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Phosphoprotein phosphatase 2A dephosphorylates eIF-4E and does not alter binding to the mRNA cap

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The phosphorylation and dephosphorylation of the 25 kDa mRNA cap binding protein eukaryotic initiation factor-4E (eIF-4E) is regulated during different physiologic and pathophysiologic states that include cell growth and the late phase of adenovirus infection. We have found that okadaic acid is much more effective in increasing the phosphorylated fraction of eIF-4E than phorbol 12-myristate 13-acetate in Hep G2 cells. Phosphoprotein phosphatase 2A dephosphorylated eIF-4E isolated from both phorbol 12-myristate 13-acetate- or okadaic acid-treated cells, whereas alkaline and acid phosphatase were relatively ineffective. The ability of purified [^{35}S]eIF-4E isolated from okadaic acid-treated cells to bind mRNA caps was compared to phosphoprotein phosphatase 2A-treated [^{35}S]eIF-4E and found to be no different. This suggests that alternative explanations for the previously observed effects of eIF-4E phosphorylation on protein synthesis must be considered. In addition, our results indicate that the in vivo phosphorylation of eIF-4E is not catalyzed solely by protein kinase C.

mRNA cap binding protein; eIF-4E; Phosphoprotein phosphatase 2A; Okadaic acid; Hep G2; Translation

1. INTRODUCTION

The 25 kDa mRNA cap binding protein eukaryotic initiation factor-4E (eIF-4E) exists in both an isolated state and as a multisubunit complex (eIF-4F) that appears to be required for efficient binding of most eukaryotic mRNA to 48 S ribosomal complexes [1–6]. Both eIF-4E and eIF-4F appear to be present in less than a 1:1 molar ratio with cellular mRNA [7,8]. Increases in the phosphorylation state of eIF-4E have been found to occur after the addition of pyrroline-5-carboxylate to reticulocyte lysates and following the treatment of cultured cells with phorbol 12-myristate 13-acetate (TPA) of the regulatory peptides, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin and tumor necrosis factor α (TNF-α) [9–17]. In addition, dephosphorylation of eIF-4E occurs during mitosis, heat shock, and the late phase of adenovirus infection which are all associated with decreases in protein synthesis [7,18,19]. Protein kinase C, casein kinase I and at least one other partially characterized protein kinase are capable of phosphorylating eIF-4E in vitro [20–23].

Abbreviations: m^{7}GTP, 7-methylguanosine triphosphate; eIF, eukaryotic initiation factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; TNF-α, tumor necrosis factor-α; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethyl sulfonyl fluoride; TPA, phorbol 12-myristate 13-acetate; IEF, isoelectric focusing.

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Although tryptic phosphopeptide map studies indicate that only protein kinase C phosphorylates an in vivo site, the phosphorylation sites of eIF-4E have been characterized under limited physiologic conditions [16,22]. At the present time it remains unclear if protein kinase C is the sole enzyme catalyzing eIF-4E phosphorylation following the activation of EGF, PDGF, insulin and TNF-α signal transduction pathways [11–14].

The in vivo effects of okadaic acid on eIF-4E phosphorylation support an involvement of either phosphoprotein phosphates 1 and/or 2A [12,24]. However, the phosphatases responsible for the rapid turnover of [^{32}P]eIF-4E intact cells have not been identified and studied with regard to eIF-4E [24]. In addition, there has been no direct evidence determining if dephosphorylation of native eIF-4E affects its interaction with mRNA caps.

2. MATERIALS AND METHODS

2.1. *Materials*

7-Methylguanosine triphosphate (m^{7}GTP) Sepharose and ampholytes were from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). [^{32}P]orthophosphate (1,201 Ci/mmol) and [^{35}S]methione/cysteine (1,134 Ci/mmol were from ICN Biomedicals Inc. (Irvine, CA). Okadaic acid was from Moana BioProducts Inc. (Honolulu, Hawaii). Protein phosphatase 2A, bovine cardiac catalytic subunit, was kindly provided by Marc Mumby of the University of Texas, Southwestern Health Sciences Center (Dallas, TX) [25]. All other reagents were from Sigma Chemical Co. (St. Louis, MO).

2.2. *Cell culture*

Human hepatoblastoma cells (Hep G2) were obtained from the

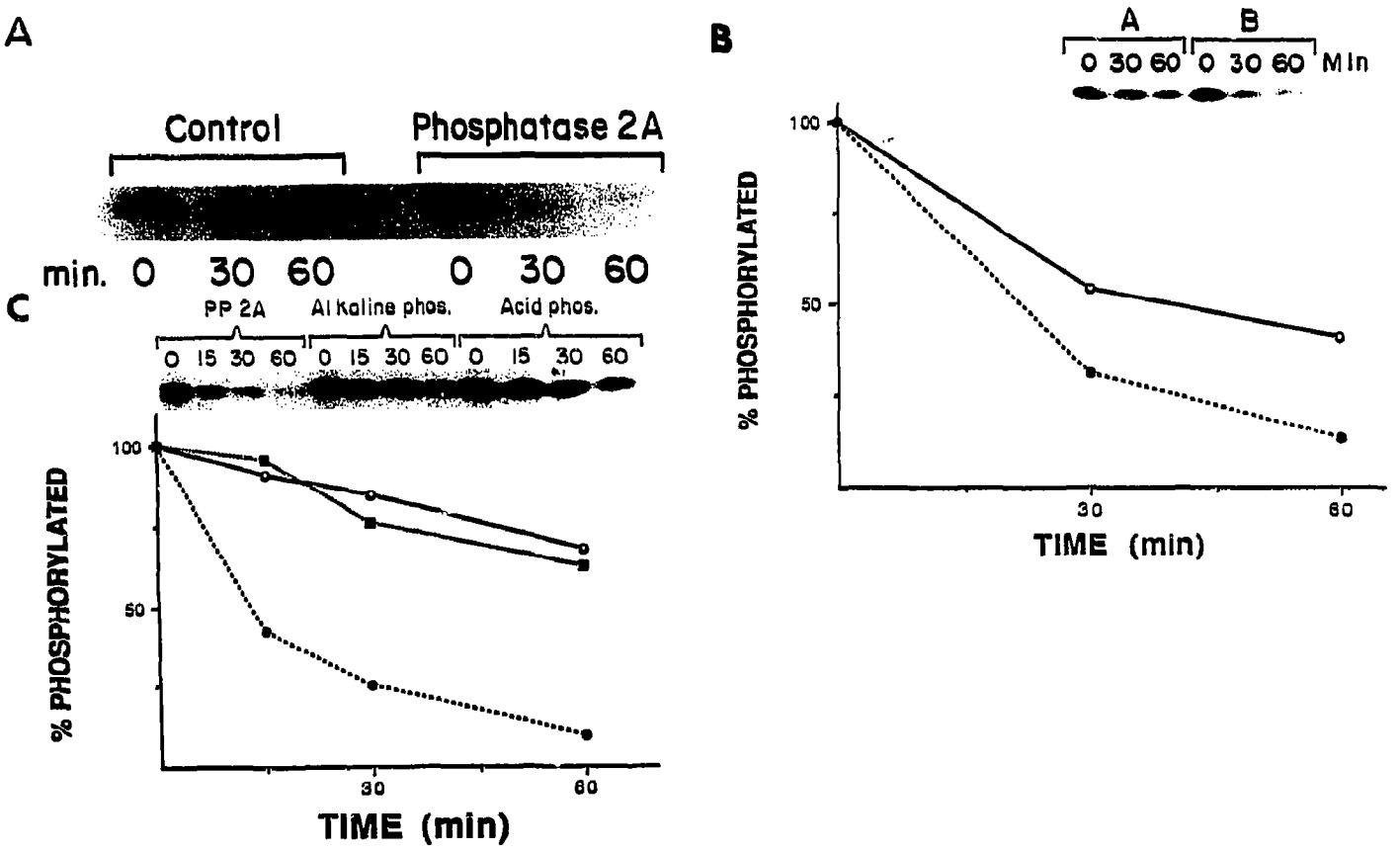

Fig. 1. Dephosphorylation of in vivo phosphorylated eIF-4E by the catalytic subunit of phosphoprotein phosphatase 2A. [^{32}P]eIF-4E was isolated from Hep G2 cells treated with 100 ng/ml of TPA for 3 h as described in Materials and Methods. (A) Time-dependent dephosphorylation of eIF-4E by phosphoprotein phosphatase 2A. (B) Densitometric quantitation of [^{32}P]eIF-4E following incubation with different quantities of phosphoprotein phosphatase 2A. The quantity of phosphatase 2A catalytic subunit in incubations were 1 μg (○) and 2 μg (●). At the indicated times samples were removed, analyzed by SDS-PAGE and autoradiography and [^{32}P]eIF-4E quantitated by densitometry. The insert in B shows an autoradiogram of samples analyzed at different incubations times in the presence of 1 μg (A) and 2 μg (B) of phosphoprotein phosphatase 2A. (C) The relative rate of eIF-4E dephosphorylation by acid phosphatase, alkaline phosphatase and phosphatase 2A. [^{32}P]eIF-4E isolated from TPA-treated Hep G2 cells was used as a substrate to assay relative rates of phosphatase activity. Incubations were at 30°C in 50 mM Tris/HCl (pH 8.0) for alkaline phosphatase, 50 mM sodium citrate (pH 5.5) for acid phosphatase and 20 mM HEPES (pH 7.5) for phosphoprotein phosphatase 2A. Incubations were 150 μl by volume and contained 2 μg of alkaline phosphatase, 2 μg of acid phosphatase and 2 μg of phosphatase 2A. Samples were removed at the indicated time and analyzed by SDS-PAGE, autoradiography and densitometry. The quantity of [^{32}P]eIF-4E given as percent of control (0 min) for each incubation is shown where (○) represents alkaline phosphatase, (■) represents acid phosphatase and (●) represents phosphatase 2A. The insert shows an autoradiogram of [^{32}P]eIF-4E removed from incubations at the indicated times.

American Type Culture Collection (Rockville, MD). Cells were grown to confluent monolayers in Eagle's minimum essential medium (MEM) with L-glutamine, non-essential amino acids, sodium pyruvate and antibiotics supplemented with 10% heat-inactivated fetal calf serum (Hyclone Laboratory Inc.) as described elsewhere [11]. Cells were washed with Dulbecco's modified Eagle's medium (minus phosphate or methionine) and incubated in this medium at 37°C for 2 h. Medium was removed following this pre-incubation and replaced with medium containing [^{32}P]orthophosphate or [^{35}S]methionine/cysteine (0.5 mCi/ml). Cells were then incubated at 37°C without other additions or with 100 ng/ml of TPA or 100 nM okadaic acid for the times indicated in the figure legends.

2.3. *Isolation of eIF-4E*

Labeled cells were washed with ice-cold phosphate buffered saline and lysed on ice by gently rocking with 1 ml of lysis buffer (20 mM HEPES, pH 7.5, 100 mM KCl, 50 mM β-glycerolphosphate, 10% glycerol, 0.2 mM EDTA, 0.2 mM sodium vanadate, 0.5% Triton X-100, 7 mM β-mercaptoethanol, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin) for 30 min. eIF-4E was isolated from lysates using m^{7}GTP Sepharose as described in detail elsewhere [11]. SDS-PAGE and equilibrium isoelectric focusing (IEF) analysis of eIF-4E was performed as described elsewhere [23]. Phosphoamino acid analysis was performed as described elsewhere [23].

2.4. *Phosphatase incubations*

[^{32}P]eIF-4E isolated from intact Hep G2 cells was incubated with or without protein phosphatase 2A at 30°C for the times indicated in the figure legends. Incubations were 150 μl and contained 20 mM HEPES, pH 7.5, 1 mM dithiothreitol and 10% glycerol. Equal volume aliquots of each incubation were taken at the indicated times and added to sample buffer. [^{32}P]eIF-4E was separated by 10% SDS-PAGE, autoradiograms prepared and [^{32}P]eIF-4E quantitated by densitometry.

3. RESULTS AND DISCUSSION

The identification of a phosphoprotein phosphatase that extensively dephosphorylates eIF-4E would be useful in determining if the in vivo phosphorylation of

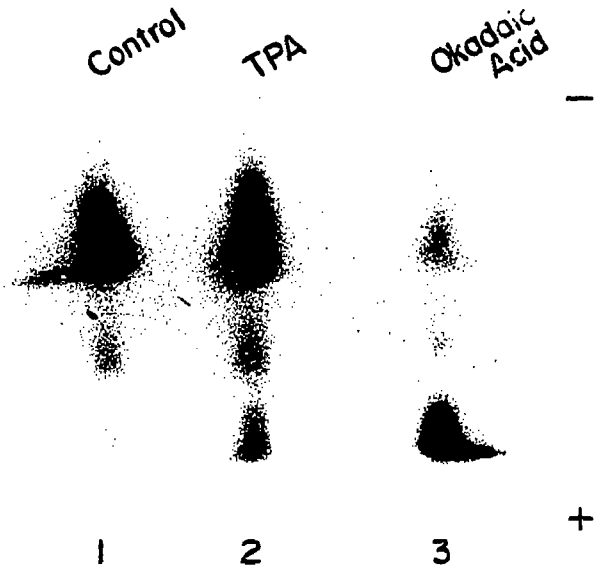

Fig. 2. Effect of TPA and okadaic acid on the recovery of phosphorylated eIF-4E from intact cells. Hep G2 cells were labeled with [^{35}S]methionine/cysteine and treated with TPA or okadaic acid for 3 h. eIF-4E was isolated as described in Materials and Methods and analyzed by equilibrium IEF. This figure shows an autoradiogram of an IEF gel of [^{35}S]eIF-4E isolated from control (lane 1), TPA (lane 2) and okadaic acid (lane 3)-treated cells. The major phosphorylated species is closest to the anode (+). Densitometry indicated that the three isoelectric species of eIF-4E (values given from cathode to anode) under each condition represented 83, 10 and 7% (control); 78, 8 and 14% (TPA); and 18, 8 and 74% (okadaic acid) of the total. The anode (−) and cathode (+) regions of the gel are indicated in the figure.

eIF-4E affects its function. Hep G2 cells were metabolically labeled with ^{32}P, treated with TPA to stimulate eIF-4E phosphorylation, and eIF-4E isolated using m^{7}GTP Sepharose as described. We found that purified phosphoprotein phosphatase 2A dephosphorylated [^{32}P]eIF-4E isolated from intact cells in a time- and dose-dependent manner (Fig. 1). In addition, relative to phosphoprotein phosphatase 2A, both alkaline and acid phosphatase were ineffective in dephosphorylating [^{32}P]eIF-4E isolated from intact cells (Fig. 1C).

To determine the optimal method of maximally phosphorylating eIF-4E at in vivo sites we compared the ability of TPA and okadaic acid to increase the phosphorylated fraction of eIF-4E. Hep G2 cells were incubated in the presence of [^{35}S]methionine/cysteine for 3 h with either TPA, okadaic acid or no additions and the phosphorylation state of eIF-4E isolated from these cells was quantitated by IEF analysis. Treatment of cells with okadaic acid proved to be effective in preparing eIF-4E that existed primarily (at least 74% in the phosphorylated state (Fig. 2). The observation that TPA treatment of cells was relatively ineffective in preparing in vivo phosphorylated eIF-4E suggests that protein kinase C is not the sole enzyme catalyzing physiologic eIF-4E phosphorylation (Fig. 2).

We used the okadaic acid treatment of cells and in vitro phosphoprotein phosphatase 2A treatment to prepare phosphorylated and dephosphorylated eIF-4E and directly determine if in vivo phosphorylation of eIF-4E altered its ability to interact with the mRNA cap structure. No difference in the ability of phosphorylated or dephosphorylated eIF-4E to bind to m^{7}GTP Sepharose was detected (Fig. 3A). IEF analysis of the eIF-4E bound to m^{7}GTP Sepharose verified that the control (lane 1) was primarily phosphorylated (denoted by B) and that the phosphatase 2A-treated eIF-4E bound to m^{7}GTP Sepharose was predominantly dephosphorylated (denoted by A) (Fig. 3B).

Based on our results we conclude that the in vivo phosphorylation of eIF-4E does not dramatically alter its ability to bind to the mRNA cap moiety in a purified system. This direct evidence argues against phosphorylated eIF-4E binding to mRNA caps with higher affinity (Fig. 4, model 1) as an explanation for the observation that in reticulocyte cell-free systems phosphorylated forms of eIF-4E stimulate protein synthesis and are preferentially incorporated into 48 S preinitiation complexes [26,27]. One possible explanation for this paradox is that phosphorylation of eIF-4E effects the assembly of the eIF-4E complex and/or the affinity by which the complex binds to the m^{7}GTP cap of mRNAs (Fig. 4, model 2). The dephosphorylation of eIF-4E that occurs during the late phase of adenovirus infection may provide a useful probe to address this question in intact cells [19]. An alternative approach to testing this hypothesis will be to use functional eIF-4E components overexpressed in *E. coli* and purified protein kinases that phosphorylate in vivo sites in reconstituted systems.

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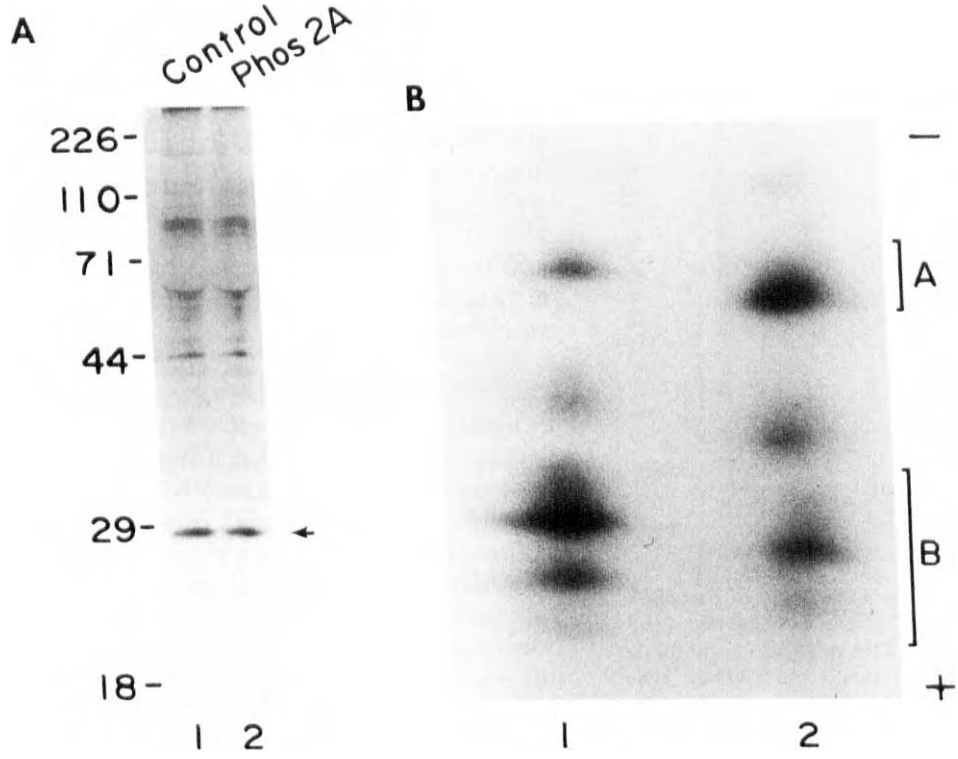

Fig. 3. Ability of dephosphorylated and phosphorylated [^{35}S]eIF-4E to bind the mRNA cap structure. Hep G2 cells were incubated with [^{35}S]methionine/cysteine and okadaic acid for 3 h. eIF-4E was isolated from cell lysates and equal quantities incubated in either the presence or absence of phosphoprotein phosphatase 2A as described in Fig. 1. After a 1 h incubation 400 μl of bovine serum albumin (1 mg/ml) was added and samples dialyzed against three exchanges (1 l each) of 20 mM HEPES, pH 7.5, 1 mM DTT and 10% glycerol over 18–20 h to remove the m^{7}GDP initially used to elute eIF-4E. Samples were mixed with 25 μl (packed volume) of m^{7}GTP Sepharose for 10 min and then washed two times with buffer. eIF-4E that remained bound to m^{7}GTP Sepharose was analyzed by SDS-PAGE and fluorography. (A) Control (lane 1) and dephosphorylated eIF-4E (lane 2) bound to m^{7}GTP Sepharose. The arrow indicates eIF-4E. (B) IEF analysis of eIF-4E that bound to m^{7}GTP Sepharose following control and phosphatase treatment (shown in A). Lane 1 contained untreated and lane 2 contained phosphatase 2A-treated eIF-4E.

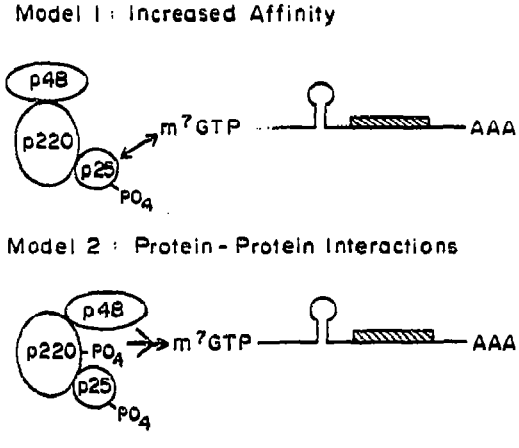

Fig. 4. Two hypothetical models for the effects of eIF-4E phosphorylation that have been reported previously [18,19,26,27]. The results we report here argue against model 1 where phosphorylation of eIF-4E simply modifies its interaction with the mRNA cap. An alternative hypothesis is that eIF-4E phosphorylation modifies protein–protein interactions within the eIF-4F complex and that this either promotes assembly of an active eIF-4E complex, increases the catalytic activity of the complex or increases the affinity with which the entire complex interacts with the mRNA cap structure.

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