with the pTP and stabilizes the pTP-polymerase complex (van Leeuwen, Rensen et al. 1997).

After the pre-initiation complex is bound to the origin of replication, elongation can begin. Chain elongation depends on three proteins, the DNA polymerase, the single-stranded DNA-binding protein and the cellular protein NFII (nuclear factor II). The DNA binding protein binds to the single stranded displaced DNA during elongation and enhances the processivity of the DNA polymerase (Field, Gronostajski et al. 1984; Lindenbaum, Field et al. 1986; Dekker, Kanellopoulos et al. 1997). The NFII protein has topoisomerase activity (Nagata, Guggenheimer et al. 1983) and is required to overcome DNA structural problems that exist after extensive replication.

1.1.8 Late Gene Expression

Adenoviral late gene expression occurs at the onset of viral DNA replication. The late coding regions are organized into a single transcription unit whose main transcript is 29,000 residues. This transcript is processed by differential poly(A) site utilization and splicing to produce various mRNAs. Based on the use of common poly(A) addition sites,

these mRNAs have been subdivided into five groups termed L1 to L5 (Chow, Roberts et al. 1977; Nevins and Darnell 1978; Ziff and Fraser 1978). The major late promoter controls the expression of L1 to L5 and is more active during late times post infection (Shaw and Ziff 1980). Two components are responsible for the delay in this promoter's activation: a cis-acting modification in the viral chromosome and generation of a virus-coded transcription factor.

The first component responsible for the delay in the activation of the late promoter is the cis-acting modification in the viral chromatin. This modification seems to enable transcription factors required for activation, such as USF or MLTF, to access the late promoter only after the onset of DNA replication (Toth, Doerfler et al. 1992). The second component responsible for stimulation of the late promoter involves a virus-coded transcription factor. Downstream of the initiation region, there are binding sites for several factors that cooperate with the upstream USF / MLTF element to activate the late promoter (Mondesert and Kedinger 1991; Mondesert, Tribouley et al. 1992). The adenovirus IVa2 gene encodes one of these factors (Tribouley, Lutz et al. 1994) which is expressed once an early gene product relieves repression of IVa2.

1.1.9 Host Cell Shut-off

The virus shuts off host protein synthesis when DNA replication commences. Host cell shut off is achieved by two mechanisms. First, the virus selectively transports viral mRNAs from the nucleus to the cytoplasm and prevents cellular mRNAs from exiting the nucleus. These events are mediated by the E1B 55 kD and E4orf6 protein complex (Babiss and Ginsberg 1984; Sarnow, Hearing et al. 1984; Babiss, Ginsberg et al. 1985; Halbert, Cutt et al. 1985; Pilder, Moore et al. 1986; Weinberg and Ketner 1986). E4orf6 shuttles between the cytoplasm and the nucleus, therefore this complex is able to transport mRNAs from the site of synthesis (Goodrum, Shenk et al. 1996; Dobbelstein, Roth et al. 1997) to the cytoplasm.

Secondly, the virus reduces the interferon response (Zhang and Schneider 1993), ensuring that viral mRNAs are translated in the cytoplasm late after infection (Zhang and Schneider 1993). Adenovirus-infected cells activate the cellular protein kinase R (PKR)

(O'Malley, Mariano et al. 1986; Maran and Mathews 1988). PKR phosphorylates and inactivates the eukaryotic initiation factor-2α (eIF-2α) blocking translation. To overcome this, the virus encodes VA RNAs which inhibit PKR activation allowing viral mRNA translation to occur. Also, the transcription factor eIF-4F plays a role in the selective translation of viral mRNAs (Huang and Schneider 1991). eIF-4F is a cap binding protein that initiates translation through its intrinsic helicase activity. During adenoviral infection, eIF-4F is inactivated and translation of cellular mRNAs is prevented (Huang and Schneider 1991). However, translation of viral mRNAs still occurs because these mRNAs contain a 5' untranslated region that does not have extensive secondary structures therefore eIF-4F's helicase activity is not required (Dolph, Huang et al. 1990).

1.1.10 Viral Assembly and Release

Viral assembly occurs once DNA replication is complete and adenoviral structural proteins are synthesized. Trimeric hexon capsomers are assembled from monomers in the cytoplasm and the late viral protein L4 acts as a scaffold (Horwitz, Scharff et al. 1969; Cepko and Sharp 1982). Penton capsomers are also assembled in the cytoplasm. After synthesis, the penton and hexon capsomers accumulate in the nucleus and form empty capsids (Raska and Gallimore 1982; Philipson 1984). The L1 52/55 kD protein enables a viral DNA molecule to enter the capsid (Hasson, Soloway et al. 1989; Hasson, Ornelles et al. 1992; Gustin and Imperiale 1998) and the L3-coded proteinase cleaves several viral proteins, stabilizing the structure and making the virus infectious (Mangel, McGrath et al. 1993; Tihanyi, Bourbonniere et al. 1993; Webster, Hay et al. 1993).

The adenovirus facilitates the escape and spread of viral progeny by disrupting the cellular cytoskeleton, leading to an apoptotic-like form of cell death. Early in the infectious cycle, the intermediate filament vimentin is cleaved which causes the extended system to collapse into the perinuclear region (Zhai, Wang et al. 1988; Defer, Belin et al. 1990). Late in the infectious cycle, the L3 proteinase cleaves the cellular cytokeratin K18 preventing polymerization and formation of filaments (Chen, Ornelles et al. 1993). These events will lead to a form of cell death that resembles apoptosis allowing the virus to escape the cell without activating an inflammatory response by the host immune system.

1.2 E4orf4

1.2.1 Overview

E4orf4 produces a 14 kD protein early after viral infection. The role of E4orf4 was first revealed from studies which demonstrated that this protein induced the hypophosphorylation of E1A and c-fos leading to the downregulation of AP-1 activity and expression of E4 adenoviral genes (Müller, Roberts et al. 1989; Muller, Kleinberger et al. 1992; Bondesson, Ohman et al. 1996; Whalen, Marcellus et al. 1997). Insight into the mechanisms underlying E4orf4 action came with the observation that this protein interacts with the Bα subunit of protein phosphatase 2A (PP2A) present in PP2A holoenzymes which also contain A and C subunits (Kleinberger and Shenk 1993). After this discovery, studies suggested that the biological effects induced by E4orf4 were a result of its association with PP2A. E4orf4 was also shown to play a role in the regulation of alternative splicing through the dephosphorylation and inactivation of SR proteins (Kanopka, Mühlemann et al. 1998). Studies have demonstrated that the interaction between E4orf4 and PP2A is required for E4orf4-mediated cell death (Shtrichman, Sharf et al. 1999; Marcellus, Chan et al. 2000; Roopchand, Lee et al. 2001). E4orf4-induced cytotoxicity is p53-independent and does not require caspase activation (Lavoie, Nguyen et al. 1998; Marcellus, Lavoie et al. 1998; Shtrichman and Kleinberger 1998; Szymborski, Miron et al. In preparation).

1.2.2 E1A-Activated Transcription

1.2.2.1 E4orf4 and AP-1

AP-1 is a transcription factor which binds to both ATF / CREB and AP-1 binding sites present in E1A-inducible promoters and activates transcription (Angel, Hattori et al. 1988; Hai, Liu et al. 1988). AP-1 is composed of proteins from the *jun* and *fos* gene families. The Jun and Fos proteins interact as homo- and heterodimers with the AP-1 binding site (Curran and Franza 1988).

Studies in S49 mouse lymphoma cells have shown that with the addition of cAMP and the expression of E1A proteins, AP-1 activity is enhanced through an increase in the cytoplasmic levels of *c-fos* and *junB* mRNAs (Müller, Roberts et al. 1989). Analysis of mutant adenoviruses in S49 cells has revealed that AP-1 activity is significantly higher in cells infected with an E4orf4 mutant virus, than in cells infected with the wild-type virus. In addition to reducing the transcriptional and translational levels of AP-1, these studies also showed that E4orf4 is responsible for inducing the hypophosphorylated form of c-fos and that this effect is specific since other proteins, like Jun B, are not affected (Muller, Kleinberger et al. 1992). The phosphorylation state of c-fos regulates its activation or repression function as part of the AP-1 transcription factor. Therefore, the hypophosphorylation of c-fos might play a role in E4orf4-mediated downregulation of AP-1.

Insight into the mechanism of E4orf4-induced hypophosphorylation of c-fos came with the observation that immunoprecipitates of S49 cells prepared using anti-E4orf4 antibodies also contained the Bα regulatory subunit, as well as the A and C subunits of protein phosphatase 2A (PP2A) (PP2A will be discussed in more detail in section 1.3) (Kleinberger and Shenk 1993). Studies revealed that the E4orf4 / PP2A complex contained phosphatase activity that was sensitive to low levels of okadaic acid (OA), an inhibitor of PP2A (Kleinberger and Shenk 1993). It was also demonstrated that the presence of OA prevented E4orf4 from inducing the hypophosphorylation of c-fos (Kleinberger and Shenk 1993) implicating the involvement of PP2A. The kinetics data of the hypophosphorylation of c-fos supported the model that PP2A-induced inactivation of a protein kinase, rather than being directly involved (Muller, Kleinberger et al. 1992; Kleinberger and Shenk 1993). Collectively, the E4orf4 / PP2A complex has been shown to reduce cAMP and E1A-mediated induction of AP-1 activity which correlates with underphosphorylated forms of c-fos.

1.2.2.1 E4orf4 and the E4 Region

The major transcription factor involved in activating the E4 promoter is E4F, not AP-1 (Raychaudhuri, Rooney et al. 1987; Rooney, Raychaudhuri et al. 1990), and E4F is

regulated through an E1A- induced phosphorylation event (Raychaudhuri, Bagchi et al. 1989). Studies have shown that E4orf4 autoregulates its expression by inhibiting E1A-induced activation of E4 transcription in both transient transfection experiments and during lytic virus growth (Bondesson, Ohman et al. 1996). The inhibitory activity of E4orf4 was specific for E1A-CR3-dependent transactivation and was relieved by OA suggesting that other transcription factors, like E4F, might be a target of the E4orf4 / PP2A complex, although this idea has not been directly studied (Bondesson, Ohman et al. 1996).

E4 transcription also requires E1A proteins, specifically those containing the CR3 domain (289R) (Bondesson, Svensson et al. 1992). Studies revealed that E4orf4 induced the hypophosphorylation of 289R E1A proteins (Muller, Kleinberger et al. 1992) on sites that were later mapped to serine residues 185 and 188 (Whalen, Marcellus et al. 1997). Studies indicated that both of these residues were hyperphosphorylated in cells expressing a constitutively active form of mitogen-activated protein kinase (MAPK) (Whalen, Marcellus et al. 1997). Furthermore it was revealed, through analysis of E1A-induced transactivation activity, that MAPK-mediated hyperphosphorylation of these residues enhanced expression of the E4 promoter, but had no affect on the E3 promoter. Therefore, although another study implicated that E1A phosphorylation was not very important (Bondesson, Ohman et al. 1996), these results suggested that the E4orf4 / PP2A complex might play a role in dephosphorylating the 289R serine residues causing a reduction in the ability of E1A to activate the E4 promoter (Whalen, Marcellus et al. 1997). As was the case with c-fos, it is not known whether PP2A is directly involved in dephosphorylating these residues or if the phosphatase has an indirect role that involves dephosphorylating the E1A kinase, assumed to be a MAPK-like enzyme (Whalen, Marcellus et al. 1997).

1.2.3 Regulation of Alternative Splicing

SR proteins are a family of required splicing factors essential for constitutive splicing and acting as regulators of alternative splicing (Fu 1995). These proteins have one or two amino terminal RNA recognition domains and a carboxy terminus abundant in

arginine/serine dipeptide repeats which facilitate protein interactions with other members of the splicing machinery (Zahler, Lane et al. 1992; Caceres and Krainer 1993). SR proteins are hyperphosphorylated *in vivo* (Roth, Murphy et al. 1990) and this modification is essential for the initiation of the spliceosome assembly (Mermoud, Cohen et al. 1994; Roscigno and Garcia-Blanco 1995; Xiao and Manley 1997). The phosphorylation state of SR proteins also plays a role in their localization. Phosphorylation of SR proteins seems to release them from their storage sites and direct them to sites of transcription and splicing in the nucleus (Colwill, Pawson et al. 1996; Caceres, Screaton et al. 1998; Misteli, Caceres et al. 1998).

The adenovirus L1 region is an alternatively spliced pre-mRNA in which a common 5' splice site can be joined to two competing 3' splice sites generating two mRNA products: the 52,55 kD mRNA from the proximal 3' splice site or the IIIa mRNA from the distal 3' splice site (Imperiale, Akusjnarvi et al. 1995). L1 splicing is regulated during the infectious cycle, where early after infection only the 52,55 kD mRNA is generated and late in the infectious cycle the IIIa mRNA is synthesized (Chow, Broker et al. 1979; Akusjarvi and Persson 1981; Nevins and Wilson 1981; Svensson and Akusjarvi 1986). Studies have shown that SR proteins purified from uninfected HeLa cells inhibit adenovirus IIIa pre-mRNA by binding to the purine-rich intronic repressor element (3RE) (Kanopka, Muhlemann et al. 1996), preventing U2 snRNP recruitment to the IIIa branch site. However, SR proteins purified from late adenovirus infection are functionally inactivated as IIIa repressors due to virus-mediated dephosphorylation, leading to an accumulation of IIIa mRNAs (Kanopka, Mühlemann et al. 1998). Furthermore, this study revealed that E4orf4 is responsible for inducing the dephosphorylation of SR proteins and E4orf4 has the capacity to stimulate IIIa pre-mRNA splicing *in vitro* and in transiently transfected cells (Kanopka, Mühlemann et al. 1998). Recently, E4orf4 was shown to interact with a subset of hyperphosphorylated SR proteins, including SF2/ASF and Srp30c, confirming its role in the dephosphorylation of SR proteins (Estmer_Nilsson, Petersen_Mahrt et al. 2001). To further investigate how E4orf4 activates a switch in the late adenovirus splicing pattern studies were performed with E4orf4 mutants that fail to bind strongly to PP2A or SF2/ASF and it was revealed that the mutants were not able to

activate IIIa pre-mRNA splicing (Estmer_Nilsson, Petersen_Mahrt et al. 2001). Taken together, these results suggest that the E4orf4 / PP2A complex might dephosphorylate SR proteins, such as SF2/ASF, enhancing IIIa mRNA production.

1.2.4 Characterization of E4orf4-induced Cell Death

Previous studies have demonstrated that E1A proteins induce p53-dependent apoptosis by elevating the levels of p53 (Braithwaite, Nelson et al. 1990; Lowe and Ruley 1993; Querido, Teodoro et al. 1997). Considerable evidence indicates that a major function of E1B is to suppress the E1A-induced cytotoxic effects. Results show that the 55 kD E1B protein binds to p53 (Sarnow, Ho et al. 1982; Yew and Berk 1992) and prevents apoptosis (Teodoro and Branton 1997), as well as p53-induced activation of gene expression (Teodoro, Halliday et al. 1994; Yew, Liu et al. 1994). Whereas, the 19 kD E1B protein blocks apoptosis by a mechanism similar to that of the proto-oncogene Bcl-2 (Rao, Debbas et al. 1992; White, Sabbatini et al. 1992; Boyd, Malstrom et al. 1994; Nguyen, Branton et al. 1994).

Studies have also shown that in the E1B deletion virus E1A 289R induces p53-independent apoptosis (Subramanian, Kuppuswamy et al. 1984; Teodoro, Shore et al. 1995). Expression of the 289R E1A protein in p53-null cells induced some DNA degradation, chromosomal condensation and cytoplasmic blebbing, all features of apoptotic cell death. These results indicated that 289R transactivated expression of an early viral gene that encodes a protein capable of mediating p53-independent apoptosis (Teodoro, Shore et al. 1995). Mutant analysis identified the early viral gene as the E4 region (Marcellus, Teodoro et al. 1996) and the protein was later mapped to E4orf4 (Marcellus, Lavoie et al. 1998). E4orf4 was shown to induce a form of p53-independent apoptotic-like cell death, either when expressed alone or as a consequence of viral infection (Marcellus, Lavoie et al. 1998; Shtrichman and Kleinberger 1998).

Apoptosis, or programmed cell death, is a genetically regulated process that leads cells to commit suicide in response to a variety of stimuli. Apoptosis is a non-inflammatory response characterized by morphological changes including condensation of the nucleus, DNA degradation, plasma membrane blebbing, cytoplasmic shrinkage and

exposure of phosphatidylserine at the outer cell surface (Bellamy, Malcomson et al. 1995). The process of apoptosis is regulated by a number of core death machinery proteins, such as caspases, that induce cell death only under certain conditions. Caspases are cysteine proteases that cleave proteins after aspartic acid residues. There are two major caspase-activating pathways that regulate apoptosis: one is triggered from the cell surface death receptors and the other is initiated by changes in the mitochondrial integrity (Ashkenazi and Dixit 1998; Green and Kroemer 1998; Kroemer and Reed 2000; Joza, Kroemer et al. 2002). The death receptor pathway involves signaling by transmembrane proteins, including Fas/APO-1/CD95 and TNF, which causes activation of caspase-8 leading to stimulation of downstream caspases such as caspase-3, -6 and -7 (Cohen 1997). The mitochondrial pathway involves depolarization of the mitochondrial membrane and cytochrome *c* release. These events lead to the activation of caspase-9 which further stimulates downstream caspases, as well as the production of reactive oxygen species (ROS) (Kim, Zamora et al. 2001; Adams and Cory 2002; Fleury, Mignotte et al. 2002).

Early studies characterizing E4orf4-induced cell death used CHO Chinese hamster ovary cells to demonstrate that the mechanism of cytotoxicity involves many of the classical hallmark features of apoptosis including some DNA degradation, condensed nuclei, membrane blebbing, externalization of phosphatidylserine and cytoplasmic blebbing. However, the results also suggested that E4orf4-induced cytotoxicity may not require activation of caspases. This conclusion was based on the failure to detect poly ADP ribose polymerase (PARP) cleavage and activation of caspase-3, as well as the inability of the wide spectrum caspase inhibitor, zVAD-fmk, to prevent E4orf4-induced cell death (Lavoie, Nguyen et al. 1998). Therefore, in CHO cells a caspase-independent cell death pathway is induced by E4orf4. These results are similar to other pathways that manifest the morphological features of apoptosis, but do not depend on the activation of caspases to mediate cytotoxicity (Xiang, Chao et al. 1996; Boise and Thompson 1997).

Another study has re-addressed this question using both H1299 human non-small cell lung carcinoma cells and 293T human embryonic kidney cells and have indicated that E4orf4 induces activation of caspase-8 through the death receptor pathway. The results

also indicated that E4orf4 mediated the release of cytochrome *c* and the accumulation of ROS (Livne, Shtrichman et al. 2001). Another group also observed cytochrome *c* release and activation of caspase-3 as features of E4orf4 cytotoxicity in human C33A cells, however, the results in this study suggested that caspase activation was not essential for E4orf4-induced cell death, as overexpression of Bcl-2 did not prevent cytotoxicity (Robert, Miron et al. 2002).

Recently our group has characterized E4orf4-mediated cell death in both H1299 and 293T cells. The results indicated that E4orf4 induces a form of cell death that is caspase-independent, accompanied by accumulation of ROS and release of cytochrome *c* (Szymborski, Miron et al. In preparation). The caspase results from this study are different from those of Livne et al. (2001) and the reason for this discrepancy is not clear, one possibility is that the tissue culture conditions were different causing the cells to be more prone to caspase activation. Nevertheless, it seems that E4orf4-induced cell death is characterized by some apoptotic hallmark features including cytochrome *c* release and accumulation of ROS, however caspase activation is not essential.

E4orf4 has also been shown to induce two distinct cell death pathways, described as the nuclear and cytoplasmic pathways. The cytoplasmic pathway involves the c-Src kinase family, as well as stimulation of calpain, a cysteine protease, and is associated with remodeling of the actin cytoskeleton in 293T and C33A cells (Robert, Miron et al. 2002). Our group has shown that E4orf4 induced rounding and swelling of H1299 cells suggesting that these cytotoxic features may be part of the cytoplasmic pathway (Szymborski, Miron et al. In preparation). The nuclear pathway has not yet been fully characterized, although it is known that c-Src is not involved. In previous studies using mammalian (Kornitzer, Sharf et al. 2001) and yeast cells (Roopchand, Lee et al. 2001) it was suggested that E4orf4 induces a G2 / M arrest, which may be a consequence of the nuclear death pathway.

Studies involving forty human cancer cell lines representing most major classes of human tumors have indicated that E4orf4-induced cell death is specific to cancer cells and does not occur in primary cells (Marcellus, Chan et al. 2001b). Therefore, E4orf4

cytotoxicity is p53-independent and cancer cell specific suggesting that this protein may be used to develop small molecule mimetics for cancer therapy.

1.2.5 Role of Protein Phosphatase 2A (PP2A) in E4orf4-mediated Cell Death

As mentioned, the first major insight into the mechanisms underlying E4orf4 action came with the observation that this protein binds to the Bα subunit of PP2A (Kleinberger and Shenk 1993). Previous studies have associated the E4orf4 / PP2A complex with the biological effects induced by E4orf4, including downregulation of stimulated transcription (Kleinberger and Shenk 1993; Bondesson, Ohman et al. 1996) and of alternative splicing (Kanopka, Mühlemann et al. 1998). To determine if this interaction also played a role in E4orf4-induced cell death several groups performed genetic analysis.

Genetic studies with E4orf4 are complex as small deletions introduced into the gene result in an unstable protein that is not able to interact with PP2A and can not be detected in cell extracts (Shtrichman, Sharf et al. 1999; Marcellus, Chan et al. 2000). Therefore, two groups introduced mutations into the E4orf4 sequence both randomly and by selective mutagenesis affecting residues that were highly conserved amongst serotypes (Shtrichman, Sharf et al. 1999; Marcellus, Chan et al. 2000). Both groups examined the functional interaction between the E4orf4 mutants and PP2A by measuring the phosphatase activity, as well as the binding of the mutants to the Bα or C subunits of PP2A. These results were compared to the effect that the mutants had on the induction of cell death. Both groups demonstrated that there was a strong correlation between the binding of E4orf4 to PP2A and mediation of cell death.

The suggestion that E4orf4-induced cell death required an interaction with PP2A was supported by another study involving an antisense PP2A-Bα construct which reduces expression of the endogenous Bα subunit (Shtrichman, Sharf et al. 1999). The effect on E4orf4 cytotoxicity was measured in the presence or absence of this construct and the results indicated that with reduced Bα expression, E4orf4-mediated cell death is decreased. This result is in contrast to recent findings in which inhibition of Bα mRNA by RNAi was found to be toxic (D. Hahn, unpublished results)

Further evidence for the requirement of Bα in E4orf4-induced cell death was presented in studies involving *Saccharomyces cerevisiae* (Roopchand, Lee et al. 2001). Yeast provides a genetically tractable model system to characterize the actions of E4orf4. Similar to mammalian cells, E4orf4 is toxic in yeast, it interacts with the Bα-like subunit, encoded by *CDC55,* and recruits an active PP2A complex (Roopchand, Lee et al. 2001). However, in a *cdc55* deletion strain, E4orf4 does not interact with the A and C subunits of PP2A and the majority of E4orf4's toxicity is abolished (Roopchand, Lee et al. 2001). These results indicate that an interaction with PP2A is required for E4orf4-induced cell death in yeast, similar to that of mammalian cells.

Further insight into this interaction was provided by a genetic study, previously mentioned, in which a large pool of E4orf4 mutants were produced. These mutants were organized into two groups termed class I and II (Marcellus, Chan et al. 2000). Class I mutants fail to bind Bα and associate with PP2A activity and they were highly defective for induction of cell death. Class II mutants bind to the Bα subunit, but they were defective for killing. These results indicated that an interaction with PP2A was necessary for E4orf4-induced cell death, but was not sufficient. Perhaps another E4orf4 function was required or Bα binding may not have been functional in terms of inducing its biological effect on PP2A.

1.2.6 Association with PP2A

Previous studies have shown that E4orf4 interacts with the Bα subunit of PP2A and this interaction is necessary for E4orf4 to elicit its biological effects. None of the studies have shown whether the interaction is direct or if it occurs through an intermediary protein; however, evidence supporting a direct interaction has recently been found using a yeast two hybrid system (Zhang, Marcellus et al. In preparation).

PP2A consists of various B regulatory subunits that define the enzyme's substrate specificity, cellular localization and tissue specificity. Presently, more than twenty mammalian subunits have been identified and they exist in four classes termed B, B', B'' and B''' (discussed in more detail in section 1.3.2). There is little sequence homology between the four classes, however the members of each class are highly homologous.

Studies were performed to determine the specificity of E4orf4's interaction with the Bα subunit, a member of the B class.

In one study 293T cells were co-transfected with plasmids expressing E4orf4 from the immediate early CMV promoter, as well as HA-tagged constructs of five cDNAs encoding the B' isoforms and of the Bα cDNA (Shtrichman, Sharf et al. 2000). E4orf4 was immunoprecipitated with specific antibodies and the immune complexes were analyzed for the presence of various B subunits. This study indicated that E4orf4 interacted with the Bα subunit, as well as several isoforms of the B' class. Mutant analysis was performed to determine if the interaction between E4orf4 and B' family members was required for E4orf4-induced cell death. The results indicated that E4orf4's association with the B' subunits did not contribute to E4orf4-mediated cell death (Shtrichman, Sharf et al. 2000).

Another study analyzed the specificity of E4orf4 binding by co-transfecting H1299 cells with plasmid DNAs expressing amino terminally-tagged forms of E4orf4 and members of the four classes of B subunits (Zhang, Marcellus et al. In preparation). E4orf4 immunoprecipitates were prepared and the immune complexes were examined for the B subunits and vice versa. The results indicated that E4orf4 binds uniquely to all of the members of the B family, but E4orf4 did not associate with any members of the B', B'' or B''' subunits (Zhang, Marcellus et al. In preparation). The reason why these groups had different results is unclear, but one possibility is that B' binding is affected by factors that differ between 293T and H1299 cells. However, it is generally accepted that E4orf4-induced cell death depends only on the interaction between E4orf4 and the Bα subunit.

1.3 PP2A

1.3.1 Protein Phosphatases

Cellular processes are regulated in response to external stimuli. Reversible protein phosphorylation is an essential regulatory mechanism that induces change in the function of intracellular proteins. Protein kinases covalently attach a phosphate group to an amino

acid side chain. Protein phosphatases remove phosphate groups. The functions of a protein can be affected by the addition or removal of a phosphate group.

Protein kinases and phosphatases are categorized into various gene superfamilies. Specifically, protein phosphatases are separated into three structurally distinct gene families, termed PPP, PPM and PTP (Table 1-2) (Barford, Das et al. 1998).

The PPP and PPM families dephosphorylate phosphoserine and phosphothreonine amino acids and include PP1, PP2A, and PP2B of the PPP family and PP2C of the PPM family. Studies suggest that these phosphatases dephosphorylate their substrates in a one step reaction that involves a metal activated water molecule or hydroxide ion.

The PTP family includes phosphatases that dephosphorylate phosphotyrosine residues, as well as a subfamily of dual-specific phosphatases that dephosphorylate all three phosphoamino acids. Most of the PTP family members include receptor-like transmembrane proteins and soluble cytosolic proteins. Previous studies indicate that PTP's dephosphorylate their substrates using a cysteinyl-phosphate enzyme intermediate.

Structural similarity exists between the members of each superfamily, however diversity is generated with the addition of regulatory subunits that define the enzyme's substrate specificity and subcellular localization.

PPM family

PP2C
Arabidopsis ABI1
Arabidopsis KAPP-1
Pyruvate dehydrogenase phosphatase
Bacillus subtilis SpoIIE phosphatase

Protein–tyrosine phosphatase family

Tyrosine-specific phosphatases
Cytosolic, nonreceptor forms
PTP1B, SHP-1, SHP-2
Receptor-like, transmembrane forms
CD45, RPTPμ, RPTPα
Dual-specificity phosphatases
CDC25
Kinase-associated phosphatase
MAP kinase phosphatase-1

PPP family

Catalytic subunit	Regulatory subunits
PP1c	G_M, G_L, M_{110} + M_{21}, NIPP-1, RIPP-1, R110, p53BP2, L5, sds22, RB gene product, inhibitor-1, DARPP-32, inhibitor-2, splicing factor, kinesin-like protein, γ134.5 (Herpes simplex), R5
PP2Ac	A subunit (PR65) B subunit (PR55, PR72, PR61), eRF1, PTPA, SET, polyoma middle and small T antigens, SV40 small T antigen
PP2B	B-subunit, calmodulin, AKAP-79

Novel protein phosphatases of the PPP family

PPP1:	PPY, Ppz1, Ppz2, Ppq1
PPP2A:	PP4, PP6, PPV 6A, sit4, Ppc1, Ppg1
PPP5:	PP5, RdgC

Table 1-2: Nomenclature of protein phosphatases.
[Adapted from (Barford, Das et al. 1998)]

1.3.2 Structure of PP2A

PP2A is a multimeric serine / threonine phosphatase and a member of the PPP family. The core enzyme is a dimer consisting of a structural A subunit that interacts with a catalytic C subunit. This dimer creates a scaffold to which the various regulatory B subunits bind and form the holoenzyme (Figure 1-3) (Janssens and Goris 2001).

The 65 kD A subunit contains 15 non-identical repeats and the 36 kD C subunit interacts with repeats 11-15. The A and C subunits in mammalian cells each have two isoforms, α and β and are ubiquitously expressed. Presently, more than twenty mammalian subunits have been identified and exist in four classes termed B, B', B'' and B'''. The 55 kD B class is encoded by four genes denoted Bα, Bβ, Bγ, Bδ which are expressed in a tissue specific manner (Healy, Zolnierowicz et al. 1991; Mayer, Hendrix et al. 1991; Zolnierowicz, Csortos et al. 1994; Strack, Chang et al. 1999). The B' class is encoded by five genes termed B'α, B'β, B'γ, B'δ, B'ε that produce approximately 13 isoforms (McCright and Virshup 1995; Csortos, Zolnierowicz et al. 1996; McCright, Rivers et al. 1996; Tanabe, Nagase et al. 1996; Tehrani, Mumby et al. 1996; Zolnierowicz S 1996). The B'' class consists of three members designated PR72/130, PR48 and PR59 (Hendrix, Mayer-Jackel et al. 1993; Voorhoeve, Hijmans et al. 1999; Yan, Fedorov et al. 2000). The B''' class contains two recently identified members, striatin and S/G2 nuclear autoantigen (Moreno, Park et al. 2000). Interestingly, all of the B classes bind to repeats 1-10 of the A subunit, but they share little or no sequence homology. Binding of the B subunit to the A/C dimer is regulated by methylation of the C subunit on the carboxy-terminal residue Leu^{309} (Bryant, Westphal et al. 1999; Wei, Ashby et al. 2001). The presence of various regulatory B subunits has been shown to define PP2A's substrate specificity, subcellular localization and tissue specificity.

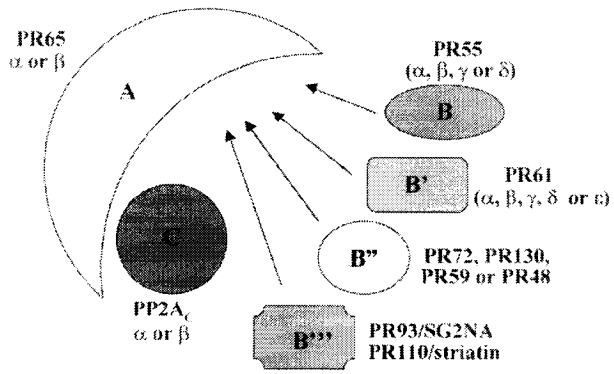


Figure 1-3: Structure of PP2A. [Adapted from (Janssens and Goris 2001)]

1.3.3 Interacting Proteins and Functions of PP2A

1.3.3.1 Overview

PP2A regulates a diverse set of cellular processes and interacts with numerous cellular and viral proteins (Figure 1-4).

PP2A is a regulator of cell cycle progression and is involved in the G2 / M transition (Janssens and Goris 2001). Studies indicate that PP2A negatively effects entry into mitosis by regulating the activity of Wee1 kinase and Cdc25 phosphatase which are responsible for regulating the activity of MPF (mitosis promoting factor) (discussed in section 1.3.3.3).

PP2A also interacts with several viral proteins and plays a role in cellular transformation. Small DNA tumour viruses such as simian virus 40 (SV40) and polyoma virus form complexes with cellular proteins that are involved in signal transduction and growth control in order to change the cells' normal activities. PP2A is one of these proteins and it is targeted by SV40 small t which interacts with the phosphatase by binding to the A/C dimer and replacing the B subunit (Yang, Lickteig et al. 1991; Pallas, Weller et al. 1992; Sontag, Fedorov et al. 1993). This interaction changes the substrate specificity of PP2A and inhibits the enzyme's activity towards certain substrates (Yang, Lickteig et al. 1991).

PP2A is also involved in signal transduction pathways by regulating the activities of several major protein kinase families, including protein kinase B, protein kinase C, the

calmodulin-dependent kinases, as well as ERK MAP kinases. In most cases, PP2A dephosphorylates the kinase and in doing so negatively regulates the protein kinase cascade (Janssens and Goris 2001).

PP2A also plays a role in apoptosis. The activity of Bcl-2, an anti-apoptotic protein, is positively regulated by phosphorylation on Ser^{70}. Phosphorylation on this residue is essential for Bcl-2 to prevent apoptosis. Studies indicate that PP2A dephosphorylates Ser^{70} on Bc1-2 and promotes apoptosis (Deng, Ito et al. 1998).

PP2A regulates many other cellular functions including transcription, RNA splicing, and translation (described in section 1.3.3.2). This phosphatase also interacts with several other viral and cellular proteins as described in Figure 1-4 (Janssens and Goris 2001).

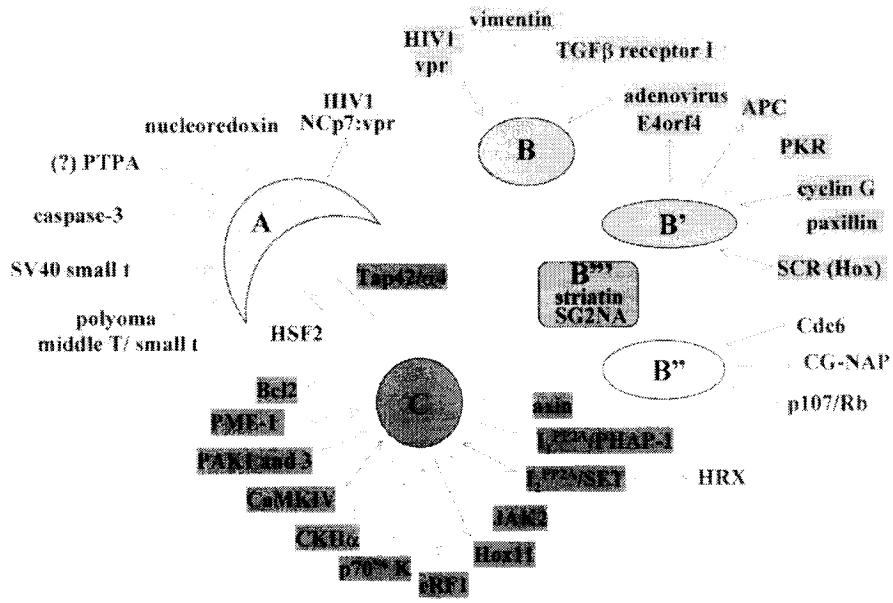


Figure 1-4: PP2A interacts with a variety of viral and cellular proteins. [Adapted from (Janssens and Goris 2001)]

1.3.3.2 PP2A and the Mammalian Target of Rapamycin (mTOR)

The mammalian target of rapamycin (mTOR) is a 289 kD serine / threonine kinase that shares 45% identity with yeast Tor proteins (Brown, Albers et al. 1994; Sabatini, Erdjument-Bromage et al. 1994; Sabers, Martin et al. 1995). The signaling of mTOR is

upregulated in response to increased availability to nutrients, although the mechanism has not yet been characterized (Proud 2002).

Rapamycin, a lipophilic macrolide isolated from a strain of *Streptomyces hygroscopicus*, inhibits the function of mTOR, hence the name target of rapamycin (Vezina, Kudelski et al. 1975). The primary intracellular binding partner of rapamycin is FKBP12 (FK506-binding protein), an abundant, ubiquitously expressed protein (Harding, Galat et al. 1989; Siekierka, Hung et al. 1989). Together this complex binds to the FKBP12-rapamycin binding (FRB) domain of mTOR and inhibits mTOR signaling to downstream targets (Cafferkey, Young et al. 1993; Kunz, Henriquez et al. 1993; Cafferkey, McLaughlin et al. 1994; Helliwell, Wagner et al. 1994).

TOR activity regulates translation initiation in both yeast and mammalian cells, specifically of certain classes of mRNAs. The ribosomal S6 kinases (S6K1 and S6K2) regulate the translation of 5' terminal oligopyrimidine tract (5'TOP) mRNAs. These mRNAs encode ribosomal proteins, as well as other members of the translation machinery (Meyuhas and Hornstein 2000). 5'TOP mRNA translation is stimulated by phosphorylation and activation of $p70^{S6K}$. $p70^{S6K}$ then phosphorylates the 40 S ribosomal protein S6 which initiates translation. TOR signaling is required for the activation of $p70^{S6K}$ and subsequently 5'TOP mRNA translation (Thomas and Hall 1997).

TOR activity also regulates cap-dependent translation. During the initiation of translation a ribosome is recruited to the 5' end of the mRNA and positioned at the start codon. The ribosome relies on a number of translation initiation factors to direct it there. The mRNA 5' end is distinguished by a "cap" structure (m7GpppN, where m is the methyl group and N is any nucleotide), which is recognized by the eukaryotic translation initiation factor 4E (eIF4E). eIF4E interacts with other translation initiation factors and forms a complex which directs the translation machinery to the 5' end of the mRNA. Translation initiation is inhibited when the hypophosphorylated form of 4EBP-1 binds to eIF4E and prevents it from forming the translation initiation complex. TOR signaling induces phosphorylation of 4EBP-1 which prevents it from binding eIF4E and results in cap-dependent translation initiation (Thomas and Hall 1997).

Studies indicate that TOR regulates mRNA translation initiation through a combination of direct phosphorylation of downstream targets and repression of phosphatase activity. PP2A has been indicated as the phosphatase responsible for dephosphorylating 4EBP-1 and $p70^{S6K}$ (Nanahoshi, Nishiuma et al. 1998; Peterson, Desai et al. 1999; Petritsch, Beug et al. 2000; Shah, Kimball et al. 2000). Interestingly, genetic screening in *S. cerevisiae* has identified several phosphatases and a phosphatase associated protein, Tap42p, as components of a rapamycin-sensitive signaling pathway (Di Como and Arndt 1996; Schmelzle and Hall 2000). Studies have shown that phosphorylation of Tap42p regulates its direct interaction with these phosphatases, specifically PP2A and a PP2A-like phosphatase, Sit4p (Jiang and Broach 1999). Tap42p is phosphorylated by the TOR pathway and in this state it competes with the A and B subunits for binding to the catalytic subunit of PP2A (Jiang and Broach 1999). It has been suggested that the interaction between Tap42p and the C subunit alters the substrate specificity and activity of PP2A. An orthologue of Tap42p has been identified in mammalian cells as the B cell receptor binding protein, α4 (Inui, Kuwahara et al. 1995; Onda, Inui et al. 1997). The ability of α4 to interact with phosphatases is conserved in mammalian cells, as this protein binds directly to the catalytic subunits of PP2A (Murata, Wu et al. 1997; Inui, Sanjo et al. 1998), PP4 and PP6 (the orthologue of Sit4p) (Chen, Peterson et al. 1998; Nanahoshi, Tsujishita et al. 1999). The α4 / PP2A complex also showed an increased activity towards specific substrates including phosphorylase *a*, MAPK-phosphorylated myelin basic protein and histone H1. Therefore, studies suggest that TOR signaling initiates translation by generating an interaction between α4 or Tap42p and the C subunit of PP2A in order to stimulate the phosphatase activity towards certain substrates and "repress" it against others, particularly 4EBP-1 and $p70^{S6K}$.

1.3.3.3 PP2A and Cell Cycle Regulation

Cell cycle progression is controlled by the activity of CDKs which are regulated by reversible phosphorylation. MPF (mitosis promoting factor) is one of the most well characterized CDK complexes and it plays a role in the G2 / M transition. MPF consists of a complex between $p34^{cdc2}$ (Cdc2), a serine / threonine protein kinase and cyclin B, a

regulatory protein that is required for Cdc2 kinase activity. Cdc2 is inactive during the S and G2 phase of the cell cycle and activated in mitosis.

The level of Cdc2 is constant throughout the cell cycle, whereas cyclin B is degraded at the end of mitosis and resynthesized during the S and G2 phase. During G2, Cdc2 associates with cyclin B and is phosphorylated on three residues, Thr^{161}, Thr^{14} and Tyr^{15}. This phosphorylated complex is inactive and termed pre-MPF. Thr^{161} is phosphorylated by CAK (CDK-activating kinase), Thr^{14} is phosphorylated by the dual specificity kinase Myt1 and Tyr^{15} is phosphorylated by Wee1 kinase and / or Myt1. The pre-MPF complex becomes active once the inhibitory Thr^{14} and Tyr^{15} residues are dephosphorylated by Cdc25, a dual specificity phosphatase. During mitosis, when the MPF complex is active it phosphorylates numerous proteins involved in various mitotic processes including vimentin, lamins and histone H1. MPF is inactivated at the end of mitosis when Thr^{161} is dephosphorylated, cyclin B is degraded and substrates of MPF are dephosphorylated (Janssens and Goris 2001) (Figure 1-5).

Studies using OA first indicated that PP2A plays a role in the regulation of the G2 / M transition. Treatment of *Xenopus* (Goris, Hermann et al. 1989; Goris, Hermann et al. 1989) or starfish oocytes (Picard, Capony et al. 1989) with OA induced an active MPF complex suggesting that PP2A activity is required to maintain MPF in its inactive form. Other studies supported this theory by demonstrating that PP2A inhibited the CAK pathway which leads to the phosphorylation of Thr^{161}, the residue required for MPF activation (Lee, Turck et al. 1994). Genetic evidence also suggested that PP2A maintains MPF in its inactive form by dephosphorylating and positively regulating Wee1, the kinase responsible for the inhibitory phosphorylation of Tyr^{15} (Kinoshita, Yamano et al. 1993). PP2A has also been implicated in negatively regulating Cdc25, the phosphatase responsible for dephosphorylating the inhibitory residues Thr^{14} and Tyr^{15}, as studies have shown that PP2A dephosphorylates the hyperphosphorylated form of Cdc25 *in vitro* (Clarke, Hoffmann et al. 1993). Taken together, these results indicate that PP2A negatively regulates the G2 / M transition by maintaining MPF in its inactive form.

PP2A has also been implicated to play a role in the exit from mitosis. Studies have shown that PP2A is the major phosphatase involved in dephosphorylating MPF substrates

Inhibitor	Source	Inhibitory potency
Okadaic acid	*Dinoflagellates*	PP2A ~ PP4 > PP1 ~ PP5 >>> PP2B
Dinophysistoxin-1	*Dinoflagellates*	PP2A > PP1 >>> PP2B
Microcystins	cyanobacteria	PP2A ~ PP1 >>> PP2B
Nodularins/Motuporin	cyanobacteria	PP2A ~ PP1 >>> PP2B
Calyculin A	isolated from marine sponges	PP2A > PP1 >>> PP2B
Tautomycin	*Streptomyces spiroventricillatus*	PP1 > PP2A >>> PP2B
Cantharidin	blister beetles	PP2A > PP1 >>> PP2B
Endothall	synthetic compound	PP2A > PP1 >>> PP2B
Fostriecin	*Streptomyces pulveraceus* subsp. *fostreus*	PP2A ~ PP4*
TF-23A	isolated from marine red alga	PP2A
Cytostatin	*Streptomyces sp.* MJ654-NF4	PP2A
I_1^{PP2A}	cellular inhibitor	PP2A
I_2^{PP2A} (SET, PHAP-II, TAF-1β)	cellular inhibitor	PP2A

Table 1-3: Inhibitors of PP2A. [Adapted from (Zolnierowicz 2000)]

1.4 Thesis Proposal

The adenoviral protein E4orf4 was shown to interact with the Bα subunit of the holoenzyme of PP2A (Kleinberger and Shenk 1993). Several studies suggested that the biological effects induced by E4orf4 were a result of its association with PP2A. Recent work also demonstrated that the interaction between E4orf4 and Bα is required for E4orf4-mediated cell death (Shtrichman, Sharf et al. 1999; Marcellus, Chan et al. 2000; Roopchand, Lee et al. 2001). Based on these studies, it was assumed that E4orf4 functions by redirecting or relocating PP2A to modify or enhance substrate specificity. Thus, the goal of this project was to determine the effects of E4orf4 on PP2A activity, as well as the role of PP2A in E4orf4-mediated cell death. Studies were performed in H1299 cells to examine the effect of E4orf4 on PP2A activity towards specific substrates. In addition, PP2A inhibitors were used to determine if E4orf4-induced cell death required PP2A activity and to study the role of PP2A in E4orf4-mediated G2 / M arrest.

The most widely used cell permeable phosphatase inhibitor is OA, a complex polyether derivative of a 38-carbon fatty acid. OA is produced by marine dinoflagellates and accumulates in filter feeding organisms, such as shell fish (Bialojan and Takai 1988). OA specifically inhibits several serine / threonine phosphatases, but each phosphatase is inhibited to a different degree. PP2A, PP4 and PP5 are the most sensitive to inhibition by OA followed by PP3, PP1 and PP2B (Cohen, Holmes et al. 1990; Brewis, Street et al. 1993; Chen, McPartlin et al. 1994). OA inhibits PP2A by binding directly to the enzyme's catalytic subunit (Zhang, Zhao et al. 1994). This inhibitor also prevents methylation of the C subunit on the carboxy-terminal residue Leu^{309}, suggesting that OA binds to the catalytic subunit and blocks access of a carboxy methyltransferase to its target site (Floer and Stock 1994; Li and Damuni 1994).

In addition to OA, there are several other naturally occurring compounds that inhibit PP2A (Figure1-7). These compounds, like OA, inhibit various serine / threonine phosphatases to differing degrees, for example PP2A is ten-fold more sensitive to inhibition by microcystin than PP1, whereas the reverse is true for tautomycin (Takai, Sasaki et al. 1995; Gupta, Ogawa et al. 1997). Studies using computational analysis have demonstrated that the majority of these inhibitors contain comparable three-dimensional structures that bind to the catalytic subunit and inhibit the phosphatase (Bagu, Sykes et al. 1997; Gauss, Sheppeck et al. 1997; Gupta, Ogawa et al. 1997).

Furthermore, two heat stable protein inhibitors of PP2A, termed I_1^{PP2A} and I_2^{PP2A}, were purified from bovine kidney. I_1^{PP2A} is a 30 kD protein that is a homologue of the human putative histocompatibility leukocyte antigen class II associated protein-1 (PHAP-1). I_2^{PP2A} is a 39 kD protein that is a homologue of the human SETα protein (Li, Makkinje et al. 1996; Li, Makkinje et al. 1996). Both of these proteins specifically inhibit all holoenzyme forms of PP2A, presumably by binding to the catalytic subunit (Li, Makkinje et al. 1996). Studies have suggested that the highly acidic C-terminus region shared by I_1^{PP2A} and I_2^{PP2A} is responsible for mediating inhibition of PP2A (Li, Makkinje et al. 1996; Li, Makkinje et al. 1996). Thus a considerable amount of information has been obtained on PP2A and the regulatory B subunits that E4orf4 targets.

Inhibitor	Source	Inhibitory potency
Okadaic acid	*Dinoflagellates*	PP2A ~ PP4 > PP1 ~ PP5 >>> PP2E
Dinophysistoxin-1	*Dinoflagellates*	PP2A > PP1 >>> PP2B
Microcystins	cyanobacteria	PP2A ~ PP1 >>> PP2B
Nodularins/Motuporin	cyanobacteria	PP2A ~ PP1 >>> PP2B
Calyculin A	isolated from marine sponges	PP2A > PP1 >>> PP2B
Tautomycin	*Streptomyces spiroventricillatus*	PP1 > PP2A >>> PP2B
Cantharidin	blister beetles	PP2A > PP1 >>> PP2B
Endothall	synthetic compound	PP2A > PP1 >>> PP2B
Fostriecin	*Streptomyces pulveraceus* subsp. *fostreus*	PP2A ~ PP4*
TF-23A	isolated from marine red alga	PP2A
Cytostatin	*Streptomyces sp.* MJ654-NF4	PP2A
I_1^{PP2A}	cellular inhibitor	PP2A
I_2^{PP2A} (SET, PHAP-II, TAF-1β)	cellular inhibitor	PP2A

Table 1-3: Inhibitors of PP2A. [Adapted from (Zolnierowicz 2000)]

1.4 Thesis Proposal

The adenoviral protein E4orf4 was shown to interact with the Bα subunit of the holoenzyme of PP2A (Kleinberger and Shenk 1993). Several studies suggested that the biological effects induced by E4orf4 were a result of its association with PP2A. Recent work also demonstrated that the interaction between E4orf4 and Bα is required for E4orf4-mediated cell death (Shtrichman, Sharf et al. 1999; Marcellus, Chan et al. 2000; Roopchand, Lee et al. 2001). Based on these studies, it was assumed that E4orf4 functions by redirecting or relocating PP2A to modify or enhance substrate specificity. Thus, the goal of this project was to determine the effects of E4orf4 on PP2A activity, as well as the role of PP2A in E4orf4-mediated cell death. Studies were performed in H1299 cells to examine the effect of E4orf4 on PP2A activity towards specific substrates. In addition, PP2A inhibitors were used to determine if E4orf4-induced cell death required PP2A activity and to study the role of PP2A in E4orf4-mediated G2 / M arrest.

Chapter 2: Materials and Methods

2.1 Cell culture.

H1299 ($p53^{-/-}$) human non-small-cell lung carcinoma cells (ATCC CRL-5803) were cultured at 37°C in Dulbecco's modified minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (Bio-Whittaker), 100 units/ml penicillin, 100 units/ml streptomycin, 0.292 mg/ml L-glutamine, in a humidified 5% CO_2 atmosphere.

2.2 DNA transfection.

H1299 cells were grown in 60 mm dishes to about 60% confluence and transfected with the liposome reagent DMRIE-C (BAbCO), according to the manufacturer's instructions.

2.3 DNA plasmids.

A plasmid DNA expressing FLAG-tagged heat stable PP2A inhibitor, I_1^{PP2A}, was cloned as follows. Reverse transcription of total RNA from MCF-7 cells was carried out using SuperScriptII (Invitrogen) according to the manufacturer's protocol, followed by polymerase chain reaction (PCR) using Taq Plus DNA Polymerase (Stratagene) to isolate coding sequences for I_1^{PP2A} and the following primers: forward – (5') ATG GAG ATG GGC AGA CGG ATT CAT TTA GAG (3') and reverse - (5') TTA GTC ATC ATC TTC TCC CTC ATC TTC AGG (3') (Invitrogen). The PCR product was then cloned into the vector pDrive (Qiagen) and sequenced. For the purpose of mammalian expression, the I_1^{PP2A} coding sequence was subcloned into pcDNA3-FLAG using the restriction enzymes *Not*I (MBI Fermentas) and *Acc*651 (MBI Fermentas).

The construct expressing the PP2A Cα subunit point mutant Y307F was created by a two-step PCR procedure. The first round of PCR involved two independent reactions, one with the primers *Bst*EII forward - (5')GCT CCA AAC TAT TGT TAT CGT TGT GG(3') and Y→F reverse - (5')CTT ACA GGA AGA AGT CTG GGG TAC G(3') and the other with the primers Y→F forward - (5')CGT ACC CCA GAC TTC TTC CTG TAA G(3') (Invitrogen) and Sp6 reverse. Then the two PCR products were mixed and another

round of PCR was carried out using the *Bst*EII forward primer and the Sp6 reverse primer. After the second round of PCR, the product was digested using the restriction enzymes *Bst*EII (New England Biolabs) and *Not*I (MBI Fermentas) and subcloned into pcDNA3 HA-PP2A-Cα to replace the wild-type region.

The construct expressing GFP-HA-E4orf4 was generated by digesting pHAE4orf4hygro with *Pme*I followed by subcloning the resulting HA-E4orf4 fragment into the multiple cloning site (MCS) of the pEGFP-C1 vector (ClonTech Laboratories) that had been previously digested with *Sma*I.

Hemagglutinin (HA)-tagged Ad2 E4orf4 and FLAG-tagged Ad2 rat Bα subunit were expressed using pcDNA3-based plasmid DNAs as described previously (Marcellus, Chan et al. 2000).

2.4 Adenoviruses vectors.

In most cases HA-E4orf4 was expressed using adenovirus vector AdTRex-HA-E4orf4 that expresses HA-E4orf4 under the control of the cytomegalovirus (CMV) promoter (described in detail in Marcellus *et al.*, in preparation). Cells were infected at a multiplicity of infection (moi) of 10-50 plaque-forming units (pfu) per cell, a level sufficient to allow expression in most cells of the culture. In some studies, a two-vector system was used, involving co-infection with adenovirus vector Ad-HA-E4orf4 that expresses HA-E4orf4 under the control of the tetracycline operator (tetO) (Gossen and Bujard 1992; Gossen, Freundlieb et al. 1995) and with Ad-rtTA that expresses the reverse tetracycline transactivator (rtTA) under the control of the CMV promoter (Marcellus *et al.*, in preparation). With this system rtTA drives gene expression from the tetO in the presence of 1 mM doxycycline, but not in its absence. Ad-rtTA alone was used in some cases as a control for virus infection. Some studies utilized adenovirus vector Ad-SV40-ST, which expresses SV40-ST under control of the CMV promoter, and Ad-CMV as a control (Porras, Bennett et al. 1996). In most studies a moi of 50 pfu/cell was employed.

2.5 Measurement of PP2A phosphatase activity *in vitro*.

To measure the activity of PP2A *in vitro*, H1299 cells were infected with AdTRex-HA-E4orf4 and at 18 hours post-infection (h.p.i.) cell extracts were prepared for immunoprecipitation. Extracts were prepared simultaneously from H1299 (HA-Bα) cells, which stably express HA-Bα. Half of each extract was immunoprecipitated using mouse monoclonal anti-HA antibody to isolate HA-E4orf4 complexes containing bound Bα and associated PP2A holoenzymes, or HA-Bα-containing PP2A holoenzymes in the absence of E4orf4, respectively. The other half of the extracts was immunoprecipitated with a control monoclonal antibody, 7-34-1. Immune complexes were washed twice in wash buffer (50 mM Tris-HCl, pH 8.0, 0.5% Triton X-100, 3 mM $MgCl_2$, 150 mM NaCl) and twice with buffer A (50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% b-mercaptoethanol, 1 mg/ml bovine serum albumin). One-third of each immunoprecipitate was analyzed by SDS-PAGE and immunoblotted with anti-HA monoclonal antibody to detect HA-E4orf4 or HA-Bα, and with anti-PP2A C subunit monoclonal antibody (BD Biosciences). The relative amounts of C subunit in the various immunoprecipitates were quantified using a Biorad Fluor S-Max chemilumimager that directly measures band intensities via a supercooled CCD camera without the use of film that provides linear data over 4.8 orders of magnitude. This method yielded highly reproducible results that did not vary with image capture times. The other two-thirds of each immunoprecipitate was divided up into two equal aliquots and used to assay PP2A activity in duplicate. The phosphatase activities of the E4orf4-associated PP2A samples were normalized to the activity of an equivalent amount of HA-Bα-associated PP2A C subunit analyzed in parallel. Phosphorylase *a* ATP(g-^{32}P)-labeled substrate was prepared from phosphorylase *b* and used according to the manufacturer's instructions (GibcoBRL). Histone H1 was phosphorylated in the presence of ATP(g-^{32}P) by $p34^{cdc2}$ enzyme (New England Biolabs) and used as described elsewhere (Pearson, Dennis et al. 1995). Assays were performed at a linear range and with sub-saturating amounts of each substrate. PP2A activity was calculated by subtracting the average phosphatase activity in duplicate 7-34-1 control immunoprecipitates, which represented the level of non-specifically associated phosphatase, from the average phosphatase activity of duplicate experimental samples.

2.6 Immunoprecipitation.

Cell extracts prepared in 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.5% NP-40, 1 μg/ml aprotinin and 1 μg/ml PMSF were clarified at 13000 x g for 10 min and then immunoprecipitations were carried out as described previously (Marcellus, Chan et al. 2000). Several studies employed mouse anti-HA.11 monoclonal antibody 12CA5 (BAbCo) that recognizes the HA tag.

2.7 Western blotting.

In most cases, whole cell extracts were prepared by resuspending cells in lysis buffer (10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 50 mM KCl, 2 mM $MgCl_2$, 100 μg/ml leupeptin, 100 μg/ml aprotinin and 100 μg/ml pepstatin A). Protein concentrations were quantified with the Bio-Rad system (Biorad Laboratories). Equal amounts of protein were resolved by SDS-PAGE using 12-16% polyacrylamide gels. Proteins were transferred to nitrocellulose or PVDF membranes (Millipore Corporation or Fisher) and immunoblotted using appropriate antibodies, including mouse monoclonal anti-HA.11 antibody (16B12, BAbCO), mouse monoclonal anti-Flag M2 antibody (Kodak/Sigma-Aldrich), mouse monoclonal anti-GFP antibody (B-2, Santa Cruz), and rabbit polyclonal anti-4EBP-1 antibody (Gingras, Svitkin et al. 1996), all at a 1/1,000 dilution. Visualization was completed using anti-mouse or anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) (Jackson ImmunoResearch Laboratories, Inc.), all at a 1/1,000 or 1/5000 dilution, followed by enhanced chemiluminescence (ECL) detection (Amersham Corporation or Pierce).

In certain cases whole cell extracts were prepared using lysis buffer (130 mM Tris-HCl, pH 7.4, 2% SDS, and 1% β-MSH) followed by sonication. Equal volumes of protein were separated by SDS-PAGE and analyzed by western blotting using appropriate antibodies, including rabbit polyclonal anti-$p70^{S6K}$ antibody (C-18, Santa Cruz, Inc.) and a rabbit polyclonal antibody that recognizes phosphothreonine-389 of $p70^{S6K}$ (Cell Signaling Technology, Inc.), both at a 1/500 dilution. To detect myosin light chain (MLC), MLC-specific mouse monoclonal antibody (Sigma) was used at a 1/1000 dilution

and rabbit polyclonal anti-MLC phospho-specific antibody (Ratcliffe, Smales et al. 1999) was used at a dilution of 1/500 to recognize the phoshorylated form. To detect the PP2A Cα subunit, mouse monoclonal anti-PP2A Cα antibody was used at a 1/10,000 dilution, and mouse monoclonal antibody 4b7 (Upstate, Inc. or Santa Cruz Biotechnologies), which recognizes unmethylated PP2A C subunit (Yu, Du et al. 2001), was used at a dilution of 1/4000. Proteins were visualized using HRP-conjugated anti-mouse or anti-rabbit secondary antibodies at a 1/1000 dilution (except for the anti-phosphoT-389 antibody that required a dilution of 1/10,000), followed by ECL. To ensure equal loading of lysates all membranes were re-probed with mouse monoclonal anti-actin antibody (C4, ICN Biomedicals, Inc.) at a 1/1000 dilution and incubated with an HRP-conjugated anti-mouse secondary antibody at a 1/1000 dilution to ensure equal loading.

2.8 Growth inhibition death assay.

To measure cell viability, H1299 cells were grown in 60 mm dishes to about 80% confluence and were co-infected with Ad-HA-E4orf4 and Ad-rtTA at a multiplicity of infection (moi) of 100 plaque-forming units (pfu) per cell in the presence of 1 mM doxycycline. Cells were also mock infected or infected with Ad-rtTA alone as a control. At the time of infection, cells were treated with 3 or 5 nM okadaic acid (OA) (LC Laboratories) dissolved in ethanol. Control cells were treated with the same level of ethanol. At 48 h.p.i. the culture supernatant was removed and cells were washed with PBS. The adherent cells were then removed by trypsin and counted. Therefore, cell killing was evaluated by a reduction in adherent cells relative to the controls.

2.9 Nuclear morphology cell death assay.

H1299 cells were grown on 0.2% gelatin-coated coverslips in 35 mm dishes to a density of 7.5 x 10^4 cells/well and transfected with appropriate plasmid DNAs expressing Green Fluorescent Protein (GFP) or GFP-HA-E4orf4 fusion products using the DMRIE-C reagent (BAbCO). At 48 hours post-transfection (h.p.t), cells were washed with PBS, fixed for 20 min in 4% formaldehyde/PBS (Polysciences, Inc.), then washed again and post-fixed overnight in 70% ethanol at 4°C in the dark. The next day the cells were

washed and stained with 4'-6-diamidino-2-phenylindole-dihydrochloride (DAPI) (Molecular Probes, Inc.) for 20 minutes, washed again, and mounted on microscope slides using VectaShield mounting media (Vector Laboratories, Inc.). The number of cells containing shrunken condensed nuclei by DAPI staining was counted only in GFP-positive cells, as detected by direct fluorescence microscopy.

2.10 Cell cycle analysis by fluorescence-activated cell sorting (FACS).

H1299 cells were plated at a density of 1.5 x 10^5 cells per 60 mm dish and after 24 h they were infected with adenovirus vectors, as described above, at a moi of 50 pfu/cell. At 48 h p.i. floating and adherent cells were collected, washed with PBS and resuspended in 500 µl of PBS. To fix the cells, the suspension was rapidly pipeted into 500 µl of 100% ethanol and left on ice for 15 min. The fixed cells were then pelleted and resuspended in 100 µl of PBS and 25 µl of RNAse (10 mg/ml). After 40 min at 37°C, the samples were resuspended in 500 µl of PBS plus 2.5 µl propidium iodide (2 mg/ml). FACS analysis was conducted using a FACScan instrument employing the cell quest program and the data was analyzed using Modfit software.

2.11 Immunoprecipitation-kinase assay.

H1299 cells were grown in 60 mm dishes to about 80% confluence and after 24 h they were infected with adenovirus vectors, as described above, at a moi of 50 pfu/cell. At 48 h.p.i. floating and adherent cells were collected, lysed as described above and 600 µg of whole cell extract was immunoprecipitated with 1 µg of mouse monoclonal cyclin B antibody (Santa Cruz) and protein A Sepharose beads (50% slurry). Precipitates were washed five times in lysis buffer and once in kinase assay buffer (50 mM HEPES, pH 7.5, 1 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT). Each precipitate was incubated for 30 minutes at 30°C in 20 µl of reaction mix containing kinase assay buffer, 5 µM cold ATP, 10 µg H1 Histone (Sigma), and 10 µCi $\gamma[P^{32}]$-ATP (6000 Ci/mmol specific activity). Kinase reactions were stopped by adding 10µl of 4 x sample buffer followed by boiling for five minutes. Reactions were subjected to SDS-PAGE and transferred to PVDF. To visualize the level of H1 phosphorylation, membranes were exposed to film (Kodak, Biomax MR).

Chapter 3: Experimental Results

3.1 Effect of E4orf4 on PP2A activity measured *in vitro*.

Previously, the effect of E4orf4 on the activity of PP2A was examined *in vitro*. HA-E4orf4 was expressed in H1299 cells using the adenovirus vector AdTRex-HA-E4orf4 and Bα was expressed using plasmid DNA expressing FLAB-Bα. E4orf4 complexes with associated Bα-containing PP2A holoenzymes were purified by immunoprecipitation using anti-FLAG antibody and PP2A activity against the small universal phosphopeptide substrate was measured in the presence or absence of E4orf4. The binding of E4orf4 did not appear to have any significant effect on PP2A activity towards this substrate (R.Marcellus, data not shown); therefore similar assays were carried out to examine the effect of E4orf4 on PP2A activity against more physiological substrates. HA-E4orf4 was expressed using the adenovirus vector AdTRex-HA-E4orf4 in H1299(HA-Bα) cells that stably express HA-tagged rat Bα, which is identical in amino acid sequence to human Bα. Extracts from the H1299(HA-Bα) cells were immunoprecipitated with anti-HA.11 antibody to purify Bα-containing PP2A holoenzymes. PP2A phosphatase activity was then measured *in vitro* and normalized to levels of the C subunit, as described in Materials and Methods. The assays were performed using either purified ^{32}P-labeled phosphorylase *a* or histone H1. Fig. 3-1A shows that the presence of E4orf4 decreased PP2A activity by about 2-fold against phosphorlylase *a*. Fig. 3-1B demonstrates that in the case of p34^{cdc2}-phosphorylated histone H1, the presence of E4orf4 decreased PP2A activity by about 4-fold. These results suggested that binding of E4orf4 to the Bα subunit inhibits Bα-mediated PP2A activity in a substrate-specific manner.

3.2 Effect of E4orf4 on PP2A substrates 4EBP-1 and p70^{S6K} *in vivo*.

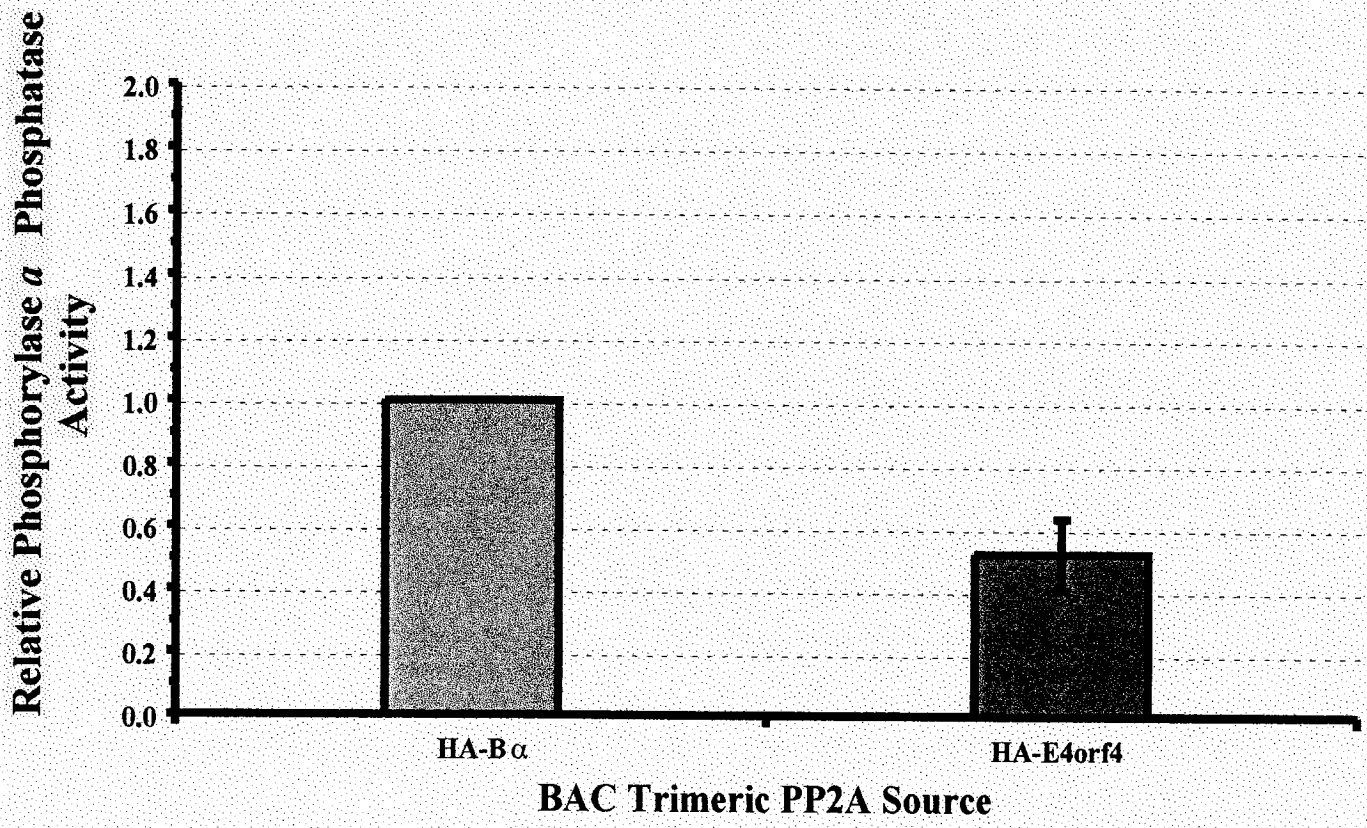
To determine the effect of E4orf4 on the dephosphorylation of two PP2A substrates *in vivo*, H1299 cells were transfected with plasmid DNA expressing HA-E4orf4 or with control pcDNA3 and 48 h.p.t. the cells were harvested. Cell extracts were separated by SDS-PAGE and immunoblotted using the appropriate antibodies. Western blotting using

Figure 3-1: E4orf4 reduces PP2A activity towards phosphorylase *a* and histone H1 *in vitro.*

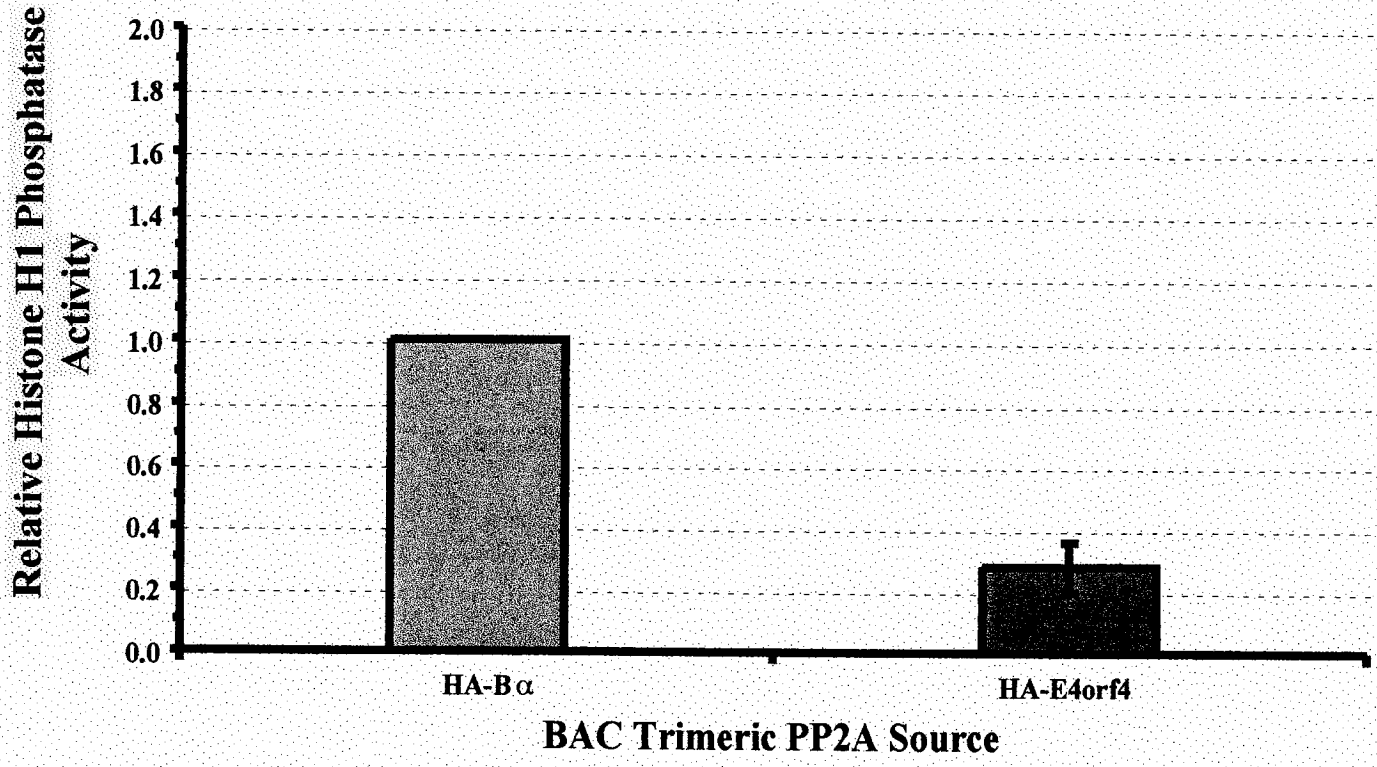
A. H1299 cells were infected with adenovirus vector AdTRex-HA-E4orf4 and collected 18 h later. Simultaneously H1299 (HA-Bα) cells were harvested. Both sets of cell extracts were immunoprecipitated using anti-HA antibody. Samples were analyzed for phosphatase activity *in vitro*. Release of $^{32}PO_4$ from ^{32}P-labeled phosphorylase *a* was measured as described in Materials and Methods. The combined results of 5 separate experiments are shown with activity obtained in the presence of E4orf4 set at 100%. The error bar indicates standard deviation.

B. Studies similar to those of Fig. 3-1A were conducted. The combined results of 5 separate experiments are shown with activity obtained in the presence of E4orf4 set at 100%. The error bar indicates standard deviation.

A


Relative Phosphorylase a Phosphatase Activity
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
HA-Bα
HA-E4orf4
BAC Trimeric PP2A Source

B


Relative Histone H1 Phosphatase Activity
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
HA-Bα
HA-E4orf4
BAC Trimeric PP2A Source

anti-4EBP-1 antibody was used to examine the migration of 4EBP-1. 4EBP-1 is inactivated by phosphorylation via the TOR pathway, thus facilitating protein synthesis. Dephosphorylation of 4EBP-1 by PP2A stimulates 4EBP-1 binding to eIF4E, thus blocking cap-dependent translation initiation (Peterson, Desai et al. 1999). Fig. 3-2A shows that greater amounts of highly phosphorylated, and thus more slowly migrating (Pause, Belsham et al. 1994; Gingras and Sonenberg 1997), 4EBP-1 species were present in cells expressing E4orf4 (upper panel, lane 2) than in the control cells (upper panel, lane 1). Similar results were obtained using cells infected with adenovirus vectors expressing E4orf4 (data not shown). The mTOR kinase also activates $p70^{S6K}$, which phosphorylates the S6 40S ribosomal protein to enhance translation of 5'TOP mRNAs. Bα-containing PP2A holoenzymes are known to act on this substrate to negatively regulate translation (Ballou, Jeno et al. 1988; Peterson, Desai et al. 1999; Petritsch, Beug et al. 2000). The phosphorylation status of $p70^{S6K}$ was determined using antibodies that recognize either all forms of $p70^{S6K}$ or uniquely the active form phosphorylated at T389 (Ferrari, Bannwarth et al. 1992; Han, Pearson et al. 1995; Pearson, Dennis et al. 1995). Fig. 3-2B shows that all cells expressed an equal level of $p70^{S6K}$ (middle panel, lane 2 compared to lane 1), but that considerably higher levels of phosphorylated $p70^{S6K}$ were present in E4orf4 expressing cells (upper panel, lane 2 compared to lane 1). These results were consistent with the idea that E4orf4 reduces the activity of Bα-containing PP2A holoenzymes *in vivo* against two components of the signaling pathway that regulates initiation of protein synthesis. Possible implications of this effect in terms of E4orf4 killing are discussed below in the Discussion. Importantly, these observations were clearly consistent with *in vitro* results shown in Figs. 3-1A and B and indicated that binding of E4orf4 appears to reduce the activity of Bα-containing PP2A holoenzymes against certain substrates.

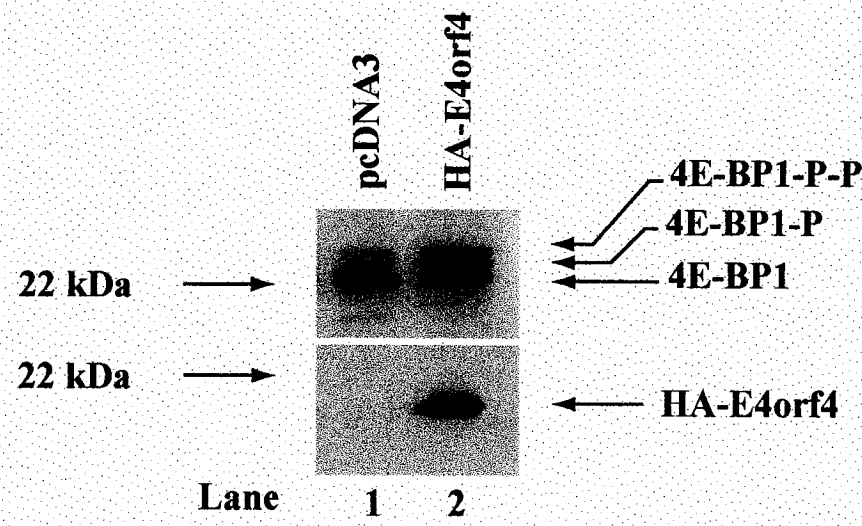
3.3 PP2A inhibitors enhance the toxicity of E4orf4.

To determine if inhibition of PP2A activity plays a role in E4orf4-induced cell killing, studies were carried out using reagents known to block PP2A activity. Although we believe that E4orf4 only binds to and inhibits Bα-containing PP2A holoenzymes, global PP2A inhibitors could still be informative as they might enhance E4orf4-induced cell

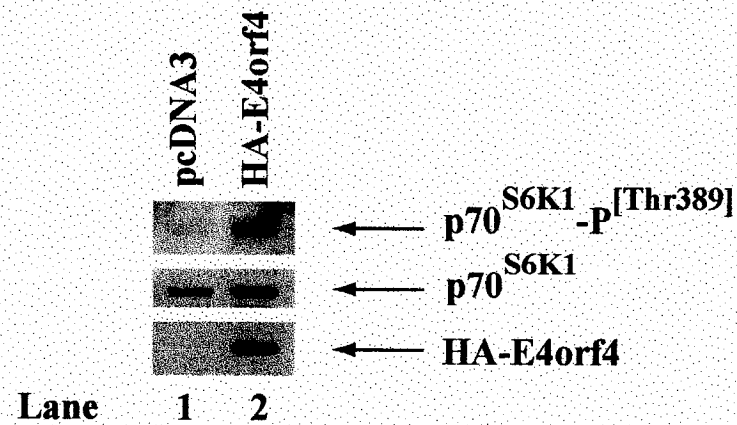
Figure 3-2: E4orf4 induces the hyperphosphorylation of 4EBP-1 and $p70^{S6K}$ *in vivo.*

A. H1299 cells were transfected with either empty vector pcDNA3 DNA (lane 1) or with a construct expressing HA-E4orf4 (lane 2). Cell extracts were prepared 48 h later and separated by SDS-PAGE. 4EBP-1 phosphorylation levels (top panel) were detected by western blotting using anti-4EBP-1 antibody. Expression of E4orf4 was confirmed by immunoblotting using anti-HA antibody (bottom panel).
B. H1299 cells were transfected with either empty vector pcDNA3 DNA or with a construct expressing HA-E4orf4. 24 h later cell extracts were prepared and separated by SDS-PAGE. The presence of total $p70^{S6K}$ (middle panel) or its phosphorylated form (top panel) was determined by western blotting using anti-$p70^{S6K}$ or anti-phosphothreonine-389 $p70^{S6K}$ antibodies, respectively. Expression of E4orf4 was confirmed by western blotting using anti-HA antibody (bottom panel).

A


pcDNA3
HA-E4orf4
4E-BP1-P-P
4E-BP1-P
4E-BP1
22 kDa
22 kDa
HA-E4orf4
Lane
1
2

B

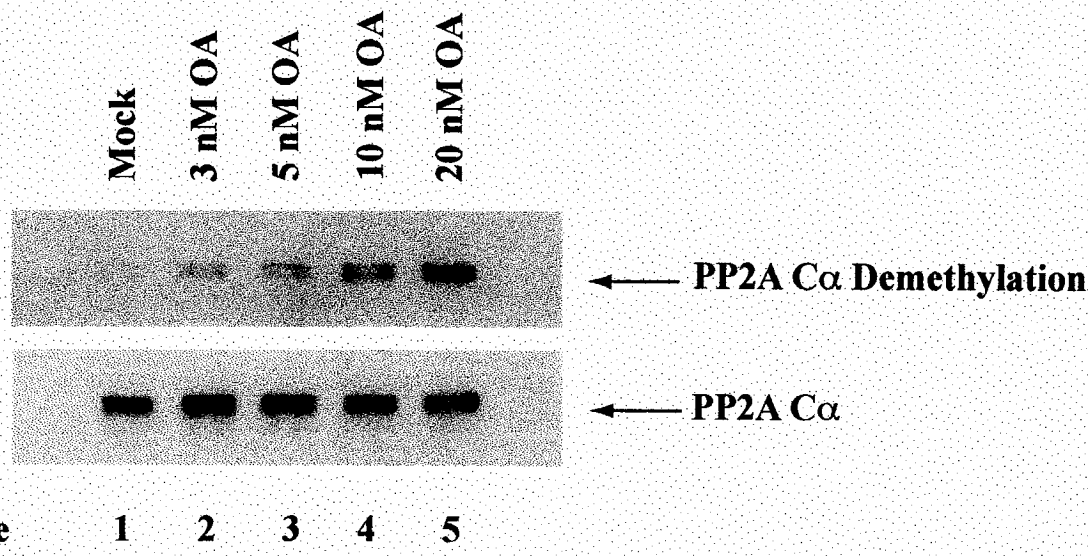

pcDNA3
HA-E4orf4
p70S6K1-P[Thr389]
p70S6K1
HA-E4orf4
Lane
1
2

death by causing an additional reduction in Bα-specific PP2A activity. Studies were performed using okadaic acid (OA), a specific inhibitor of PP2A at low concentrations (Bialojan and Takai 1988). To confirm that OA inhibited PP2A at the concentrations used in these studies, use was made of the fact that inhibition of PP2A by OA is accompanied by demethylation of the PP2A catalytic C subunit (Floer and Stock 1994; Li and Damuni 1994). Thus extracts from cells treated with 3, 5, 10 or 20 nM OA were separated by SDS-PAGE and western blotting was performed using either antibodies that recognize the Cα subunit of PP2A or those that recognize only the demethylated form. Figure 3-3A shows that all cells expressed equivalent levels of PP2A Cα (lower panel); however, higher levels of the demethylated form were present upon increasing concentrations of OA (upper panel, lane 2, 3, 4, 5 compared to lane 1), indicating that PP2A was clearly affected. To measure the effect of OA on E4orf4-induced cell death, H1299 cells were co-infected with Ad-E4orf4 plus Ad-rtTA, and HA-E4orf4 expression was induced by addition of 1 mM doxycycline. Cells were also mock-infected or infected with AdrtTA as controls, and all cultures were treated immediately following infection with 0, 3 or 5 nM okadaic acid. Fig. 3-3B shows that OA treatment of mock infected and AdrtTA infected cells had no effect on cell viability, as measured at 48 h.p.i. by counting the number of cells that remained adherent to the culture dishes. Expression of E4orf4 in the absence of OA induced considerable cell death, as shown previously (Marcellus, Lavoie et al. 1998). Interestingly, increasing low concentrations of OA enhanced cell killing by E4orf4. The effects of OA were confirmed using another cell death assay. We have shown previously that E4orf4-induced cell death is associated with the formation of condensed and irregular shaped nuclei, a response that closely correlates with cell death as measured by other techniques (Lavoie, Nguyen et al. 1998; Lavoie, Champagne et al. 2000; Robert, Miron et al. 2002). Fig. 3-4A shows representative results obtained with cells transfected with plasmid DNAs expressing either Green Fluorescent Protein (GFP) or a polypeptide composed of GFP fused to the amino terminus of HA-E4orf4. Direct fluorescence microscopy indicated that GFP was present throughout the cell while GFP-HA-E4orf4 was somewhat more concentrated in the nucleus. Staining of these same cells with DAPI indicated that the nucleus in the GFP-HA-E4orf4-expressing cell was

Figure 3-3: Okadaic acid inhibits PP2A as shown by demethylation of the Cα subunit and enhances the toxicity of E4orf4.

A. H1299 cells were either untreated or treated with 3, 5, 10, or 20 nM OA. Cell extracts were prepared 48 h later and separated by SDS-PAGE. The level of total Cα subunit (bottom panel) or its demethylated form (top panel) was measured by western blotting using anti-Cα antibody or 4b7 antibody, respectively.
B. H1299 cells were infected with adenovirus vectors Ad-HA-E4orf4 plus Ad-rtTA and the expression of HA-E4orf4 was induced by the addition of 1 mM doxycycline. Cells were also mock infected or infected with Ad-rtTA as a control. Immediately after infection the cells were treated with 0, 3 or 5 nM OA. Expression of E4orf4 was confirmed by western blotting (data not shown). Cell viability was determined by counting the number of adherent cells, with values found in mock-infected cells in the absence of OA set arbitrarily at 1. The results are from 5 experiments. Treatment of E4orf4-expressing cells with 3 and 5 nM OA significantly enhanced cell death, compared to E4orf4 alone ($p<0.01$, t-test).

A


Mock
3 nM OA
5 nM OA
10 nM OA
20 nM OA
PP2A Cα Demethylation
PP2A Cα
Lane 1 2 3 4 5

B

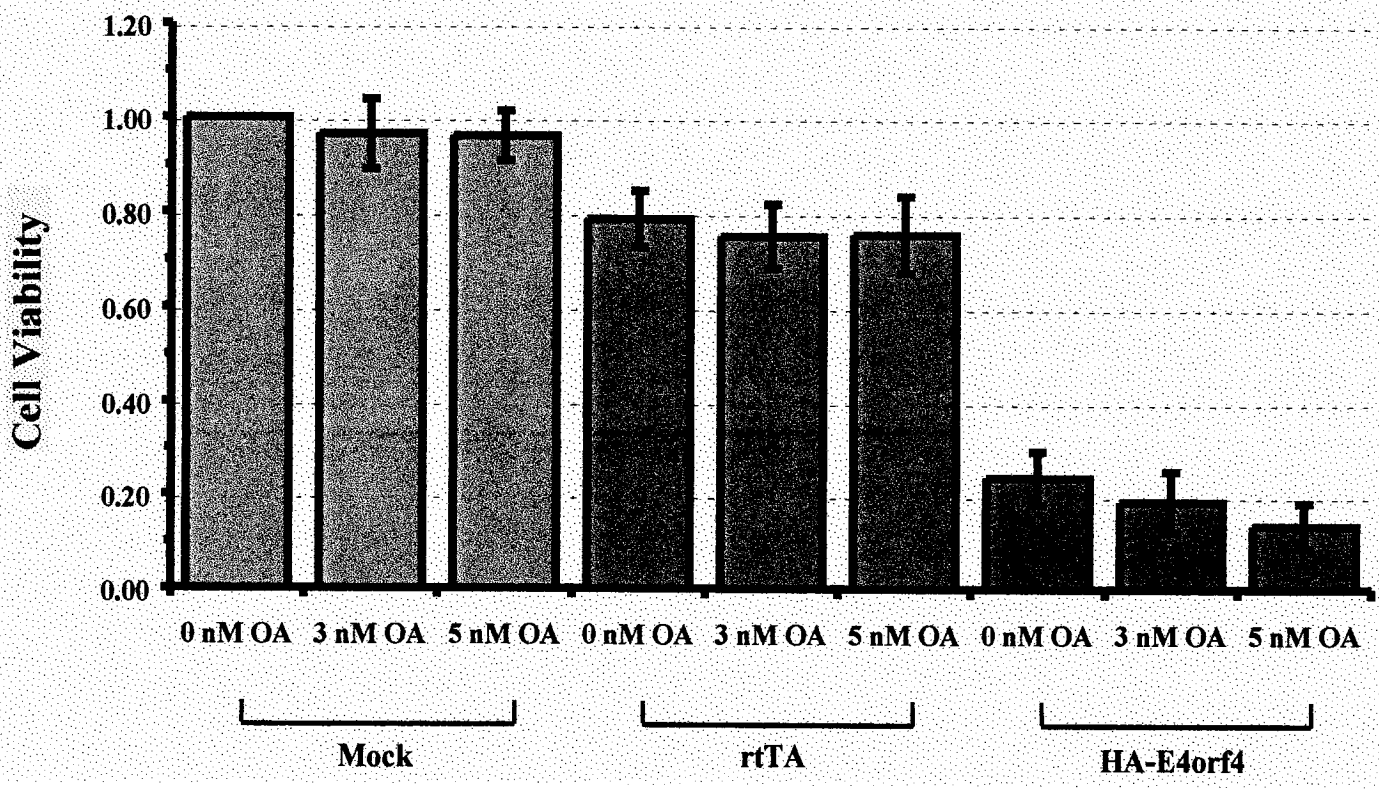
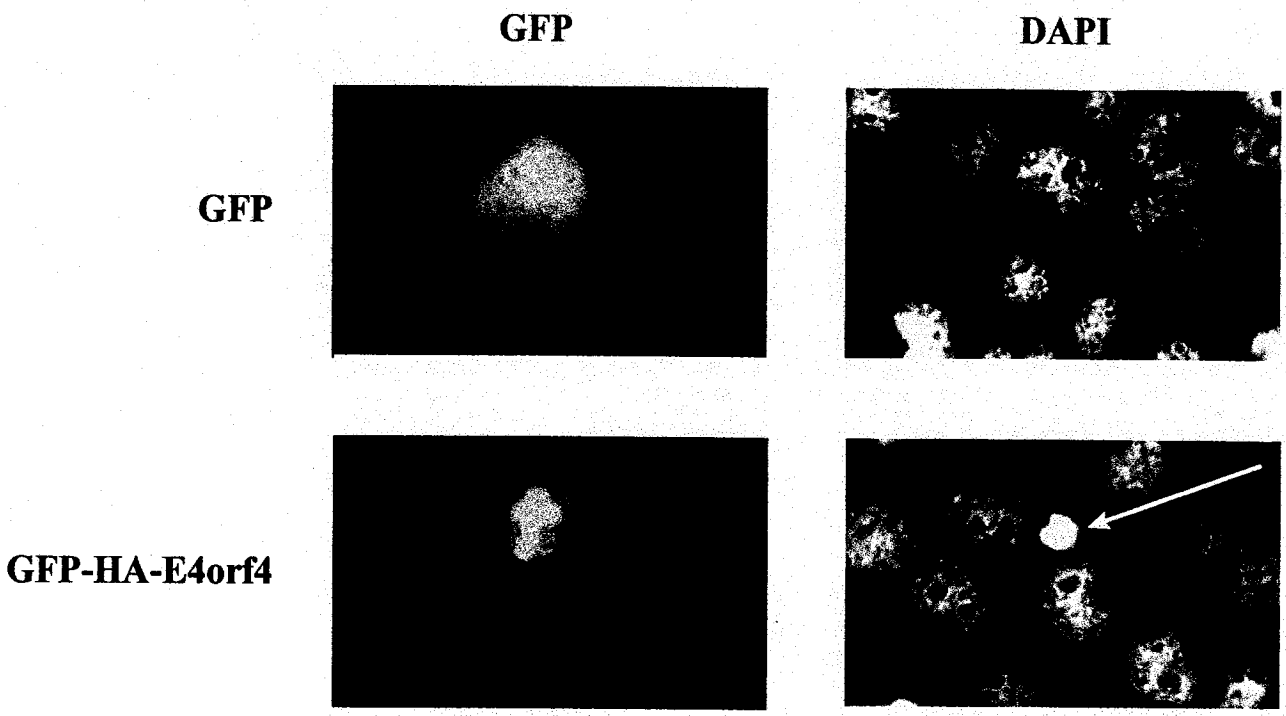

Cell Viability
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0 nM OA
3 nM OA
5 nM OA
0 nM OA
3 nM OA
5 nM OA
0 nM OA
3 nM OA
5 nM OA
Mock
rtTA
HA-E4orf4

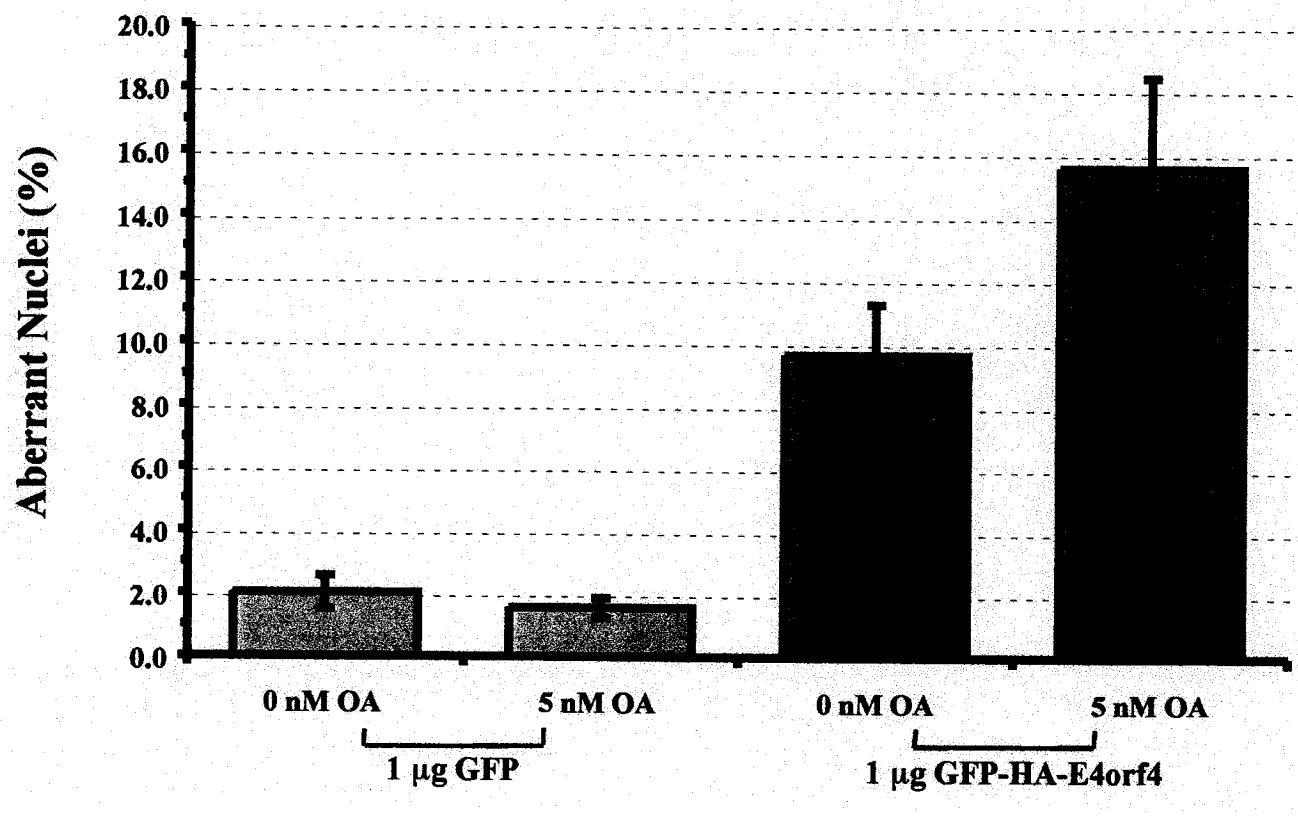
Figure 3-4: Okadaic acid augments the number of condensed nuclei in E4orf4-expressing cells.

A. H1299 cells were transfected with plasmid DNA expressing either GFP or GFP-HA-E4orf4 and after 48 h stained with DAPI. Representative examples are shown of GFP- and GFP-HA-E4orf4-expressing cells by direct fluorescence microscopy. Panels are as indicated in the figure.
B. H1299 cells were transfected with pcDNA3 constructs expressing either GFP or GFP-HA-E4orf4. Immediately after transfection the cells were either untreated or treated with 5 nM OA. 48 h later the cells were stained with DAPI and examined using direct fluorescence microscopy. The percentage of GFP-positive cells containing aberrant nuclei was measured in four experiments each involving the counting of 250 random cells. Treatment with 5 nM OA significantly enhanced the number of condensed nuclei in E4orf4-expressing cells, compared to E4orf4 alone ($p<0.05$, t-test).

A


GFP
DAPI
GFP
GFP-HA-E4orf4

B

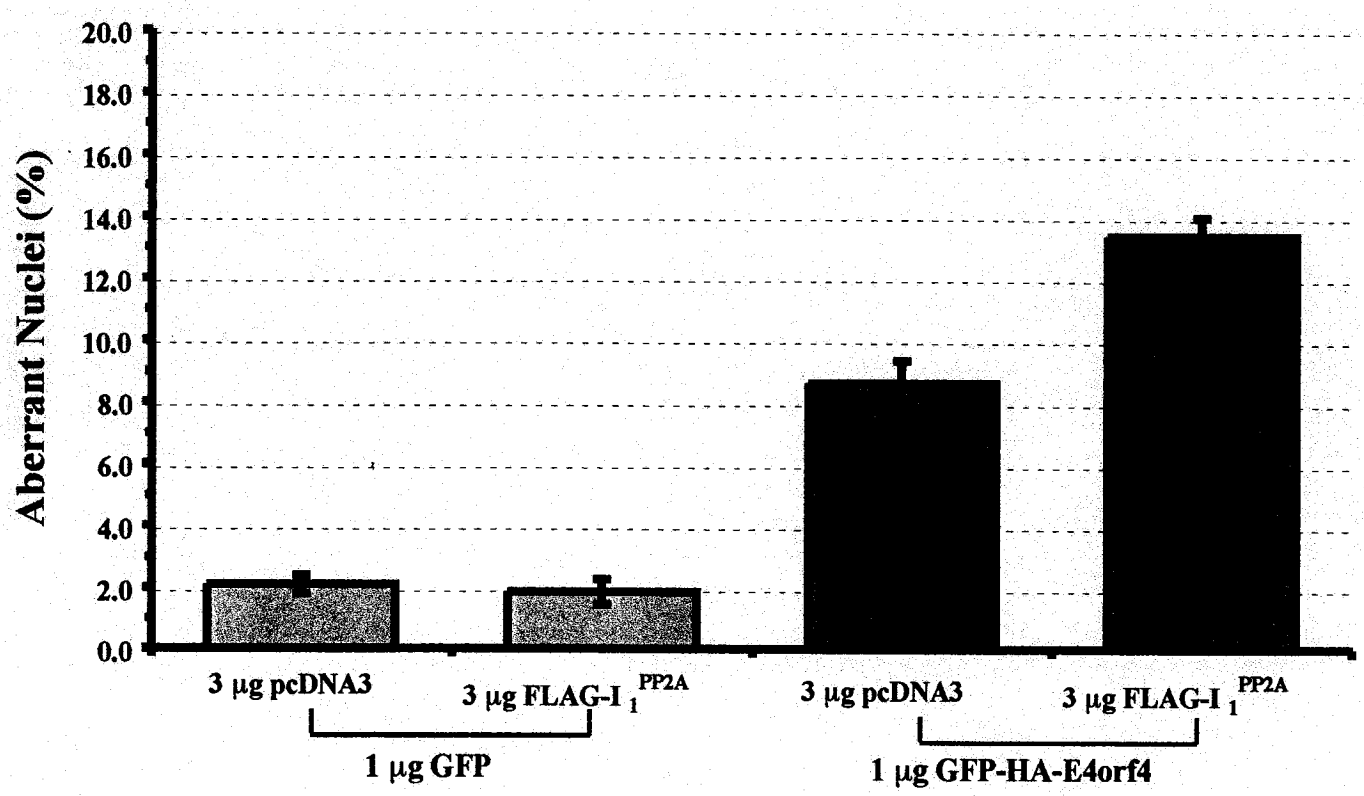

Aberrant Nuclei (%)
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
0 nM OA
5 nM OA
0 nM OA
5 nM OA
1 µg GFP
1 µg GFP-HA-E4orf4

considerably more condensed and had an irregular shape. Thus measurement of the percent of cells displaying this characteristic morphology provides an accurate measure of induction of the cell death process. Fig. 3-4B shows that cells expressing GFP displayed only low levels of aberrant nuclei and that treatment with 5 nM OA alone had no effect. On the other hand, GFP-HA-E4orf4 expression alone caused an approximate 5-fold increase in the appearance of such nuclei, while treatment with 5 nM OA during GFP-HA-E4orf4 expression increased this effect to about 8-fold. It is known that at somewhat higher concentrations OA also affects other phosphatases, including protein phosphatase 1 (PP1) (Bialojan and Takai 1988). To determine if inhibition of PP1 affected E4orf4 killing, an experiment similar to that described in Fig. 3-4B was performed but this time in the presence of 200 nM tautomycin (BIOMOL Research Laboratories Inc.), an inhibitor known to inhibit PP1 with a higher affinity than PP2A (MacKintosh and Klumpp 1990). No enhancement of E4orf4-induced cell killing was evident, suggesting that the increase in aberrant nuclei is specific to inhibition of PP2A (data not shown). Taken together, the results in Figs. 3-3 and 3-4 are consistent with the idea that E4orf4 inhibition of Bα-dependent PP2A activity is important for inducing cell death.

A second approach to this question was to utilize the 249-residue heat-stable I_1^{PP2A} polypeptide inhibitor to reduce PP2A activity. I_1^{PP2A} inhibits the catalytic activity of all forms of PP2A holoenzymes probably by binding to the C subunit (Janssens and Goris 2001). We isolated a human cDNA encoding FLAG-tagged I_1^{PP2A} using PCR-based cloning (see Materials and Methods) and used it to co-transfect H1299 cells along with plasmid DNAs expressing either GFP or GFP-HA-E4orf4. FLAG-I_1^{PP2A} was shown by western blotting using anti-FLAG antibodies to be expressed at high levels in these cells (data not shown). Fig. 3-5 shows that cells expressing GFP displayed only low levels of aberrant nuclei and that expression of I_1^{PP2A} alone had little effect. GFP-HA-E4orf4 expression caused an approximate 4-fold increase in the appearance of such nuclei, and co-expression of I_1^{PP2A} increased this effect to 7-fold. Again, these data supported previous results suggesting that E4orf4 induces cell death through the inhibition of Bα-containing PP2A holoenzymes against certain substrates.

Figure 3-5: I_1^{PP2A} increases the number of condensed nuclei in E4orf4-expressing cells.

An experiment similar to that of Fig. 3-4B was performed except that some cells were co-transfected with plasmid DNA expressing FLAG-I_1^{PP2A} instead of being treated with OA. The percentage of GFP-positive cells containing aberrant nuclei was measured in three experiments each involving the counting of 250 random cells. Expression of I_1^{PP2A} significantly enhanced cell killing by E4orf4 ($p<0.002$, t-test). In all cases expression of GFP-HA-E4orf4 and FLAG-I_1^{PP2A} was confirmed by western blotting (data not shown).


Aberrant Nuclei (%)
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
3 μg pcDNA3
3 μg FLAG-I 1 PP2A
1 μg GFP
3 μg pcDNA3
3 μg FLAG-I 1 PP2A
1 μg GFP-HA-E4orf4

3.4 Co-expression of a constitutively active PP2A C subunit reduces E4orf4 killing.

The results have suggested that inhibitors of PP2A activity, such as OA and I_1^{PP2A}, would enhance cell killing by reducing activity in the pool of Bα-containing PP2A holoenzymes not associated with E4orf4. Similarly, stimulation of the activity of this pool might reduce E4orf4 killing. To address this latter possibility use was made of a mutant form of the catalytic Cα subunit in which Tyr^{307} had been altered to a phenylalanine residue (Y307F). Tyrosine phosphorylation at Y307 by c-Src family and transmembrane receptor kinases is known to inhibit catalytic activity (Chen, Parsons et al. 1994). Thus PP2A holoenzymes containing the Y307F mutant C subunit may display constitutively higher catalytic activity *in vivo*. Therefore, H1299 cells were co-transfected with different combinations of plasmid DNAs expressing pcDNA3, GFP, GFP-HA-E4orf4, FLAG-Cα or FLAG-Cα(Y307F). Fig. 3-6 shows that expression of Cα(Y307F) significantly reduced E4orf4-induced cell killing, as measured by the number of aberrant nuclei, which is consistent with previous results that suggest E4orf4 inhibits PP2A activity.

3.5 E4orf4 induces a mitotic arrest.

Previous studies conducted by our group (Roopchand, Lee et al. 2001) and others (Kornitzer, Sharf et al. 2001) suggested that E4orf4-induced killing of yeast and mammalian cells was accompanied by increased numbers of G2 / M cells. To verify this result, H1299 cells were mock infected or infected with adenovirus vectors Ad-rtTA or AdTRex-HA-E4orf4. At 48 h.p.i. both the adherent and non-adherent cells were harvested and analyzed by FACS to determine the cell cycle profile. Fig. 3-7A shows that the mock and Ad-rtTA infected cells contained about 15% G2 / M cells, whereas the proportion following E4orf4 expression was significantly higher (27%). Fig. 3-7A also demonstrates that when the non-adherent E4orf4 expressing cells were examined alone, this proportion increased to 40%. These results confirmed that E4orf4-induced cell death is associated with a G2 / M arrest. However, to determine if the cells expressing E4orf4

Figure 3-6: A constitutively active C subunit reduces E4orf4 killing.

H1299 cells were transfected with plasmid DNAs expressing GFP and either pcDNA3 DNA or pcDNA3 expressing the wild-type FLAG-Cα or the FLAG-Cα(Y307F) mutant of the PP2A Cα subunit that is insensitive to downregulation by tyrosine phosphorylation. Other dishes of H1299 cells were transfected with plasmid DNAs expressing GFP-HA-E4orf4 and either pcDNA3 DNA or pcDNA3 expressing wild-type Cα or Cα(Y307F). The percentage of GFP-positive cells containing aberrant nuclei was measured in three experiments each involving the counting of 250 random cells. Expression of Cα(Y307F) significantly enhanced E4orf4-induced cell death ($p<0.05$, t-test). Western blot analysis confirmed the expression of GFP-HA-E4orf4, FLAG-Cα and FLAG-Cα(Y307F) (data not shown).

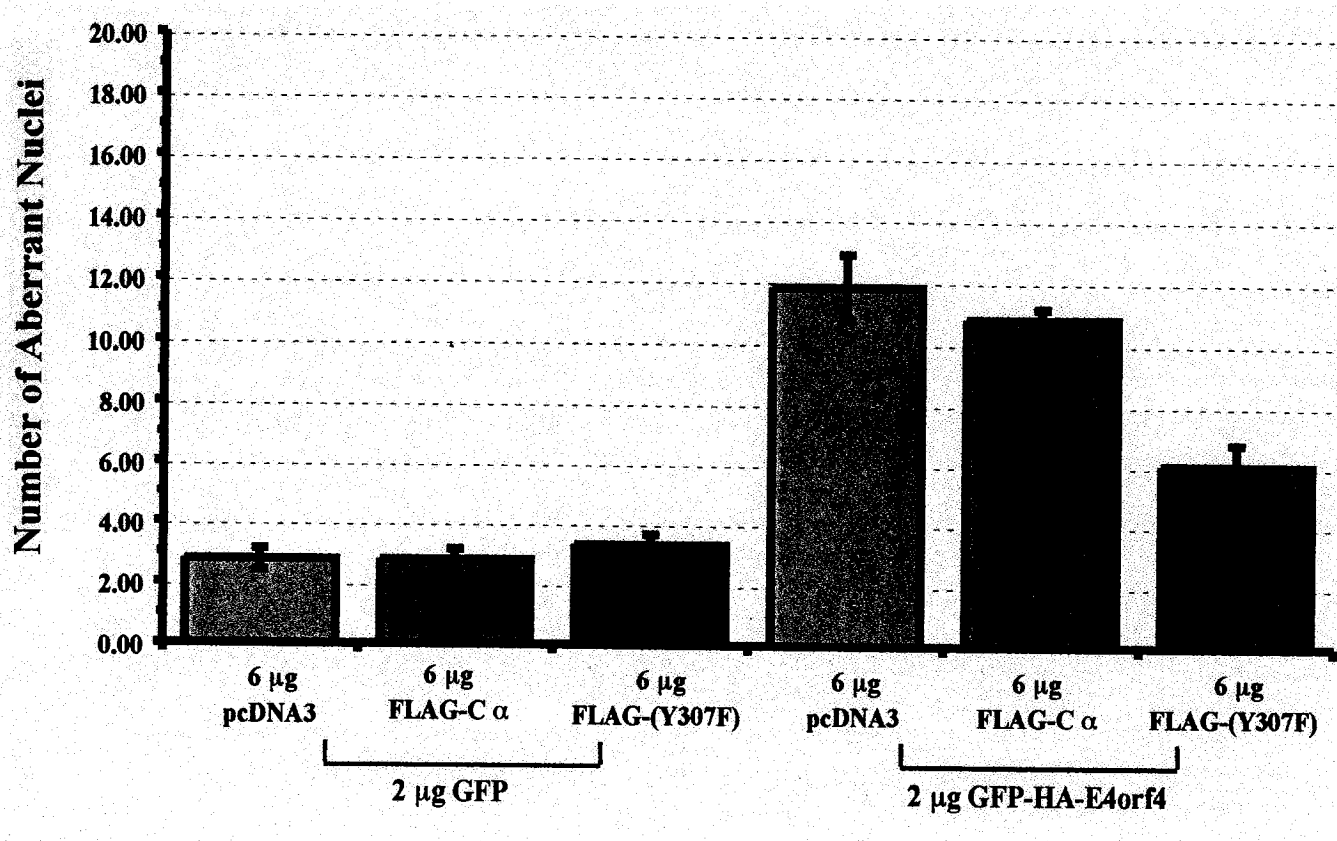
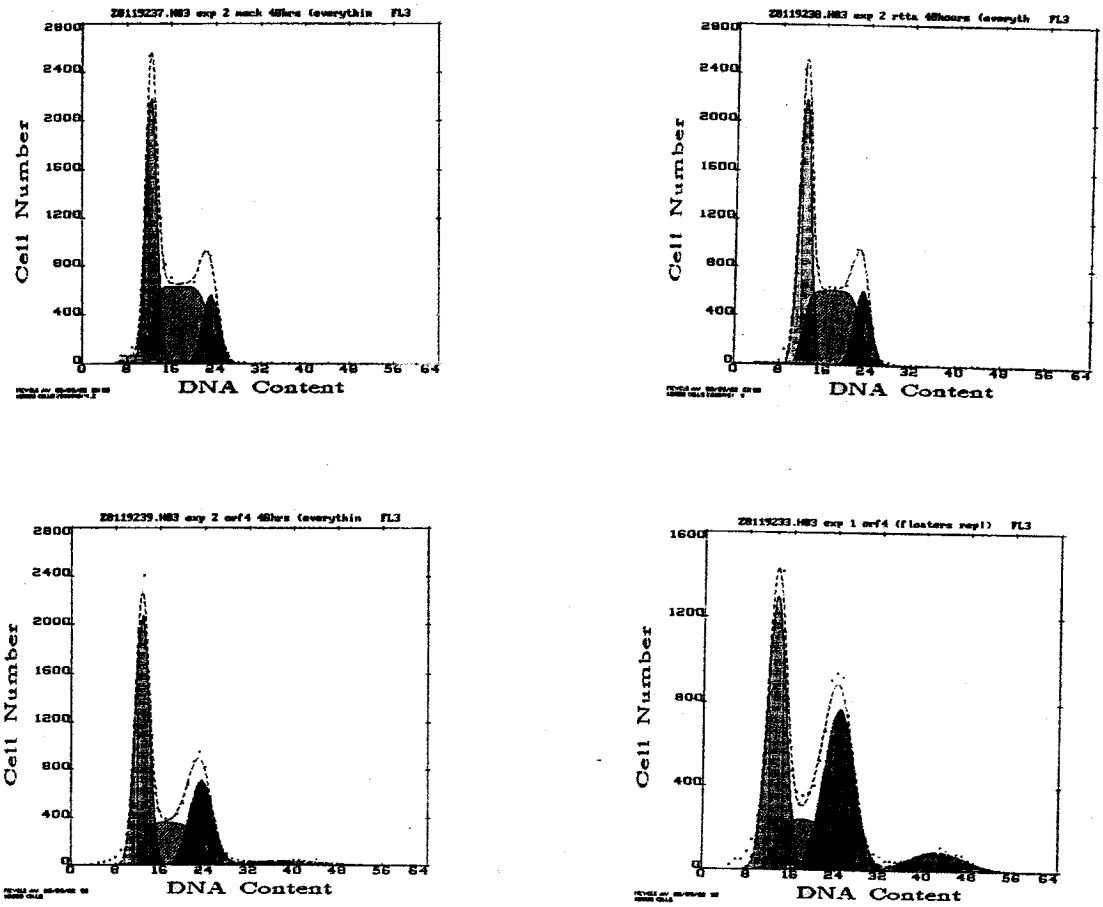

Number of Aberrant Nuclei
20.00
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
6 µg pcDNA3
6 µg FLAG-C α
6 µg FLAG-(Y307F)
6 µg pcDNA3
6 µg FLAG-C α
6 µg FLAG-(Y307F)
2 µg GFP
2 µg GFP-HA-E4orf4

Figure 3-7: E4orf4 induces a G2 / M arrest and increases CDK1 activity.

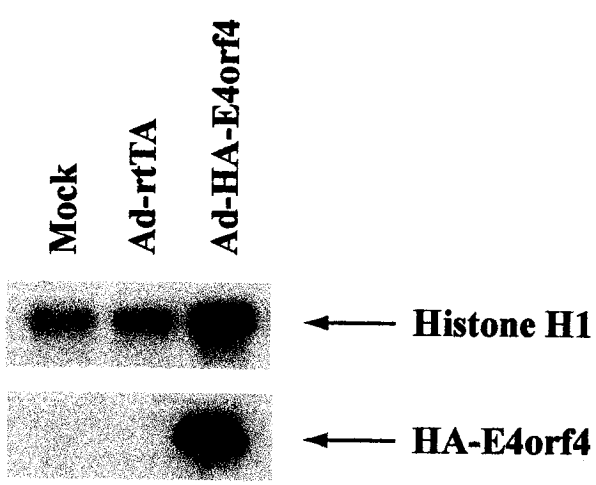
A. H1299 cells were mock infected or infected with adenovirus vectors Ad-rtTA or AdTRex-HA-E4orf4. 48 h p.i. the cells were collected, prepared for staining with propidium iodide and subjected to FACS analysis. The cell cycle profile was measured for mock infected cells (top-left panel), cells expressing Ad-rtTA (top-right panel), and cells expressing AdTRex-HA-E4orf4 either as a whole population (bottom-left panel) or as floating cells only (bottom-right panel). The first peak denotes cells in G1 phase, the second peak represents cells in S phase and the third peak denotes cells in G2 / M phase. In all cases expression of E4orf4 was confirmed by western blot analysis (data not shown).

B. Cell extracts from the samples described in Fig. 3-6A were immunoprecipitated with cyclin B antibody. Kinase assays were performed to determine the activity of the Cdc2-cyclin B complex towards histone H1 *in vitro*. The samples were separated by SDS-PAGE and exposed to film.

A


Cell Number
DNA Content
Cell Number
DNA Content
Cell Number
DNA Content
Cell Number
DNA Content

B


Mock
Ad-rtTA
Ad-HA-E4orf4
Histone H1
HA-E4orf4

were arrested in the G2 phase or mitosis, the MPF (Cdc2 / cyclin B complex) activity needs to be examined. During mitosis the MPF is active (Janssens and Goris 2001), therefore to determine the activity in the presence of E4orf4 expressing cells, cell extracts from the samples in Fig. 3-7A were immunoprecipitated with cyclin B antibody and were tested for their ability to phosphorylate histone H1 *in vitro*. As shown in Fig. 3-7B, cyclin B immunoprecipitates from cells expressing E4orf4 phosphorylated histone H1 and displayed a much higher Cdc2 / cyclin B kinase activity compared to the Ad-rtTA or mock infected cells. These results suggest that E4orf4-induced cell death is associated with a mitotic arrest.

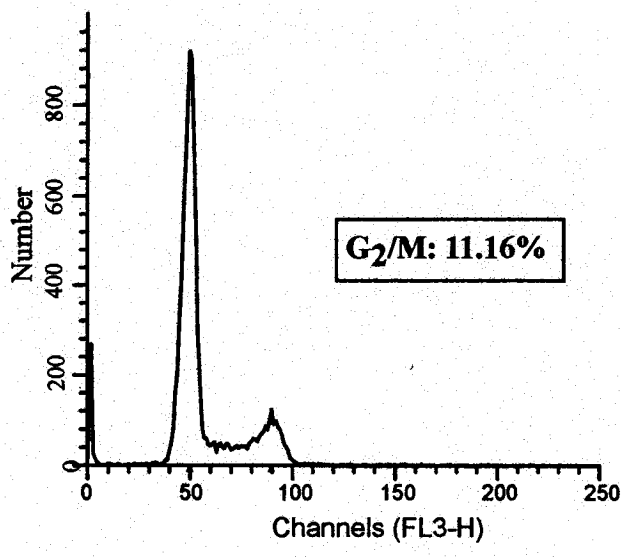
3.6 E4orf4 inhibition of PP2A induces G2 / M arrest.

As shown in Fig. 3-7, E4orf4-induced cytotoxicity in H1299 cells was accompanied by an increased number of G2 / M cells. Because certain substrates of PP2A holoenzymes, including Wee1 kinase and Cdc25 phosphatase, are known to regulate the G2 / M checkpoint (Clarke, Hoffmann et al. 1993; Kinoshita, Yamano et al. 1993), studies were carried out to examine the effects of E4orf4 and OA on the cell cycle. E4orf4 was again expressed in H1299 cells using AdTrex-HA-E4orf4 with Ad-rtTA as a control in the presence or absence of 20 nM OA. At 48 h.p.i the adherent cells were removed by trypsinization, combined with detached cells and the mixture was analyzed by FACS. Fig. 3-8 shows that treatment with 20 nM OA, which is sufficient alone to induce some cell toxicity (data not shown), induced a small increase in the G2 / M cell population from 11.2 to 15.7%. Expression of E4orf4 alone increased the G2 / M population to 21.7%, and in the presence of OA, 38.4% of the cells were in G2 / M. This enhancement of E4orf4-induced G2 / M arrest by OA did not appear to be related to its effects at high concentrations on PP1. Studies similar to those in Fig. 3-8 were conducted in the presence of 200 nM tautomycin, a PP1-specific inhibitor, instead of OA. Fig. 3-9A shows that this drug had no effect on the G2 / M population of cells expressing E4orf4. To ensure that this level of tautomycin inhibited PP1 but not PP2A, cell extracts from treated and untreated cells were analyzed by western blotting using antibodies against MLC, a known PP1 substrate (Alessi, MacDougall et al. 1992) and with those against

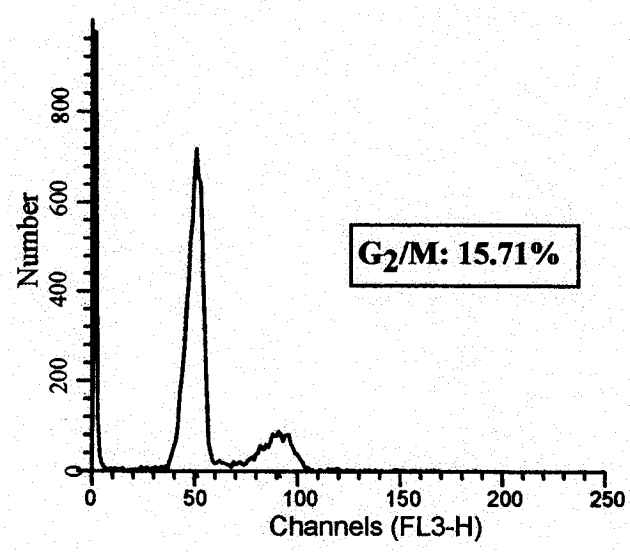
Figure 3-8: Okadaic acid enhances E4orf4-mediated G2 / M arrest.

H1299 cells were infected with adenovirus vectors Ad-rtTA or AdTRex-HA-E4orf4 in the presence or absence of 20 nM OA. 48 h later the cells were collected, prepared for staining with propidium iodide and subjected to FACS analysis. The percentage of cells in G2 / M was measured for cells expressing Ad-rtTA or AdTRex-HA-E4orf4 alone (left panel), as well as those expressing Ad-rtTA or AdTRex-HA-E4orf4 in the presence of 20 nM OA (right panel). In all cases expression of E4orf4 was confirmed by western blot analysis (data not shown).

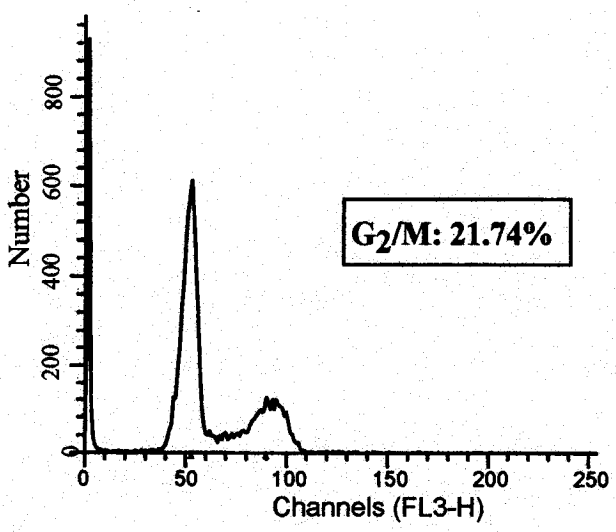
Ad-rtTA



Ad-rtTA + 20 nM OA



Ad-HA-E4orf4



Ad-HA-E4orf4 + 20 nM OA

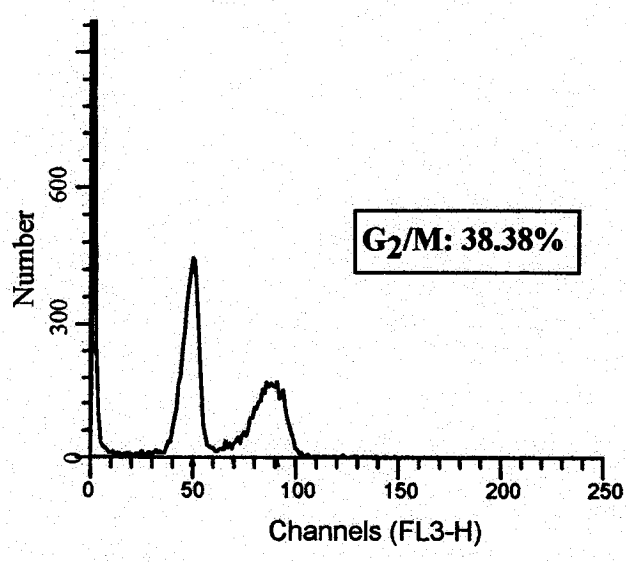
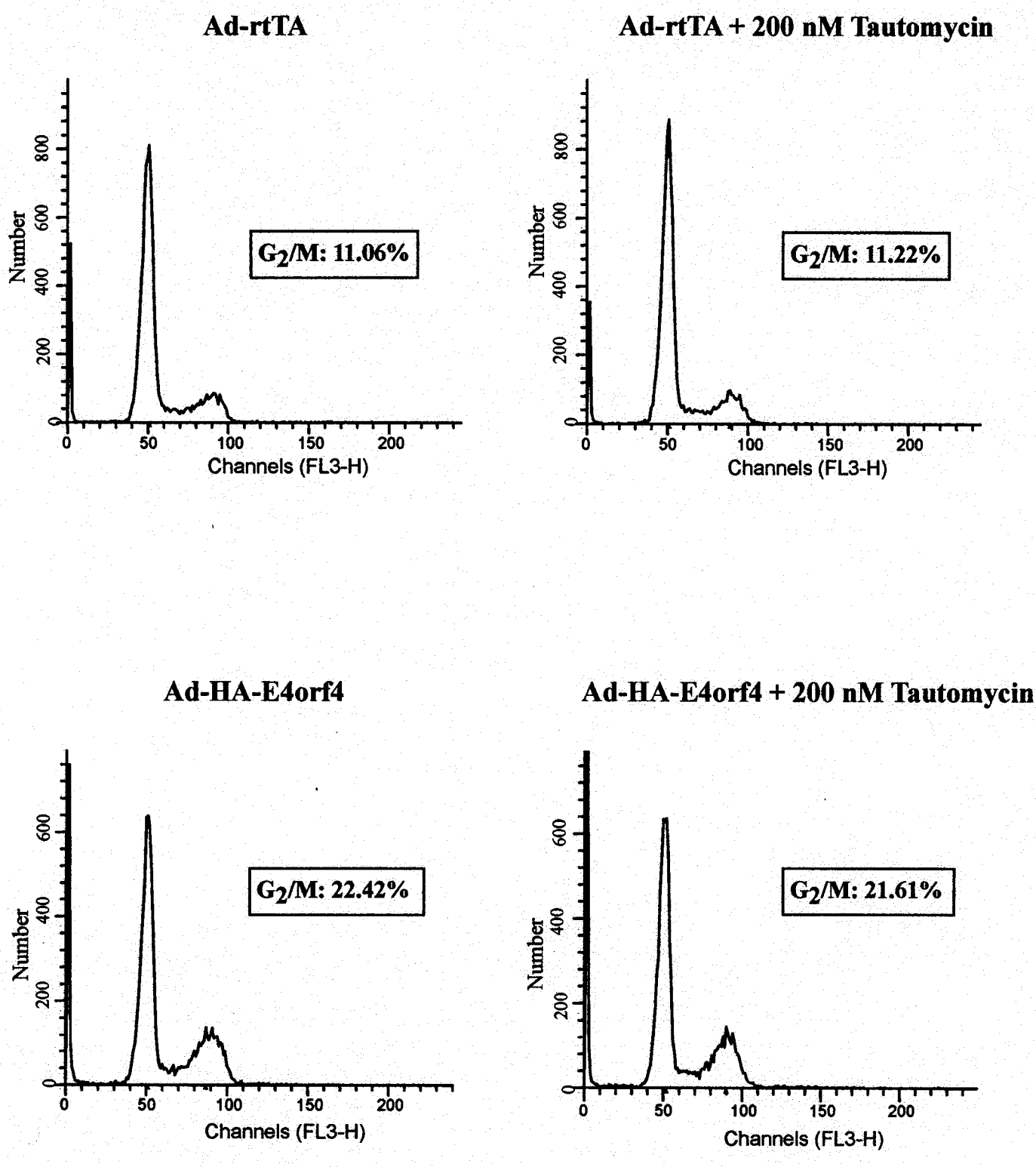


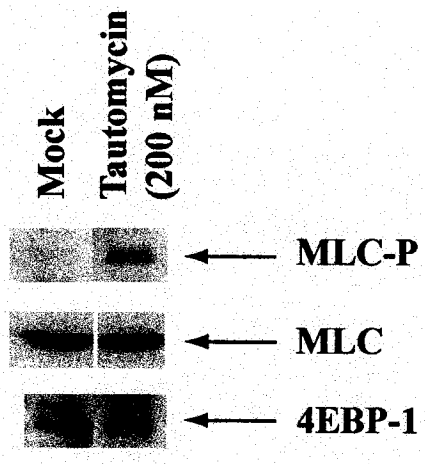
Figure 3-9: Levels of tautomycin that inhibit the dephosphorylation of a substrate of PP1, but not PP2A, do not augment E4orf4-induced G2 / M arrest.

A. H1299 cells were infected with adenovirus vectors Ad-rtTA or AdTRex-HA-E4orf4 in the presence or absence of 200 nM tautomycin. 48 h later the cells were collected, prepared for staining with propidium iodide and subjected to FACS analysis. The percentage of cells in G2 / M was measured for cells expressing Ad-rtTA or AdTRex-HA-E4orf4 alone (left panel), as well as those expressing Ad-rtTA or AdTRex-HA-E4orf4 in the presence of 200 nM tautomycin (right panel). In all cases expression of E4orf4 was confirmed by western blot analysis (data not shown).
B. H1299 cells were either untreated or treated with 200 nM tautomycin. Cell extracts were prepared 48 h later and separated by SDS-PAGE. The protein level of MLC (middle panel) and its phosphorylated form (top panel) was examined by western blot analysis using anti-MLC antibody or anti-MLC phospho-specific antibody, respectively. The phosphorylation level of 4EBP-1 (bottom panel) was measured by immunoblotting with anti-4EBP-1 antibody. Results shown are representative of results from three separate experiments.

A


Ad-rtTA
Ad-rtTA + 200 nM Tautomycin
Ad-HA-E4orf4
Ad-HA-E4orf4 + 200 nM Tautomycin
G2/M: 11.06%
G2/M: 11.22%
G2/M: 22.42%
G2/M: 21.61%
Number
Channels (FL3-H)
0
50
100
150
200
200
400
600
800

B



4EBP-1, a known PP2A substrate. Fig. 3-9B shows whereas tautomycin increased levels of hyperphosphorylated MLC, no change was seen in the case of 4EBP-1. Therefore, these results suggested that one mechanism for E4orf4-mediated cell killing may involve the induction of G2 / M arrest through the specific inhibition of PP2A holoenzymes against substrates involved in regulation of G2 / M transition (see Discussion).

3.7 Small T antigen of SV40 enhances E4orf4-induced killing and G2 / M arrest.

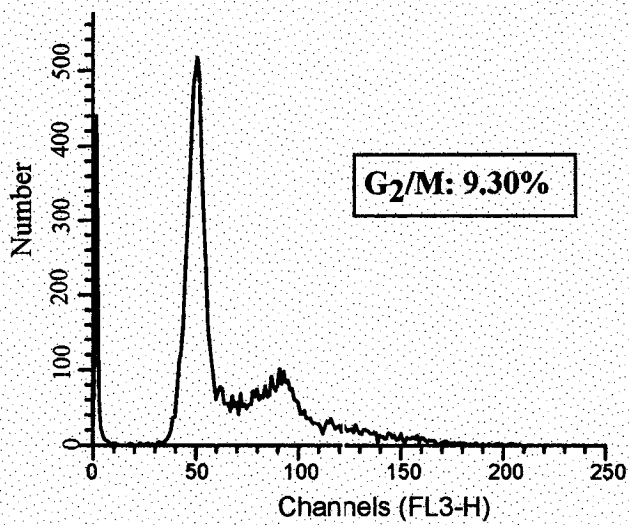
The small tumor antigen of simian virus 40 (SV40-ST), as well as the middle and small T antigens of mouse polyoma virus are known to interact with PP2A, and such interactions

are important for cell transformation by these viruses (Janssens and Goris 2001). Furthermore, expression of high levels of SV40-ST alone has recently been shown to induce apoptotic-like cell death (Gjoerup, Zaveri et al. 2001). Interactions with PP2A holoenzymes result in the replacement of B subunits by these viral products (Pallas, Shahrik et al. 1990; Walter, Ruediger et al. 1990). In the case of SV40-ST the interaction has been reported to take place preferentially with Bα-containing holoenzymes (Yang, Lickteig et al. 1991; Pallas, Weller et al. 1992; Sontag, Fedorov et al. 1993), however more recently SV40-ST has been shown to displace the B56 / B' class of B subunit with equal affinity (D. Pallas and T. Roberts, personal communication). Although the phosphatase activity of the resulting SV40-ST-A-C heterotrimers may be functionally important, one effect of the interaction would be to reduce the population of Bα-containing holoenzymes, and it has been reported that SV40-ST expression reduces PP2A activity towards Bα specific PP2A substrates such as MEK or MAP kinase (Sontag, Fedorov et al. 1993). Based on the previous results, we would predict that this reduction could in itself be toxic, and that co-expression of SV40-ST should enhance E4orf4 toxicity. This prediction was confirmed using colony formation assays which demonstrated that the expression of E4orf4 reduced the number of colonies relative to the control plates, indicating that it was highly toxic; however, co-expression of SV40-ST and E4orf4 eliminated almost all cell growth (data not shown, R. Marcellus). To

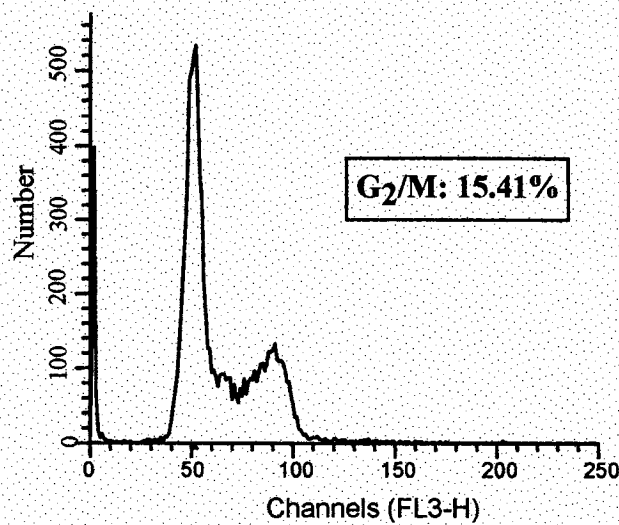
Figure 3-10: SV40 small t antigen enhances E4orf4-mediated G2 / M arrest.

H1299 cells were infected with adenovirus vectors Ad-rtTA or AdTRex-HA-E4orf4 (50 pfu / cell) followed 24 h later by a second infection at 10 pfu / cell with Ad-CMV (control) or Ad-SV40-ST, which expresses the ST antigen of SV40 (see Materials and Methods). 24 h after the second infection, the cells were collected, prepared for staining with propidium iodide and subjected to FACS analysis. The percentage of cells in G2 / M was measured for cells expressing Ad-rtTA or AdTRex-HA-E4orf4 with Ad-CMV (left panel), as well as those expressing Ad-rtTA or AdTRex-HA-E4orf4 with Ad-SV40-ST (right panel). In all cases expression of E4orf4 and SV40-ST was confirmed by western blot analysis (data not shown).

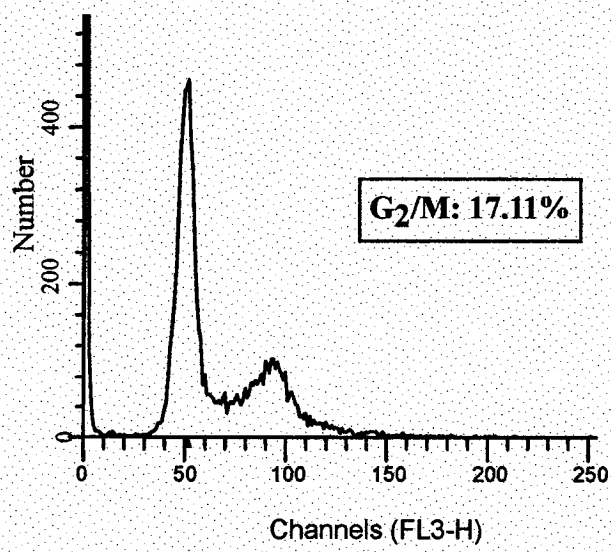
Ad-rtTA + Ad-CMV



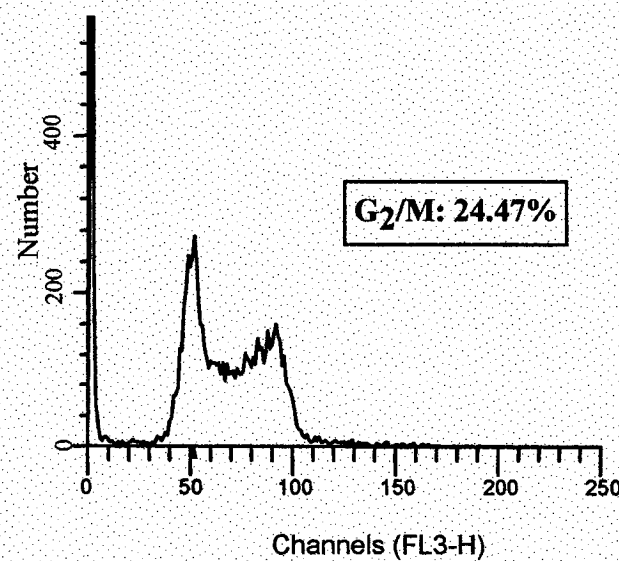
Ad-rtTA + Ad-SV40-ST



Ad-HA-E4orf4 + Ad-CMV



Ad-HA-E4orf4 + Ad-SV40-ST



determine if the enhancement of cell killing was associated with enhanced G2 / M arrest, an experiment similar to that described in Fig. 3-8 was performed in which adenovirus vectors were employed to express E4orf4 or SV40-ST, either alone or in combination. Fig. 3-10 shows the results of FACS analysis of these cells and indicated that co-expression of SV40-ST with E4orf4 increased the fraction of G2/M cells from 17.1% with E4orf4 alone to 24.5%. These results also support the idea that E4orf4 induces G2 / M arrest and cell killing through an inhibition of Bα specific PP2A activity.

Chapter 4: Discussion

The present studies have shed considerable light on the molecular basis of cell killing by the human adenovirus E4orf4 protein. Although we have found that E4orf4 interacts with all four members of the mammalian B / B55 family of B subunits (Zhang, Marcellus et al. In preparation), Bα is the most widely expressed and thus presumably of most importance for E4orf4-mediated cell death. As mentioned, it had generally been assumed that E4orf4 functions by redirecting or relocating PP2A to enhance or modify substrate specificity. Some of the reasons for this assumption originated from the observed hypophosphorylation of AP-1 transcription factor subunits, SR splicing factors, and the adenovirus early region 1A (E1A) protein in the context of E4orf4 expression in cells infected with wild-type or mutant adenoviruses (Muller, Kleinberger et al. 1992; Whalen, Marcellus et al. 1997; Kanopka, Mühlemann et al. 1998). Thus in effect, by binding to Bα E4orf4 might create a novel B-type subunit that could exhibit altered substrate specificity. It should be noted, however, that the hypophosphorylation of these proteins in adenovirus-infected cells is generally believed not to result directly from changes in PP2A activity, but rather from a reduction in their phosphorylation by the appropriate protein kinases.

Although this model may still be correct for certain substrates (as yet unidentified), such was not the case for any of the PP2A substrates examined in the present studies in which E4orf4 was expressed alone in the absence of other viral products. Rather, our results suggested that E4orf4 can inhibit Bα-directed PP2A activity against certain substrates by E4orf4 and induce cell death. This conclusion was based on two main observations. Firstly, the dephosphorylation of phosphorylase *a* and histone H1 *in vitro* using purified Bα-containing PP2A complexes was inhibited in the presence of E4orf4. Secondly, two known substrates of PP2A, 4EBP-1 and $p70^{S6K}$ (Bα specific), were hyperphosphorylated in E4orf4-expressing cells. E4orf4 does not appear simply to inhibit the catalytic activity of associated C subunits, as purified Bα-containing PP2A complexes dephosphorylated the small universal phosphopeptide substrate *in vitro* equally well in the presence or absence of E4orf4 (R.Marcellus, data not shown). Activity against this

substrate is known to be independent of B subunit regulation (Janssens and Goris 2001). Thus inhibition of PP2A activity by E4orf4 is unlike that induced by the I_1^{PP2A} heat stable inhibitor, which probably binds to the C subunit and inactivates catalytic activity (Li, Guo et al. 1995). It is also different from the inhibition promoted by phosphorylation of Tyr307 of the C subunit, which is catalyzed by c-Src family kinases and by certain transmembrane tyrosine kinases, including the epidermal growth factor receptor (Chen, Parsons et al. 1994). It is more likely that E4orf4 binding inhibits the ability of Bα to direct PP2A against its selective range of substrates.

The role of PP2A inhibition in E4orf4-induced cell death was demonstrated by the fact that two inhibitors of global PP2A activity (including of course Bα-dependent activity), enhanced induction of cell death by E4orf4. Treatment of E4orf4-expressing cells with low levels of OA or expression of the I_1^{PP2A} inhibitor, each of which alone caused no effect on cell viability under the same conditions, enhanced E4orf4-induced cell death. Further, co-expression of the SV40 ST antigen, which has been reported to preferentially replace Bα subunits in PP2A complexes, thus eliminating Bα regulation of substrate selection, also enhanced E4orf4-induced cell death (R.Marcellus, data not shown) and G2 / M arrest (Yang, Lickteig et al. 1991). SV40 ST has recently been shown to induce p53-independent cell death when expressed at high levels (Gjoerup, Zaveri et al. 2001), and thus it is possible that the mechanism of its toxicity parallels that of E4orf4.

The fact that expression of a constitutively active Y307F mutant form of the PP2A catalytic Cα subunit, which is insensitive to downregulation by tyrosine phosphorylation, reduced the toxicity of E4orf4 is also consistent with the idea that PP2A activity is not required for E4orf4-induced cell death. We have shown previously that cell killing correlates with the level of E4orf4 expression (Marcellus *et al.*, in preparation). Therefore, taken together the results suggest that induction of cell death requires E4orf4 to interact with a critical proportion of Bα-containing PP2A complexes to attain sufficient reduction in substrate dephosphorylation to elicit cell death. The identity of such substrates and the mechanism of inhibition of Bα regulatory activity are not known.

Although it is well known that B subunits are responsible both for intracellular localization and substrate selection of PP2A enzymes, little is known about their mode of action (Janssens and Goris 2001). Unlike some of the other B subunits, B / B55 family members contain six degenerate WD repeats and thus likely resemble 'propeller' proteins structurally (Neer, Schmidt et al. 1994; Smith, Gaitatzes et al. 1999). Therefore, they comprise stable binding platforms able to interact with multiple proteins. Our group has recently mapped the region involved in E4orf4 binding to the Bα subunit (Zhang, Marcellus et al. In preparation). We propose that binding of E4orf4 blocks the ability of this region of the Bα subunit to interact with substrates or proteins involved in substrate acquisition. Furture studies could be carried out to examine these possibilities.

The identities of critical PP2A substrates involved in inducing cell death are unknown. Two PP2A substrates that we know are affected by E4orf4 expression are 4EBP-1 and $p70^{S6K}$. Dephosphorylation of these proteins by PP2A is known to negatively regulate the mTOR pathway and thus initiation of protein synthesis (Peterson, Desai et al. 1999). Therefore, expression of E4orf4 might be predicted to promote protein synthesis, or at least components of this signaling pathway, and this effect could result in toxicity by autophagy or some other process. Future studies could be undertaken to examine these possibilities.

E4orf4 was also found to induce a considerable G2 / M arrest in E4orf4-expressing cells. Cell death due to mitotic arrest followed by mitotic catastrophe is known to induce a response not unlike that seen in E4orf4-dependent cell death (Roninson, Broude et al. 2001). Both E4orf4 and high levels of OA induced such an arrest and other studies have suggested that the arrest may occur during the late stages of mitosis prior to cytokinesis (Szymborski, Miron et al. In preparation). Studies suggest that several components involved in the G2 / M transition are regulated by PP2A, including Wee1 kinase and Cdc25 phosphatase which regulate the Cdc2 / cyclin B complex (MPF) (Clarke, Hoffmann et al. 1993; Kinoshita, Yamano et al. 1993). PP2A seems to negatively regulate entry into mitosis by positively regulating Wee1 and negatively regulating Cdc25. Therefore, inhibition of Bα-containing PP2A by E4orf4 would be predicted to block inactivation of the Cdc2 / cyclin B complex and possibly

result in progression through mitosis. Interestingly the results have demonstrated that human tumor cells undergoing E4orf4-induced death display elevated levels of Cdc2 kinase activity, as measured *in vitro*. Future studies could be performed to link E4orf4-mediated cell death with the effects on components of the G2 / M transition.

Finally, the identification of the mechanism of action of E4orf4 is of some importance for the development of new cancer therapies. E4orf4 displays a remarkable specificity for killing human cancer cells but not normal human primary cells (Marcellus, Lavoie et al. 1998; Shtrichman, Sharf et al. 1999). Although the E4orf4 gene could prove useful in cancer gene therapy (Marcellus *et al.*, in preparation), small molecules that mimic E4orf4 activity or that act on critical downstream PP2A substrates could provide entirely novel approaches for the development of cancer therapeutics.

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