Phosphorylation of eIF4E at a Conserved Serine in Aplysia*

We have cloned eIF4E from the marine mollusk, Aplysia californica. The sequence of eIF4E from Aplysia is more similar to vertebrate eIF4Es than to other invertebrate sequences. Aplysia eIF4E is encoded by two tissue-specific RNAs. Antibodies raised to the carboxyl terminus of eIF4E recognize a 29-kDa protein that can bind to 7-methyl-GTP caps. The phosphorylation site identified in mammalian eIF4E is conserved in the Aplysia homologue, and an Aplysia eIF4E fusion protein is phosphorylated well by both Aplysia protein kinase C isoforms. However, protein kinase C phosphorylates both Ser-207 and Thr-208 in vivo, while only Ser-207 is phosphorylated in vitro. We have confirmed that Ser-207 is phosphorylated in vivo by raising a phosphopeptide antibody to this site. This antibody will be useful in determining the signal transduction pathways leading to eIF4E phosphorylation in Aplysia.

Synaptic strength is modulated in the short term by post-translational modifications, such as protein phosphorylation, and in the long term by changes in gene expression. Recently, the regulation of translation has been suggested to be important in regulating synaptic strength at a time between short and long term periods (1–4). We are therefore interested in the biochemical pathways that regulate translation in the nervous system.

Translation is regulated by a number of factors, both eukaryotic initiation factors (eIFs) and eukaryotic elongation factors (eEFs). In the nervous system, a large decrease in translation rate during ischemia in vertebrates is correlated with a Ca2+-dependent phosphorylation of eEF2 (5). The translation rate can be increased by synaptic activation (6) or by the actions of growth factors (1); however, the translation factors involved in these pathways have not been identified.

In other cell types growth factors increase the translation rate through a rapamycin-sensitive pathway involving phosphorylation of two regulators of translation, the S6 kinase and the eukaryotic initiation factor 4E (eIF4E)-binding protein (7–9). Phosphorylation of the eIF4E-binding protein releases eIF4E, the subunit of the eIF4F initiation complex that binds directly to the 5′-7-methyl-G(5′)ppp(5′)N mRNA cap, from its complex with the eIF4E-binding protein (9–11). eIF4E is also regulated by phosphorylation at Ser-209 (12–14), increasing its ability to bind to caps (15) and to form the eIF4F complex (16, 17). This phosphorylation is correlated with translational rate in a number of cell types (10). The kinase that phosphorylates eIF4E has been proposed to be PKC (18, 19) and/or the MAP kinase-activated Mnk (14, 20, 21).

In the marine mollusk Aplysia californica, the facilitating neurotransmitter serotonin (5-HT) induces both short and long term facilitation of the sensory-motorneuron synapse (22). While short term facilitation does not require protein synthesis and long term facilitation requires the activation of transcription, at intermediate times facilitation requires translation, but not transcription (23). Furthermore, when 5-HT is applied only to the synaptic region, long term facilitation requires local protein synthesis at the synapse (24). 5-HT increases the rate of translation in sensory neurons and in the entire pleural ganglia (25, 26). 5-HT-mediated activation of translation in the pleural ganglia can be blocked by rapamycin (26), suggesting that it may involve regulation of eIF4E. To study the regulation of translation at the molecular level in this system, we have cloned and characterized the Aplysia homologue of mammalian eIF4E. The Aplysia eIF4E shares many properties with the vertebrate molecule, including phosphorylation at a conserved serine in vivo.

EXPERIMENTAL PROCEDURES

Cloning—Degenerate PCR primers were designed from eIF4E regions of high conservation and low degeneracy (Fig. 1) and used to synthesize by PCR (template = Aplysia nervous system cDNA plasmid library (27); Taq DNA polymerase (Life Technologies, Inc.) = 0.5 unit/µl; MgCl2 = 2.5 mM; annealing temperature = 40 °C) a 217-base pair region that is highly homologous to other eIF4E sequences. Two opposing specific PCR primers were made based on the sequence of this subclone and used in conjunction with primers hybridizing to 5′ ends of the Aplysia eIF4E clone. This screening yielded a clone that includes a possible initiating methionine with a good match to the Kozak consensus sequence.

NORTHERN BLOTTING—RNA was isolated from Aplysia using Trizol. Five µg of total RNA was loaded in each lane (equivalent amounts verified by methylene blue staining of the nylon filters). The

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‡ Medical Research Council scholar and a scholar of the EJLB Foundation. To whom correspondence should be addressed: Dept. of Neurology and Neurosurgery, McGill University, Montreal Neurological Institute, Montreal, Quebec H3A 2B4, Canada. The abbreviations used are: eIF, eukaryotic initiation factor; eEF, eukaryotic elongation factor; PKC, protein kinase C; 7-methyl-GTP, 5′-7-methyl-G(5′)ppp(5′); MAP, mitogen-activated protein; PCR, polymerase chain reaction; NTA, nitritotriaetic acid; PAGE, polyacrylamide gel electrophoresis; 5-HT, serotonin.

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Fusion Protein Synthesis—A six-histidine fusion protein (His-eIF4E) was expressed in bacterial Top10 cells from a pTrcHis plasmid (Invitrogen, Carlsbad, CA) containing an insert of eIF4E amino acids 16 to 215 synthesized using Vent DNA polymerase (New England Biolabs, Beverly, MA), primers specific for the ends of the Aplysia eIF4E ORF, and the Aplysia GT10 library. The ST-AA mutant was created by PCR using the same 5′ primer, a different 3′ primer (GGGGTACCTTAGGAAAAACGTTTTCTCAGCTGCTCCAGCCTGGTGC), and the pTrcHis-eIF4E plasmid. The His-eIF4E fusion proteins were purified on nickel-NTA-agarose beads (Qiagen, Mississauga, ON).
Phosphorylation of eIF4E in Aplysia

RNA was transferred to Hybond-N nylon filters (Amersham Pharmacia Biotech, Oakville, ON), UV-cross-linked, and hybridized overnight at 42 °C with an end-labeled antisense oligonucleotide probe (CATTCCTCCTCCTTACTTCTGGTCTCCCCATACAGG-CCTGATGC- GCTTTAACACCGT) corresponding to amino acids 77–99 (Fig. 1) in 6× SSC, 0.5% sodium pyrophosphate, 1% SDS, and 100 μg/ml tRNA. The blots were washed twice for 15 min in 1× SSC, 0.1% SDS and exposed for 6 days at 20 °C. The filters were then washed in 3 mM ATP. The filters were washed for 5 min in 100 mM NaF and 1 mM dithiothreitol. For tryptic mapping of the peptides, the bovine serum albumin-peptide conjugates were phosphorylated under similar conditions, separated on a 12% polyacrylamide gel, transferred to nitrocellulose membranes, and exposed to film and/or a PhosphorImager screen and quantitated using ImageQuant (Molecular Probes).

In Vivo Phosphorylation—The His-eIF4E fusion protein was tested as a substrate for phosphorylation by purified ApiI and ApiII, the two Aplysia PKC isoforms (29, 30). 40 nm to 3 μM His-eIF4E was incubated with either 1.5 nm ApiI or 0.5 nm ApiII in the presence of 20 mM DTT, 1 mM dithiothreitol, 50 μM phosphatidylethanolamine, 50 μM ATP, including 1 μM [γ-32P]ATP at 37 °C for 30 min. The reaction was stopped by adding Laemmli buffer, separated on a 12% SDS-polyacrylamide gel, and transferred to 0.2-μm nitrocellulose. The nitrocellulose membranes were exposed to film and/or a PhosphorImager screen and quantitated using ImageQuant (Molecular Probes).

In Vivo Phosphorylation—Three adult Aplysia nervous systems were incubated in resting medium (26) containing [32P]orthophosphate at 2 μCi/ml for 20 h at 15 °C, washed in resting medium three times, homogenized on ice in homogenizing microtubes at 500 μl of homogenization buffer (20 mM HEPES, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 5 mM benzamidine, 0.1 mM leupeptin, 5 μM phenylmethylsulfonyl fluoride) and centrifuged at 5000 × g for 5 min at 4 °C. The supernatant was added to 40 μl of 1 M KCl and 150 μl of 7-methyl-GTP-Sepharose 4B beads and then handled as described in the cap binding section above, except that the beads were washed twice in 500 μl of homogenization buffer plus 100 mM KCl before the GDP washes, and the beads were eluted with 200 μl of 7-methyl-GTP, all of which was loaded onto the SDS-polyacrylamide gel. After transfer, the nitrocellu-
loose membrane was exposed to Kodak BioMax MS film for 2 days at \(-80\,^\circ\text{C}\) with enhancing screens.

**Analysis of Tryptic Peptides by Two-dimensional Thin Layer Chromatography**—Phosphopeptide analysis was performed as described with several modifications (31). Membrane fragments containing protein bands were preblocked in 0.5% PVP-360 in 100 mM acetic acid, washed, and subsequently digested overnight with 10 \(\mu\text{g}\) of trypsin in 10 mM Tris (pH 7.5) and 5% acetonitrile. An additional 10 \(\mu\text{g}\) of trypsin was added to each band for 3 h the following day and the supernatant solution containing desired peptides was lyophilized to completion. Peptides were resuspended in pH 1.9 buffer (0.6 M formic acid and 1.4 M acetic acid) and analyzed by two-dimensional TLC using a Hunter-3000 HTLC. Labeled peptides were visualized by autoradiography using Kodak BioMax MS film on the plate.

**Phosphoamino Acid Analysis**—After tryptic digestion and lyophilization, eIF4E peptides derived from in vitro or in vivo phosphorylations were hydrolyzed to constituent amino acids using constant boiling 6\(\times\)HCl for 2.5 h at 110 \(^\circ\text{C}\). Sample amino acids were mixed with phosphoserine, phosphothreonine, and phosphotyrosine standards prior to running the mixture in the primary TLC dimension using standard buffers (31). Phosphoamino acid standards were stained with 0.25% ninhydrin in acetonitrile, and autoradiography was performed on the completed plate.

**RESULTS**

**Cloning of Aplysia eIF4E and Sequence Comparison**—We used a degenerate PCR strategy to clone a fragment of eIF4E from an Aplysia nervous system cDNA library and then used PCR and library screening to extend this clone (see “Experimental Procedures”). Eventually a 2000-base pair DNA fragment was cloned containing an ORF of 215 amino acids 30–59% identical to eIF4E from other species (Fig. 1). Interestingly, the clone is more homologous to the vertebrate sequences (59%) than to other invertebrate sequences. The ORF begins at a putative initiating methionine which has a good match to the Kozak consensus and whose position is similar to that in vertebrate eIF4E. In-frame stop codon at the carboxyl-terminal is present at a position analogous to that of other eIF4E proteins. Residues necessary for eIF4E binding to the 5’ cap of mRNA (32–34) are completely conserved, as is the phosphorylation consensus sequence near the carboxyl-terminal.

**Tissue Distribution**—The distribution of the eIF4E message in three Aplysia tissues was investigated by Northern blot. A 3000-nucleotide message was detected in the gill, while a 6000-base pair message was seen in the nervous system and ovotestis (Fig. 2A). The different relative amounts of the two transcripts in different tissues indicates that the transcripts are regulated in a tissue-specific manner. The large size of the message suggests that there are considerable uncharacterized 5’- and/or 3’-untranslated sequences on the messages.

We raised an antibody to a synthetic peptide derived from the carboxyl-terminal of Aplysia eIF4E. This antibody recognized a major 29-kDa protein in all the tissues examined (Fig. 2B), including gill where the eIF4E message was smaller. The 29-kDa protein is specifically eluted from 7-methyl-GTP beads, whereas other cross-reacting bands are not (Fig. 2C), demonstrating that this immunoreactive 29-kDa protein has the properties expected of eIF4E. Although 29 kDa is larger than the predicted molecular size of eIF4E (24.6 kDa), it is very similar to the apparent molecular size of vertebrate eIF4E run on SDS-PAGE which also has a similar predicted molecular size (15, 35).

**Aplysia eIF4E Is Phosphorylated in Vivo**—We determined whether eIF4E is phosphorylated in vivo by examining the incorporation of radioactive phosphate into Aplysia eIF4E in intact ganglia. Two proteins which specifically eluted from 7-methyl-GTP-Sepharose beads were labeled (Fig. 2D), one at 29 kDa that aligns exactly to the location of Aplysia eIF4E as seen on the corresponding Western blot (Fig. 2D, lane 3), and the other at 22 kDa, which is more heavily phosphorylated. The 22-kDa protein is likely to be an eIF4E-binding protein, but we cannot rule out a protein that binds independently to caps, such as the cap-binding protein involved in cap-dependent mRNA transport (36, 37).

**Aplysia eIF4E Is Phosphorylated in Vitro by PKC at Ser-207 and Thr-208**—In mammals, eIF4E is phosphorylated by PKC in vitro at Ser-207, which is also the in vivo phosphorylation site (12–14). This site is conserved in Aplysia (Ser-207) (Fig. 1). To assess Aplysia eIF4E as a substrate for phosphorylation by PKC, we expressed Aplysia eIF4E in bacteria as a fusion pro-
tein with an amino-terminal six histidine tag. This recombinant protein (His-eIF4E) was readily phosphorylated in vitro by both Aplysia PKC isoforms, ApI and ApII (data not shown). Lineweaver-Burk analysis of the data yielded \( K_m \) values of 215 ± 22 nM, S.E., \( n = 4 \) (ApI) and 123 ± 18 nM, S.E., \( n = 4 \) (ApII). Maximal phosphorylation of eIF4E incorporated 0.4 mol of phosphate/mol of eIF4E, similar to results from vertebrates (14).

To determine whether the carboxyl-terminal PKC consensus sequence in Aplysia eIF4E can be phosphorylated, an in vitro phosphorylation reaction was performed using the carboxyl-terminal peptide (Fig. 1) containing the consensus sequence. Unfortunately, analysis of the phosphorylated carboxyl-terminal peptide revealed no detectable phosphotyrosine or phosphoserine in the tryptic peptides generated after phosphorylation of His-eIF4E (data not shown). To confirm this, we synthesized the carboxyl-terminal peptide with serine converted to phosphoserine. Consistent with the hypothesis that PKC phosphorylates Thr-208, we generated an antibody to the carboxyl-terminal peptide phosphorylated at Ser-207. This antibody did not recognize the His-eIF4E fusion protein, but did recognize this fusion protein after phosphorylation by PKC (Fig. 4A). After mutating Ser-207 and Thr-208 of His-eIF4E to alanines, the antibody was unable to recognize the mutated eIF4E treated with PKC, and phosphorylation of the

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**Fig. 3.** Phosphopeptide mapping and phosphoamino acid analysis of eIF4E. Proteins were phosphorylated, digested, and analyzed as described under “Experimental Procedures.” A–C, the His-eIF4E fusion protein is phosphorylated at the carboxyl-terminal site. A, phosphopeptide map of His-eIF4E fusion protein. B, phosphopeptide map of the carboxyl-terminal eIF4E peptide. C, phosphopeptide map of co-applied His-eIF4E and carboxyl-terminal eIF4E peptide illustrating that the spots from His-eIF4E are the same as those from phosphorylating the carboxyl-terminal peptide. D and E, both Ser-207 and Thr-208 can be phosphorylated in vitro. D, phosphopeptide map of the carboxyl-terminal eIF4E peptide with Ser-207 changed to phosphoserine reveals only one phosphopeptide, doubly phosphorylated at Ser-207 and Thr-208. E, phosphopeptide map of co-applied carboxyl-terminal eIF4E phosphoSer-207 peptide and His-eIF4E illustrating that the slower migrating phosphopeptide from the His-eIF4E is phosphorylated at Ser-207 and Thr-208. F and G, eIF4E is only singly phosphorylated in vivo. F, phosphopeptide map of in vivo labeled eIF4E. G, phosphopeptide map of co-applied in vivo labeled eIF4E and His-eIF4E showing that the singly phosphorylated peptide generated in vitro is identical to the peptide generated by phosphorylation in vivo. H, the in vivo phosphorylation site of eIF4E is a serine by phosphoamino acid analysis. + and − refer to the polarity of the primary electrophoresis dimension in buffer pH 1.9; vertical arrows indicate direction of liquid chromatography; O signifies origin of sample application or coapplication; S, T, and Y indicate the location of phosphoserine, phosphothreonine, and phosphotyrosine standards, respectively.

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We hypothesized that the two spots seen in the two-dimensional phosphopeptide analysis may be due to PKC phosphorylation at Thr-208, as well as Ser-207, as has been reported for vertebrate eIF4E (13). Indeed, phosphoamino acid analysis of the tryptic peptides generated after phosphorylation of His-eIF4E showed phosphorylation at both serine and threonine (data not shown). To confirm this, we synthesized the carboxyl-terminal peptide with serine converted to phosphoserine. Consistent with the hypothesis that PKC phosphorylates Thr-208 in vitro, this peptide was also a substrate for PKC. Two-dimensional tryptic analysis of this product revealed the slower migrating spot demonstrating that this spot corresponded to the doubly phosphorylated peptide (Fig. 3, D and E). This is consistent with its predicted charge of −1 compared with the predicted charge of 0 for the singly phosphorylated peptide.

**Aplysia eIF4E Is Phosphorylated at Ser-207 in Vivo**—Only one spot that co-migrated with the singly phosphorylated peptide was seen after in vivo phosphorylation of eIF4E (Fig. 3, F and G). The in vivo labeled eIF4E contained only serine by phosphoamino acid analysis (Fig. 3F). These results are consistent with phosphorylation of Ser-207 in vivo. To confirm this site of phosphorylation, we generated an antibody to the carboxyl-terminal peptide phosphorylated at Ser-207. This antibody did not recognize the His-eIF4E fusion protein, but did recognize this fusion protein after phosphorylation by PKC in vitro (Fig. 4A). After mutating Ser-207 and Thr-208 of His-eIF4E to alanines, the antibody was unable to recognize the mutated eIF4E treated with PKC, and phosphorylation of the
invertebrate isoforms (30–40% identical), suggesting that the function and regulation of *Aplysia* eIF4E will be similar to that of eIF4E in vertebrates. The closer relationship to vertebrate isoforms is primarily due to a higher degree of identity in the carboxyl-terminal region, including the consensus phosphorylation site (Fig. 1). This is somewhat surprising, as mollusks are at least as distant from vertebrates as are insects, and for many other *Aplysia* sequences that are present in both vertebrates and insects, the *Aplysia* sequence is more similar to *Drosophila* homologues than to vertebrate ones (data not shown). This suggests that regulation of translation in *Aplysia* may be more similar to vertebrates than to these other invertebrates.

There are two transcripts of eIF4E in *Aplysia* detected on Northern blots. There are mRNAs for multiple eIF4E isoforms in humans (38), *Drosophila* (39), *Xenopus* (40), *Caenorhabditis elegans* (41), and wheat germ (42, 43). The alternatively spliced *Drosophila* isoforms differ only slightly at their amino termini, and the *Xenopus* isoforms differ only in containing one or two copies of a short insert. However, the two types of eIF4E in wheat germ arise from different genes, have quite different amino acid sequences (50% identity), and exist in different initiation complexes. In *Aplysia*, the two *Aplysia* eIF4E mRNAs differ greatly in size and tissue distribution, although it is unlikely that the proteins encoded by the mRNAs differ proportionately in size, because our antibody recognizes proteins which migrate identically on SDS-PAGE from tissues expressing either the larger or the smaller mRNA. Rather, there are probably additional domains in the untranslated regions of the longer mRNA, making it tempting to speculate that the messages are translated in response to different cellular factors, such as is the case for insulin-like growth factor II (44).

The initiation of translation is regulated by the availability and phosphorylation of eIF4E, which enhances mRNA cap binding (15) and stabilizes the interaction between eIF4E and eIF4G (16). In mammals, eIF4E is phosphorylated at Ser-209 in vivo (12, 13), and the same residue is phosphorylated by PKC in vitro (14). This serine is conserved in *Aplysia* (Ser-207), and *Aplysia* eIF4E in vitro is a good substrate for the *Aplysia* PKC isoforms. However, while PKC phosphorylates both Ser-207 and Thr-208 in vitro, eIF4E is phosphorylated in vivo only at Ser-207. Furthermore, activation of PKC in the *Aplysia* nervous system decreases translation (26), while an increase in eIF4E phosphorylation would be predicted to increase the translation rate. Recently, activation of the MAP kinase-activated Mnk has been proposed to be associated with phosphorylation of eIF4E (20), and Mnk phosphorylates eIF4E in vitro at Ser-209 (21). Interestingly, the MAP kinase pathway is also involved in regulating long term facilitation in *Aplysia* (45) and may be involved in regulating translation.

The eIF4E phosphopeptide antibody will be useful for dissecting out the pathway controlling eIF4E phosphorylation in *Aplysia*. If the antibody is also useful in immunocytochemistry, it should be possible to localize the activation of eIF4E and determine whether the local protein synthesis required for synapse-specific long term facilitation (24) involves local phosphorylation of *Aplysia* eIF4E.

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**REFERENCES**


**DISCUSSION**

eIF4E is the least abundant component of the translation initiation complex. Consequently, its activity and availability regulate the complex’s function and, in turn, the rate of translation in eukaryotes (10). We have cloned eIF4E from *Aplysia* to study its potential role in changing the translation rate during learning and memory formation in this organism. We demonstrate here that *Aplysia* eIF4E is a true eIF4E homologue based on its high homology to eIF4E in other species and its ability to bind specifically to a mRNA cap analogue. In addition, *Aplysia* eIF4E is phosphorylated in vivo at the serine homologous to the serine phosphorylated in vertebrate cell lines.

The amino acid sequence of *Aplysia* eIF4E is closer to that of vertebrate eIF4E isoforms (almost 60% identical) than to other
Phosphorylation of eIF4E in Aplysia


Phosphorylation of eIF4E at a Conserved Serine in Aplysia
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