Phosphorylation of eIF-4E on Serine 209 by Protein Kinase C Is Inhibited by the Translational Repressors, 4E-binding Proteins*

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Translation initiation in eukaryotes is facilitated by the mRNA 5′ cap structure (m7GpppX, where X is any nucleotide) that binds the multisubunit initiation factor eIF-4F through one of its subunits, eIF4E. eIF4E is a phosphoprotein whose phosphorylation state positively correlates with cell growth. Protein kinase C phosphorylates eIF4E in vitro, and possibly in vivo. Using recombinant eIF4E incubated in vitro with purified protein kinase C and analyzed by solid-phase phosphopeptide sequencing in combination with high performance liquid chromatography coupled to mass spectrometry, we demonstrated that the third amino acid of the peptide SGSTK (Ser209) is the major site of phosphorylation. This finding is consistent with the newly assigned in vivo phosphorylation site of eIF4E (Joshi, B., Cal, A. L., Keiper, B. D., Minich, W. B., Mendez, R., Beach, C. M., Stepinski, J., Stolarski, R., Darzynkiewicz, E., and Rhodea, R. E. (1995) J. Biol. Chem. 270, 14597–14603). A S209A mutation resulted in dramatically reduced phosphorylation, both in vitro and in vivo. Furthermore, the mutant protein was phosphorylated on threonine (most probably threonine 210) in vivo. Here we show that in the presence of the recently characterized translational repressors 4E-BP1 or 4E-BP2, phosphorylation of eIF4E by protein kinase C is strongly reduced. This suggests a two-step model for the phosphorylation (and activation) of eIF4E by growth factors and hormones: first, dissociation of eIF4E from 4E-BPs, followed by eIF4E phosphorylation.

In eukaryotes, translation initiation is rate-limiting and is highly regulated. The initiation factor eIF4F plays a key role in regulating translation initiation rates. eIF4F1 is composed of three subunits: eIF4A, the cap-binding protein; eIF4B, an RNA helicase; and eIF4G (formerly p220), which bridges between eIF4A and eIF4E. eIF4F binding to the cap structure (m7GpppX, where X is any nucleotide) is mediated by the eIF4E subunit, eIF4F, in conjunction with another initiation factor, eIF4F, is thought to unwind the mRNA 5′-secondary structure to facilitate the binding of ribosomes. eIF4E, and hence eIF4F, is present in limiting amounts in cells relative to other initiation factors (1, 2), and plays an important role in control of cell growth and development (3). eIF4E overexpression in rodent cells induces cellular transformation (4). eIF4F also exhibits mitogenic activity, as microinjection of eIF4E into serum-starved cells activates DNA synthesis (5). Conversely, expression of eIF4E antisense RNA diminishes translation rates, reduces cellular proliferation, and partially reverts the phenotype of transformed cells (6, 7).

Recently, two proteins which interact with eIF4E, 4E-BP1 and 4E-BP2 (for eIF4E Binding Proteins 1 and 2; also known as PHAS-I for Phosphorylated Heat- and Acid-Stable protein, Insulin stimulated), were characterized (8–10). These proteins, which share 56% identity, inhibit cap-dependent, but not cap-independent, translation (9). The two 4E-BPs inhibit eIF4E function by competing with eIF4G for a common binding site on eIF4E (11, 12). 4E-BP1 and 4E-BP2 binding to eIF4E is regulated by phosphorylation: the underphosphorylated species possess a high affinity for eIF4E, whereas the hyperphosphorylated species do not bind to eIF4E (9, 10). In unstimulated cells, a significant amount of 4E-BP1 is underphosphorylated and is bound to eIF4E (9). Following activation, either by insulin or growth factors, 4E-BP1 becomes hyperphosphorylated, and dissociates from eIF4E (9, 10). Subsequently, eIF4E binds to eIF4G to form an active eIF4F complex. Conversely, upon infection with some picornaviruses, 4E-BP1 is rendered completely underphosphorylated enabling 4E-BP1 to bind strongly to eIF4E, thus contributing to the shut-off of host protein synthesis (13). 4E-BP1 is a substrate for MAP kinase in vitro, and data suggest that it could be phosphorylated in vivo by the same enzyme (10). However, the mechanism of phosphorylation now appears to be more complex, since insulin-stimulated phosphorylation is inhibited by rapamycin, an immunosuppressant which has no effect on the MAP kinase pathway (14–16). In more recent experiments, using other inhibitors, such as wortmannin and SQ20006 and mutants in the platelet-derived growth factor receptor, it has been demonstrated that 4E-BP1 phosphorylation is effected by the phosphatidylinositol-3-OH (PI3) kinase wortmannin-sensitive pathway (17). As eIF4E is limiting in cells (1, 2), the regulation of 4E-BP1 binding activity by phosphorylation may explain some of the
modulation of eIF4E-dependent translation. However, the activity of eIF4E itself is also regulated by phosphorylation. The phosphorylation status of eIF4E positively correlates with translation and cellular growth rates. Phosphorylation of eIF4E increases in response to treatment with phorbol esters, hormones and growth factors (3). eIF4E also becomes phosphorylated upon maturation of T and B cells or differentiation of PC12 cells into neurons. Conversely, eIF4E is dephosphorylated in cells blocked in mitosis, or following heat-shock or infection by adenovirus, concomitant with a decrease in translation rates (for reviews, see Refs. 3 and 18). A biochemical function for eIF4E phosphorylation has been indicated by the reports that phosphorylation of eIF4E increases its affinity for eIF4G (19), as well as for the mRNA cap structure (20).

The major phosphorylated amino acid of eIF4E is serine, but some reports also demonstrated phosphorylation, under certain conditions, on threonine (19). Early experiments assigned the phosphorylation site to Ser53 (21). However, subsequent experiments showed that a mutant in which serine 53 was replaced by an alanine (S53A) was phosphorylated in vivo to the same extent as wild type eIF4E, suggesting that eIF4E was phosphorylated on another site (22). Recently, the in vivo eIF4E phosphorylation site has been reassigned to Ser209 (23–25).

Protein kinase C (PKC) (26–28) and an insulin-stimulated protamine kinase (29) phosphorylate eIF4E in vitro. Activation of PKC with phorbol esters enhances the phosphorylation state of eIF4E in vivo (18, 30). Furthermore, coinjection of PKC and eIF4E into quiescent NIH 3T3 cells leads to a synergistic effect on eIF4E mitogenic activity (31).

In this report we identified the PKC phosphorylation site on eIF4E and described the regulation of eIF4E phosphorylation by 4E-BP1 in vitro. We first confirmed that the amino acid phosphorylated on eIF4E in vivo and that phosphorylated by PKC in vitro are the same. We assigned the PKC phosphorylation site to Ser209, and showed that Thr210 is phosphorylated to some extent. We also showed that, when complexed to 4E-BP1 or 4E-BP2, eIF4E does not serve as substrate for PKC. Thus, it is possible that dissociation of eIF4E from 4E-BPs is a prerequisite for eIF4E phosphorylation. A model for the regulation of eIF4E phosphorylation is presented.

MATERIALS AND METHODS

In Vivo Labeling and Immunoprecipitation of eIF4E—NIH 3T3 cells (ATCC) at 80% confluence were labeled with [32P]orthophosphate (1 mM/l; DuPont NEN; 300 Ci/mmol) in 2.5 ml of phosphate-free Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) for 4 h. Oka-daic acid (10 mM; LC Technologies) was added 20 min prior to cell harvest. The medium was removed, and cells were rinsed with cold phosphate-buffered saline and lysed in radiimmune precipitation buffer containing phosphatase inhibitors (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% Triton X-100, 100 Kallikrein inhibitor units/ml aprotinin, 500 μM sodium orthovanadate, 50 mM NaF, 1 mM EDTA, and 1 mM β-glycerophosphate) Debris was spun down and the supernatant was precleared with preimmune serum preadsorbed on protein A-agarose (Repligen) for 1 h at 4°C. The supernatant was immunoprecipitated for 1.5 h using rabbit polyclonal antibody 5853 against murine eIF4E (32) preadsorbed on protein-A agarose beads. Beads were washed three times in radiimmune precipitation buffer and twice in 200 mM LiCl and 1 mM β-mercaptoethanol. The beads were resuspended in Laemmli sample buffer, and boiled and the proteins were resolved by SDS-polyacrylamide gel electrophoresis.

Bacterially Expressed Proteins and In Vivo Phosphorylation—Wild type eIF4E and mutants S53A and S209A (4) were expressed by the pET3b system and purified on a cap column as described previously (33). His-tagged PHAS-I was a kind gift from J. C. Lawrence, Jr. The coding sequence of 4E-BP2 was inserted in the vector pQE-31 (QIAGEN Inc.) to generate a fusion protein with a histidine tag at its N terminus. The protein was purified under denaturing conditions according to the manufacturer’s instructions. Purified myelin basic protein was obtained from Upstate Biotechnology, Inc. Bovine brain protein kinase C (a kind gift from M. Walsh) was a mixture of α, β, and γ isozymes. eIF4E (1 μg) was phosphorylated by 0.001 units of protein kinase C for 15 or 30 min at 30°C in kinase buffer (20 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 0.1 mM CaCl2, 10 mM dithiothreitol, 5 μM ATP, 10%, v/v, mixed micelles) in the presence of 10 μCi of [γ-32P]ATP. The mixed micelles consisted of a solution of 3.1 mg/ml L-α-phosphatidylylserine, 0.6 mg/ml bovine brain phosphatidylethanolamine, and 0.3% Triton X-100 in 20 mM Tris-HCl, which was sonicated twice for 30 s. Kinase reactions were terminated by the addition of Laemmli sample buffer followed by analysis on SDS, 12.5% polyacrylamide gels. Alternatively, the product of the kinase reaction was spotted onto a phosphocellulose (P81) paper which was washed extensively in 75 mM phosphoric acid and dried. Bound radioactivity was measured by Cerenkov counting in a scintillation counter.

Analysis of Tryptic Peptides by Two-dimensional Thin Layer Chromatography and Phosphoamino Acid Analysis—For tryptic mapping, 32P-labeled eIF4E was eluted from preparative SDS-polyacrylamide gels, oxidized, and digested with trypsin (Worthington) as described previously (34). Peptides were analyzed by two-dimensional TLC using Polygram CEL 300 thin layer plates (Bodman Chemical, Aston, PA) as described previously (34). Plates were exposed to a Kodak X-AR film. Phosphoamino acid analysis was performed with eIF4E eluted from preparative SDS-polyacrylamide gels as described previously (35).

V8 Digest and HPLC Analysis—Proteins were eluted from the SDS-polyacrylamide gels, digested with Staphylococcus aureus V-8 protease (Boehringer Mannheim), and peptides were analyzed by reverse phase HPLC as described elsewhere (34). Briefly, peptides were reconstituted in 200 μl of 20% formic acid and passed through a 0.22-μm membrane (Micron Separations Inc.). Samples were injected into a Waters dual pump HPLC system with a 600E controller using a 4.6 × 250-mm Ultrasphere ODS (C18) reverse phase column, which had been pre-equilibrated with buffer A (5% formic acid in HPLC grade water from Fisher). Peptides were eluted with a linear 1 to 63% gradient of buffer B (5% formic acid in ethanol) for 95 min at a flow rate of 1 ml/min. Detection of the labeled peptides was achieved with an on line isotope detector (Berthold, LB507A).

Phosphopeptide Sequencing, HPLC, and Electrospray Ionization Mass Spectrometry—Phosphopeptide sequencing was essentially performed as described (36). Briefly, phosphopeptides were eluted by reverse phase HPLC or extracted from TLC plates were covalently coupled to Immobilon AA discs (Millipore). After extensive washing of the sample discs, phosphopeptides were sequenced in a model 477A protein sequencer (Applied Biosystems) equipped with a model 120A phenylthiohydantoin analyzer (Applied Biosystems). For amounts in excess of an estimated 1 pmol, two-thirds of the phenylthiohydantoin sample was analyzed by UV absorbance detection and one-third of the sample was used for scintillation counting. If less than an estimated 1 pmol of the phosphopeptide was available, the total extract from the sequencer cartridge was collected without conversion and analyzed by liquid scintillation counting.

For capillary HPLC, a 0.1-μm inside diameter × 15× cm C18 reversed phase HPLC column (Micr-O Trait Scientific, Saratoga, CA) was connected to the electrospray ionization interface of the MS with a 50-cm-long × 50-μm inside diameter × 150 outside diameter, fused silica capillary. The solvent gradient was delivered by a Michrom Ultrafast Microprotein analyzer. The chromatography mobile phases were 0.05% trifluoroacetic acid, 2% acetonitrile in water (phase A) and 0.045% trifluoroacetic acid, 80% acetonitrile in water (phase B). Samples (10 μl) were introduced onto the column isocratically for 5 min with phase A followed by a gradient from 0% phase B to 50% phase B over another 35 min at a flow rate of 5 μl/min. A pre-column flow split was installed to reduce the flow rate from 100 μl/min being delivered by the pumps in the HPLC. The capillary mass spectrometer was an API-III triple quadrupole instrument equipped with an electrospray ionization interface (PE/Sciex, Thornhill, Ontario, Canada). The nebulization sheath gas was supplied via a 1-mm inside diameter Teflon tubing at a rate of 2 liters/min while a drying nitrogen curtain gas at ambient temperature was supplied at a flow rate of 1.8 liters/min to the region between the ionspray ionization source and the MS orifice. Survey scans were acquired either with polyprenylglycol or myoglobin. Typically, the quadrupole was scanned from m/z 300 to 2000 at 2.9 s/scan unless otherwise stated. Background was subtracted from all mass spectra data.

Plasmids and Transfections—Mutants S53A and S209A of murine eIF4E were generated by polymerase chain reaction mutagenesis; codon sequences were introduced in frame in the vector pACTAG-2 (a kind gift from A. Charest and M. Tremblay, McGill University) to express a fusion protein with three HA-tags. DNA was transiently transfected in...
The PKC Phosphorylation Site Resides on the in Vivo Phosphorylated Peptide—The major phosphorylation site of eIF4E in vivo had originally been assigned to Ser\(^{53}\) (21). Recently, the phosphorylation site was reassigned to Ser\(^{209}\) (23–25). Because the PKC phosphorylation site in vitro was reported to reside in the same tryptic peptide as the in vivo site (26), we wished to directly map the PKC phosphorylation site.

We first performed a time course of PKC kinase assay to determine the extent of incorporation of phosphate into eIF4E (Fig. 1A). At maximal incorporation, up to 0.32 pmol of phosphate were incorporated into 1 pmol of eIF4E. However, most of the experiments described were performed in the linear portion of this curve (at 15 min), where 0.15 pmol of phosphate are incorporated into eIF4E. This value is higher than that reported earlier with native eIF4E from rabbit reticulocytes (28).

We reproduced earlier results (26) showing that the eIF4E peptide that is phosphorylated in vivo and that phosphorylated in vitro by PKC comigrate on two-dimensional tryptic maps. For in vivo labeling, NIH 3T3 cells were incubated with \([^{32}P]\)orthophosphate, treated with okadaic acid, and eIF4E was immunoprecipitated. For in vitro labeling, bacterially expressed eIF4E was phosphorylated by PKC in vitro in the presence of \([^{32}P]ATP\) (Fig. 1B). Phosphoserine was the only detectable species in both the in vivo and in vitro samples (Fig. 1C). A single major spot was detected for the tryptic maps of both samples (Fig. 1D). When the two samples were mixed together prior to the analysis, a single phosphorylated species was observed (Fig. 1D). To further establish that the two peptides are identical rather than having fortuitously the same migration, reverse phase HPLC was performed. Peptides from both in vivo and in vitro samples were eluted from SDS-polyacrylamide gels, digested with V8 protease, and analyzed by HPLC. The radioactive material eluted at the same time (50–53 min) for both in vivo and in vitro labeled samples (Fig. 1E).

Ser\(^{209}\) Is the Major PKC Phosphorylation Site in eIF4E—To determine the amino acid that is phosphorylated by PKC in eIF4E, phosphopeptides isolated by TLC after in vivo and in vitro labeling were subjected to solid-phase phosphopeptide sequencing. The major peak of radioactivity eluted in cycles 3 and 4 for both in vivo and in vitro tryptic phosphopeptides (Fig. 2; some radioactivity in the first cycle is commonly observed in this procedure and represents the material which is not covalently attached to the disc). The radioactivity in cycle 4, which was particularly high in the in vitro sample, could be due either to carryover from cycle 3 or to authentic phosphorylation of the amino acid in cycle 4. As shown in Table I, three peptides generated by tryptic cleavage of eIF4E (indicated by asterisks) have a serine at position 3: Ser\(^{24}\), Ser\(^{64}\), and Ser\(^{209}\).

Next, bacterially expressed eIF4E was extensively phosphorylated in vitro by PKC and digested with trypsin. One major phosphopeptide (1) and a minor phosphorylated form (2) were detected by TLC tryptic phosphopeptide analysis (Fig. 3A). In contrast to the samples analyzed in Fig. 1, this tryptic digest contained a significant amount of phosphorylated threonine, as determined by two-dimensional phosphoamino acid analysis (Fig. 3B). In earlier reports, it was noted that PKC phosphorylation of eIF4E yielded two phosphorylated peptides and that radioactive phosphate was incorporated into threonine in addition to serine (40). In our hands, this occurred when the reaction was carried out for an extended period of time or with excess kinase (conditions used for Fig. 3A). The tryptic digest was also analyzed by liquid chromatography coupled with mass spectrometry (LC-MS). The LC-MS chromatogram is shown in Fig. 3C. Two peaks (a and b) accounted for all the radioactivity in the sample that was injected onto the HPLC column. The mass spectra of peaks a and b are shown in Fig. 3, D and E, respectively. The mass spectra of peak a shows an intense peak at an m/z ratio of 659 (Fig. 3D), which corresponds to the m/z ratio of the molecular ion of the tryptic peptide \(207^\text{SGSTTK}^{212}\) of eIF4E, bearing one phosphate group. The spectrum also contains smaller peaks at m/z values corresponding to molecular ions resulting from partial fragmentation of the parent phosphopeptide \(207^\text{SGSTTK}^{212}\). From this fragmentation pattern we have narrowed down the site of phosphorylation to either Ser\(^{209}\), Thr\(^{210}\), or Thr\(^{211}\), since the first serine and the glycine can be cleaved from the parent phosphopeptide without

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**Fig. 1. PKC phosphorylates eIF4E at the site that is phosphorylated in vivo.** A, time course of incorporation of phosphate into eIF4E. eIF4E was performed as described under “Materials and Methods” using 10 pmol of eIF4E bacterially expressed and 1 pmol of phosphate (ratio hot/cold phosphate is 1/2000) and radioactivity bound to the phosphocellulose paper was quantified by scintillation counting (the incorporation in PKC alone was subtracted from the total counts before the conversion into picomoles of phosphate); results presented here are the mean of two experiments with different purifications of eIF4E. B, immunoprecipitated eIF4E from orthophosphatelabeled NIH 3T3 cells and murine recombinant eIF4E phosphorylated by PKC for 15 min were analyzed by SDS-PAGE and autoradiography as described under “Materials and Methods.” C, phosphoamino acid analysis was performed on the samples shown in B, as described under “Materials and Methods.” Positions of cold amino acid standards are indicated. D, samples eluted from the gel in B were digested with trypsin and analyzed on two-dimensional phosphopeptide maps as described under “Materials and Methods.” Arrows indicate the origin of application. E, Samples eluted from the gel in A were digested with V8 protease and separated by HPLC, as described under “Materials and Methods.”

293 cells using electroporation as described previously (39). Expression of the constructs was assessed 30 h post-transfection by Western analysis using anti-HA monoclonal antibody (12CA5). Cells were labeled with \([^{32}P]\)orthophosphate 48 h post-transfection, as described above. Tagged eIF4E protein was immunoprecipitated with an anti-HA-tag antibody, subjected to electrophoresis, and processed for tryptic mapping and phosphoamino acid analysis as described above.

**RESULTS**

The PKC Phosphorylation Site Resides on the in Vivo Phosphorylated Peptide—The major phosphorylation site of eIF4E in vivo had originally been assigned to Ser\(^{53}\) (21). Recently, the phosphorylation site was reassigned to Ser\(^{209}\) (23–25). Because the PKC phosphorylation site in vitro was reported to reside in the same tryptic peptide as the in vivo site (26), we wished to directly map the PKC phosphorylation site.

We first performed a time course of PKC kinase assay to determine the extent of incorporation of phosphate into eIF4E (Fig. 1A). At maximal incorporation, up to 0.32 pmol of phosphate were incorporated into 1 pmol of eIF4E. However, most of the experiments described were performed in the linear
the loss of the phosphate group. The mass spectra of the component in peak b in Fig. 3 corresponds to that of the same tryptic peptide 207SGSTTK212, but bearing two phosphate groups. The radioactivity in this fraction was, however, much lower than that of the material in peak a (data not shown), suggesting that phosphorylation occurs predominantly at only one amino acid residue. By combining the data obtained from the solid phase phosphopeptide sequencing with that from the mass spectra analysis, we conclude that Ser209 is the major PKC phosphorylation site on eIF4E, while a minor site of phosphorylation may occur on Thr210.

The assignment of Ser209 as the phosphorylation site in eIF4E was further substantiated with mutants of Ser209 in vivo experiments. HA-epitope tagged eIF4E wild type and S209A were transiently transfected into 293 cells followed by labeling with [32P]orthophosphate. Immunoprecipitation with anti-HA antibody showed a 30% decrease in the 32P incorporation in the mutant relative to the wild type protein (Fig. 4A).

Similar studies were done in vitro. Wild type eIF4E or S53A and S209A mutants were expressed in bacteria and purified on a cap column (33). The S53A mutant protein was phosphorylated by PKC to the same extent as the wild type (Fig. 5). In contrast, eIF4E S209A mutant was phosphorylated to a much lesser extent than wild type eIF4E (Fig. 5), further indicating that serine 209 is the main site phosphorylated by PKC.

DISCUSSION

eIF-4E Phosphorylation by PKC on Serine 209

TABLE I

| Amino acid sequence | Amino acid position | Position of Ser/Thr |
|---------------------|---------------------|--------------------|
| MATVEPETPTNPPAAEEK  | 1                   | 3, 8, 9, 11, 12    |
| TESNQEVEANPEHYIK*   | 22                  | 22, 24             |
| SK                  | 53                  | 53                 |
| TWQANLR             | 55                  | 55                 |
| LISK*               | 62                  | 62                 |
| FDTVEFALYNHIQLSSNLMPCDYSLFK | 66     | 68, 82, 83, 92 |
| WLITLNK            | 113                 | 116                |
| SDLDR              | 124                 | 124                |
| FWLETLLCLIGESFDDYSDDVCAGVNNR | 129    | 133, 141, 146    |
| IAIWTTCECN         | 163                 | 168, 169           |
| DAVTHIUGR          | 174                 | 177                |
| IVIGYQSHADTATK     | 193                 | 199, 203, 205      |
| SGSTTK*            | 207                 | 207, 209, 210, 211 |

* Asterisks indicate peptides generated by tryptic cleavage of eIF4E that have a serine at position 3 (see “Results”).
tion modulates eIF4E activity, as the phosphorylated form of eIF4E binds more tightly to the cap structure and has an enhanced affinity for eIF4G (19, 20, 41).

Several reports demonstrated the phosphorylation of eIF4E by PKC in vitro (26–28). The peptide phosphorylated in vitro co-chromatographed with the in vivo phosphorylated peptide (26). However, the PKC phosphorylation site has not been identified directly. Smith et al. (31) have shown that a bacterially expressed S53A mutant could not be phosphorylated in vitro by PKC. However, here we found no differences in PKC phosphorylation between S53A and wild type eIF4E. It is possible that there is a variability in the renaturation of S53A mutant and, as a result, a change of the mutant protein to serve as substrate for PKC. We determined directly by LC-MS analysis that the main phosphorylation site of PKC in vitro is Ser209. This agrees well with the recent findings that Ser209 is the major site of phosphorylation of eIF4E in vivo (23). Also, in agreement with this assignment is our finding that bacterially expressed mutant S209A was not phosphorylated to a significant extent. In addition, Ser209 conforms very well with the PKC consensus phosphorylation site (42). When the kinase reaction was performed for an extended period of time, an additional amino acid residing on the same peptide, most probably Thr210, became phosphorylated. When expressed in mammalian cells, the mutant S209A was not phosphorylated on serine, but rather on threonine, probably Thr210. Taken together, these data provide direct and unambiguous evidence that the phosphorylation site of PKC on eIF4E is Ser209.

There are many lines of evidence that support a role for PKC in phosphorylation of eIF4E in cells. Treatment of cells with phorbol esters that activate PKC induces eIF4E phosphorylation (30, 32, 43). In many cell types, down-regulation of PKC by phorbol esters prevents stimulation of eIF4E phosphorylation by phorbol 12-myristate 13-acetate or insulin (reviewed in Ref. 3). Furthermore, PKC co-injection with eIF4E into NIH 3T3 cells has a synergistic effect on eIF4E mitogenic activity (31).

**Fig. 3. Identification of the PKC phosphorylated peptide on eIF4E by LC-MS.** eIF4E was phosphorylated in vitro by PKC for 30 min and resolved by SDS-PAGE. eIF4E was eluted from the gel and digested with trypsin. A, a tryptic phosphopeptide map was performed as in Fig. 1. B, two-dimensional phosphoamino acid analysis was performed as described under “Materials and Methods.” C, tryptic digests, prepared as for A, were fractionated on an HPLC column. Peaks containing radioactivity are indicated by arrows a and b. D and E, phosphorylated peptides from peaks a and b, respectively, were analyzed directly by mass spectrometry as described under “Materials and Methods.” The position of two peaks resulting from partial fragmentation of the parent phosphopeptide SGSTTK is indicated by letters. S indicates the peak resulting from the cleavage of the first serine of the peptide, and G indicates that from the cleavage of the glycine.

**Fig. 4. Mutant S209A is phosphorylated on threonine in vivo.** HA-tagged eIF4E wild type and S209A mutant were expressed in 293 cells. Following labeling of the cells with [32P]orthophosphate, HA-tagged eIF4E was immunoprecipitated using an anti-HA antibody as described under “Materials and Methods.” A, immunoprecipitated proteins were separated on an SDS-polyacrylamide gel and autoradiographed. B and C, two-dimensional phosphoamino acid analysis was performed on proteins eluted from the polyacrylamide gel, for the wild type and the S209A mutant, respectively.

**Fig. 5. In vitro phosphorylation of eIF4E wild type, S53A, and S209A by PKC.** Recombinant eIF4E was expressed in Escherichia coli and purified by cap affinity chromatography as described under “Materials and Methods.” Proteins were phosphorylated in vitro by PKC and separated on an SDS-polyacrylamide gel followed by autoradiography as described under “Materials and Methods.”
eIF4E Phosphorylation by PKC on Serine 209

Fig. 6. **4E-BPs prevent in vitro phosphorylation of eIF4E**. A, 4E-BP1 (0.5 µg) or 4E-BP2 (0.5 µg) were mixed with eIF4E (1 µg) and incubated in the kinase buffer (without lipids) on ice for 30 min prior to the kinase reaction. Control reactions consisting of either rat 4E-BP1 (PHAS-1), 4E-BP2, myelin basic protein (MBP), 4E-BP1 and MBP, or 4E-BP2 and MBP were processed in parallel. The in vitro kinase assay was performed for 15 min as described under “Materials and Methods.” Phosphorylated proteins were separated by SDS-PAGE and autoradiographed.

However, in PC12 cells, down-regulation of PKC with phorbol insensitive isoform of PKC, such as PKC ε, could be mediated at least partly through a phorbol ester-phosphorylation. 4E-BP1 associates with eIF4G and eIF4A and can be phosphorylated by PKC. Consequently eIF4E becomes free to associate with eIF4G and eIF4A and can be phosphorylated by PKC. However, it is not known what rapamycin-sensitive kinase phosphorylates the 4E-BPs in vivo, since 4E-BP1 is not a substrate for the rapamycin-sensitive kinase p70S6k (45). Also, phosphorylation of 4E-BP1 by ERK1 and ERKII is prevented in vitro when eIF4E is bound to 4E-BP1 (15). This result is also compatible with the model presented in Fig. 7, since the kinase that phosphorylates 4E-BP1 in the rapamycin-sensitive pathway might still be able to phosphorylate 4E-BP1 when bound to eIF4E. The model in Fig. 7 posits that, following phosphorylation of 4E-BP1 and disruption of the complex between eIF4E and 4E-BP1 (step 1), eIF4E associates with eIF4A and eIF4G to form the eIF4F complex (step 2). Two subunits of eIF4F, eIF4E and eIF4G, are phosphorylated by PKC (step 3). Phosphorylation of eIF4E by PKC might occur on the eIF4F complex, since eIF4E as a part of eIF4F was reported to be a better substrate for protein kinase C than free eIF4E (28). Phosphorylation of eIF4E (or of eIF4A and eIF4G) by PKC enhances binding to the cap structure of the mRNA and stabilizes the interaction between eIF4G and eIF4E (19, 20, 39), leading to an increase in the translation (step 4).

Future studies will be necessary to define the biological significance of Ser209 phosphorylation. Joshi et al. (23) reported that a mutant S209A retained the ability to associate with 4E S preinitiation complex, in contrast to a S53A mutant of eIF4E. It would be important to test the activity of the double mutant S209A/T210A, since, as shown here, Thr210 is phosphorylated for protein kinase C than free eIF4E (28). Phosphorylation of eIF4E (or of eIF4A and eIF4G) by PKC enhances binding to the cap structure of the mRNA and stabilizes the interaction between eIF4G and eIF4E (19, 20, 39), leading to an increase in the translation (step 4).

![Diagram](image.png)

Fig. 7. **Proposed model for the events leading to eIF4E phosphorylation.** In resting cells, a significant portion of eIF4E is bound to 4E-BP1. Insulin first causes the dissociation of the 4E-BP1/eIF4E complex by phosphorylating 4E-BP1. Consequently eIF4E becomes free to associate with eIF4G and eIF4A and can be phosphorylated by PKC. However, in PC12 cells, down-regulation of PKC with phorbol esters has no effect on the nerve growth factor-induced phosphorylation of eIF4E (43). There are two possible explanations for this finding: (a) there exists an alternative pathway leading to phosphorylation of eIF4E and (b) phosphorylation of eIF4E could be mediated at least partly through a phorbol ester-insensitive isoform of PKC, such as PKCζ (44).

The results in this report indicate that phosphorylation of eIF4E can be regulated by 4E-BPs in vitro. We demonstrated here that the phosphorylation of eIF4E by PKC is prevented when 4E-BP1 is bound to eIF4E. Thus, phosphorylation of 4E-BP1 or of 4E-BP2 might be an obligatory step for eIF4E phosphorylation. Consistent with this finding, the phosphorylation of eIF4E and that of 4E-BP1 follow similar kinetics in response to serum stimulation. Phosphorylation of eIF4E is thus likely to depend on the activation of two kinases: one, unknown as yet, that phosphorylates 4E-BPs and releases eIF4E, and the second, PKC, which phosphorylates eIF4E.

Fig. 7 shows a model for the phosphorylation of eIF4E. Insulin treatment activates one or more pathway(s) that leads to the phosphorylation of 4E-BP1. Earlier results suggested that the phosphorylation of 4E-BP1 is carried out by the MAP kinases ERK1 and ERK2 (45). Recent evidence (reviewed in 3) indicate that this pathway is incorrect and that the major 4E-BP1 phosphorylation pathway is the PI3K/p70S6k rapamycin-sensitive pathway, rather than the MAP kinase pathway.

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