Serotonin Increases Phosphorylation of Synaptic 4EBP through TOR, but Eukaryotic Initiation Factor 4E Levels Do Not Limit Somatic Cap-Dependent Translation in *Aplysia* Neurons[▽]

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The target of rapamycin (TOR) plays an important role in memory formation in *Aplysia californica*. Here, we characterize one of the downstream targets of TOR, the eukaryotic initiation factor 4E (eIF4E) binding protein (4EBP) from *Aplysia*. *Aplysia* 4EBP contains the four critical phosphorylation sites regulated by TOR as well as an N-terminal RAIP motif and a C-terminal TOS site. *Aplysia* 4EBP was hypophosphorylated in synaptosomes, and serotonin addition caused a rapamycin-sensitive increase in 4EBP phosphorylation both in synaptosomes and in isolated neurites. *Aplysia* 4EBP was regulated in a fashion similar to that of mammalian 4EBPs, binding to eIF4E when dephosphorylated and releasing eIF4E after phosphorylation. Overexpression of 4EBP in the soma of *Aplysia* neurons caused a specific decrease in cap-dependent translation that was rescued by concomitant overexpression of eIF4E. However, eIF4E overexpression by itself did not increase cap-dependent translation, suggesting that increasing levels of free eIF4E by phosphorylating 4EBP is not important in regulating cap-dependent translation in the cell soma. Total levels of eIF4E were also regulated by 4EBP, suggesting that 4EBP can also act as an eIF4E chaperone. These studies demonstrate the conserved nature of 4EBP regulation and its role in cap-dependent translation and suggest differential roles of 4EBP phosphorylation in the soma and synapse.

An increase in the rate of translation that is sensitive to the pharmacological agent rapamycin was recently shown to be an important event underlying lasting phases of synaptic plasticity (2, 5, 16, 58). The primary target of rapamycin in cells is the target of rapamycin (TOR) (9, 24, 30). One way in which TOR controls translation is by regulating the phosphorylation state of the eukaryotic initiation factor 4E (eIF4E) binding protein (4EBP). Dephosphorylated 4EBP tightly binds eIF4E and represses cap-dependent protein synthesis, and TOR-dependent phosphorylation removes this repression (6, 11, 46). The amount of eIF4E available in the cell can be a rate-limiting factor in cell growth since its overexpression causes an increase in the expression of growth-promoting proteins, such as ornithine decarboxylase, cyclin D1, and c-Myc (15, 49, 51, 55). Therefore, activation of TOR may contribute to enduring forms of synaptic plasticity through phosphorylation of 4EBP and an increase in translation of molecules that contribute to synaptic growth and/or reorganization.

4EBP is located at synaptic sites in mammalian dendrites (58) and is phosphorylated in a TOR-dependent manner after brain-derived neurotrophic factor treatment in dendrites or late-phase long-term potentiation (LTP)-inducing stimuli (3, 35, 57). Moreover, knocking out the gene for 4EBP2, the major 4EBP isoform in adult neurons, converts early LTP into late LTP in the Schaffer collateral pathway (3), suggesting that

activating translation through 4EBP phosphorylation is a critical step in generating long-term changes. Long-term depression induced by the addition of an mGLUR agonist depends on local protein synthesis and is rapamycin sensitive (2, 31, 33). Interestingly, in 4EBP2 knockout mice, mGLUR long-term depression is no longer sensitive to rapamycin, indicating that the critical downstream target of TOR for this form of plasticity is 4EBP2 (2). These findings underscore the importance of the role of local 4EBP activation for late phases of synaptic plasticity. To date, however, no direct examination of the link between 4EBP activity and its effect on translation rates in neurons has been carried out.

In *Aplysia californica*, increases in the strength of sensory-to-motor neuron synapses, termed facilitation, underlie behavioral sensitization of the gill and siphon withdrawal reflexes (34). Experiments on cultured *Aplysia* sensory-motor neuron synapses have revealed that a 5-min synaptic application of serotonin (5HT) to the synapse is sufficient to cause a persistent (72-h) phase of long-term facilitation, whose induction requires local TOR-dependent translation, when gene expression is activated independently (16). Rapamycin applied to the whole cell also blocked 24-h long-term facilitation, suggesting that activation of TOR in the cell body is important for this form of plasticity (32). Soma activation of translation through TOR has also been implicated in LTP in hippocampal neurons (36). We have previously shown that 5HT can activate S6 kinase and eukaryotic elongation factor 2 (eEF2) through a rapamycin-sensitive pathway in *Aplysia* synaptosomes (14, 37). In the present study, we characterize *Aplysia* 4EBP, demonstrate its regulation by 5HT and TOR at the synapse, and determine its effects on soma translation.

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TABLE 1. List of primers and templates for PCR

Construct name	Primer and sequence	Template(s)	Introduced site
4EBP	05, 5'-GGGGGATCCATGGCAGGGCAAGATCAG-3' 03, 5'-GGGGGTACCTCAGATGTCCATCTCGAACT-3'	<i>Aplysia</i> cDNA library	BamHI KpnI
4EBP-E62/67	05, 5' 4EBP primer I5, 5'-GAGCCCTTGGCCAGAGAGCCTCCACCCAAT ATGGCCAAA-3' I3, 5'-CTCTCTGGCCAAGGGCTCATTTCTTAGTTGC ATAAGACG-3'	pGEX-4EBP	MscI
4EBP-E34/43	03, 3' pGEX primer 05, 5' 4EBP primer I5, 5'-GGTACCTTGTCTTCCACAGAACCTGGTGGAA CTAGATT-3' I3, 5'-AAACAAGGTACACCTGGCTCCGAAGTGA ATCTGAGG-3'	pGEX-4EBP	EcoRI KpnI
4EBP-4xE	03, 3' pGEX primer 05, 5' 4EBP primer I5, 5' E34/43 primer I3, 3' E34/43 primer 03, 3' pGEX primer	pGEX-E62/67	
4EBP-E34	05, 5' 4EBP primer I5, 5'-GAGCCAGGTACCTTGTCTTCCACAGAACCTG GTGGAAGT-3' I3, 5'-GGTACCACCTGGCTCCGAAGTGAATCTGA GGGAAGCTGAGA-3'	pGEX-E43 and pGEX-E43/62/67	KpnI
4EBP-dTOS	03, 3' pGEX primer 05, 5' 4EBP primer 03, 5'-GGGTACCTCACTGTGGGTGCTCGTCGTGA-3'	pGEX-4EBP	KpnI

MATERIALS AND METHODS

Animals. *Aplysia californica* (70 to 200 g) were obtained from Marine Specimens Unlimited (Pacific Palisades, CA) or the University of Miami National Institute of Health *Aplysia* resource facility (Miami, FL) and were maintained in an aquarium for at least 3 days before experimentation. Prior to dissection, animals were placed in a bath of isotonic $MgCl_2$ artificial seawater (1:1, vol/vol) and then anesthetized by injection with isotonic $MgCl_2$ solution. Ganglia were isolated from the animal and placed in ice-cold dissecting medium (460 mM NaCl, 10 mM KCl, 11 mM CaCl₂, 55 mM MgCl₂, 10 mM HEPES, pH 7.5) before use.

Cloning of *Aplysia* 4EBP and mutagenesis. An initial sequence for *Aplysia* 4EBP was donated, courtesy of Eric Kandel, from an *Aplysia* expressed sequence tag library. We designed exact primers to the 5' and 3' ends of 4EBP, including restriction sites for BamHI (5' primer) and KpnI (3' primer). A 363-bp fragment was amplified by PCR from an *Aplysia* cDNA library, digested with restriction enzymes BamHI and KpnI, and inserted into pNEX-3. pNEX-3-4EBP was subsequently digested with BamHI and EcoRI, and the excised fragment was inserted into pGEX-2T.

All mutants were generated using the two-step overlap PCR procedure described previously (39), using different pGEX-2T-4EBP constructs as templates. In order to generate the desired mutagens, the 5' 4EBP primer and a 3' primer designed against pGEX-2T (5'-GCGCGAGGCAGATCGTCAGTCAGTCACGATCAATTCCC-3') were used as outside primers in combination with inside primers containing serine- or threonine-to-glutamic acid (S/T-E) mutations (see Table 1 for the specific primer sequences and restriction enzymes used). The initial primers used to generate T-E mutations at T34/43 failed to generate such changes at T34. Consequently, all constructs meant to contain this mutation were used as templates in additional rounds of site-directed mutagenesis, using a set of inside primers with a T-E change only at T34 (Table 1). A 4EBP-dTOS construct was generated using the 5' 4EBP primer with a 3' primer missing the sequence coding for the TOS domain. All products were cut with the appropriate enzymes (Table 1) and inserted back into pGEX-2T and/or into pNEX-3. Clones were sequenced over the entire amplified product when generated by PCR to confirm that no additional changes were made.

Antibody production. (i) **Anti-PS62/67.** A 15-amino-acid peptide containing residues 58 to 72 of *Aplysia* 4EBP with S62 and S67 converted to phosphoserine (pS) and an amino-terminal cysteine (CQLRN[pS]PLAR[pS]PPN-amide) was synthesized (Biosource, Camarillo, CA), conjugated to maleimide-coupled bovine serum albumin (Pierce, Rockford, IL), and injected into rabbits, using the

adjuvant TiterMax Gold (CytRx, Norcross, GA) three times at 4-week intervals. Rabbit serum was passed over an affinity column of the immunizing phosphopeptide coupled to SulfoLink (Pierce). After each passage, specifically retained antibodies were eluted from the column and concentrated in an Amicon Ultra-15 centrifugation column (Millipore, Billerica, MA). In order to eliminate the fraction of antibody that might recognize unphosphorylated isoforms of 4EBP, nonphosphorylated peptide was incubated with the phosphoantibody at a molar ratio of 1:400 (antibody/peptide) for 30 min before each primary incubation for immunoblots.

(ii) **Anti-4EBP.** Antibody recognizing endogenous levels of 4EBP was generated against a peptide containing residues 108 to 122 (CFGHDEHPQFEMD IOH) at the C-terminal end of 4EBP, using the same procedure as that described above.

(iii) **Anti-PT34/43.** A commercial antibody mix recognizing only isoforms of 4EBP phosphorylated at T37 and/or T46 (T34/43 in *Aplysia*) (Cell Signaling Technologies, New England Biolabs, Beverly, MA) was used to measure phospho-T34/43-4EBP levels.

(iv) **Anti-eIF4E.** Antibody recognizing endogenous levels of eIF4E and antibody recognizing only isoforms of eIF4E phosphorylated at S207 were generated and used as described previously (20).

(v) **Anti-GST.** A commercial glutathione *S*-transferase (GST) antibody (Amersham, Piscataway, NJ) was used to recognize GST and GST-tagged 4EBP constructs.

Synaptosomes. Synaptosomes were prepared according to the procedure developed by Chin et al. (17). This protocol gives a P1 pellet containing particulate proteins, two soluble fractions (S2 and S3), and two synaptosome-containing fractions, P3, which contains the purest synaptosomes, and P2, which contains the greatest amount of synaptosomes but of lesser purity (17). The P2 and P3 fractions were combined in 600 μ l of Ca^{2+} -free seawater (460 mM NaCl, 10 mM KCl, 55 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4, 0.1% glucose). Synaptosomal preparations were treated for 10 min with various combinations of the drugs: 20 μ M 5HT (Sigma, St. Louis, MO), 20 nM rapamycin (Calbiochem, San Diego, CA), 100 nM calyculin (Calbiochem), and 20 μ M LY294002 (LY; Calbiochem). Samples were treated with Laemmli buffer, boiled, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting was performed as previously described (22) with 4EBP and eIF4E antibodies. Blots were scanned and quantitated using the NIH image uncalibrated optical density feature. This helps to retain linearity over a wide range of intensity levels for enhanced chemiluminescence films (45). For quantitation of 4EBP

and eIF4E phosphorylation, a phosphoratio was determined as described previously (37).

Immunoprecipitation. Ovary or total nervous system extracts were homogenized in 200 μ l homogenization buffer (1% Tergitol NP-40 [Sigma], 10 mM MgCl₂, 15 mM Tris, pH 7.5, 1 mM EGTA, 10% glycerol, 0.5% aprotinin, benzamide, and leupeptin, 0.5 mM β -mercaptoethanol, 10 mM NaF), and nonsolubilized proteins were spun out at 100,000 $\times$ g. Supernatants were then incubated overnight with preimmune serum or anti-4EBP serum which was precoupled to protein A-Sepharose beads (Sigma). Beads were spun down and washed with homogenization buffer, and Laemmli buffer was added to the samples, which were subsequently boiled and subjected to SDS-PAGE. Beads were washed extensively (four washes, $\sim$ 10 min/wash) for immunoprecipitations from ovary. Protein was transferred onto nitrocellulose membranes and subjected to immunoblotting with 4EBP and eIF4E antibodies.

mRNA cap binding with nervous system extracts. Extracts from two *Aplysia* nervous systems were homogenized on ice in a low-salt-concentration buffer (LCB) (20 mM HEPES, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 100 mM KCl, 10 mM β -mercaptoethanol, 0.5% benzamide, leupeptin, and aprotinin, 10 mM NaF) and centrifuged at 5,000 $\times$ g for 5 min at 4°C, and the supernatant was incubated with 7-methyl-GTP-Sepharose 4B beads (Amersham) for 60 min at 15°C. Beads were pelleted and washed with LCB, and Laemmli buffer was added to the samples, which were subsequently subjected to SDS-PAGE. Protein was transferred onto nitrocellulose membranes and subjected to immunoblotting with 4EBP and eIF4E antibodies.

mRNA cap in vitro binding assay. (i) **Preparation of GST-tagged 4EBP and His-tagged eIF4E protein.** All 4EBP constructs present in pGEX-2T were transformed and overexpressed in DH5 α cells. The resultant protein was purified with GST-Sepharose 4B beads (Amersham), eluted with GST elution buffer (20 mM glutathione, 100 mM HEPES, 120 mM NaCl, pH 8), and concentrated to the smallest volume possible using Amicon Ultra-15 centrifugation columns (Millipore). This solution was resuspended in $\sim$ 15 ml of 1 $\times$ phosphate-buffered saline (PBS) and reconcentrated to $\sim$ 1 μ g/ μ l. Purified His-eIF4E was generated as previously described (20).

(ii) **Binding assay.** In order to reduce background signal from both His-eIF4E and GST-4EBP constructs, 7-methyl-GTP beads were preincubated in LCB with 5% bovine serum albumin for 30 min at 4°C. Beads were subsequently incubated with roughly stoichiometric amounts of His-eIF4E and GST-4EBP constructs in LCB with 5% bovine serum albumin for 1 h at 25°C. The beads were pelleted and washed three times for 10 min with LCB at 25°C. Laemmli's buffer was added to samples, which were subsequently boiled, subjected to SDS-PAGE, transferred onto nitrocellulose, and immunoblotted with eIF4E, 4EBP, or GST antibody.

Immunocytochemistry. (i) **Experiments with isolated processes.** *Aplysia* sensory neurons were mechanically dissociated in 3-ml culture dishes and left for recovery overnight in 10% hemolymph-enriched L15 culture medium (modified for *Aplysia*) (39). Cells with resorbed processes were then plated on poly-D-lysine-coated glass coverslips in dishes containing 30% hemolymph-enriched L15. Alternatively, neurons were pulled from the ganglia with a sharp glass microelectrode tip and plated immediately with their primary axons intact. All cells were left for 3 days at 19°C. Cells with healthy processes were then selected for axotomy. This consisted of either cell body removal with a sharp glass electrode or individual axon incision. Incisions were made proximal to the cell body, roughly 50 to 100 microns away, and avoiding the initial axon segment (40). Cultures were left to recover for 2 h after cutting, and processes were then treated with 5HT and/or rapamycin. Cells were then fixed on coverslips in 4% paraformaldehyde in PBS with 30% sucrose and left for 30 min. After fixing, cultures were permeabilized in 0.1% Triton X-100 in PBS with 30% sucrose, washed in PBS, blocked with 10% normal goat serum (NGS; Sigma), and incubated with anti-PT34/43 in 10% NGS overnight. Cultures were then washed with PBS and incubated with Cy3 goat anti-rabbit antibody (Pierce) in 10% NGS for 2 h. Cultures were washed again, and fluorescence images were obtained with an Eclipse TE200 fluorescence microscope (Nikon, Tokyo, Japan) using the Metavalue Imaging program (Universal Imaging Co., Pennsylvania).

(ii) **Experiments with coinjection of 4EBP and bicistronic constructs.** A fluorescent bicistronic expression pNEX3 vector was used to monitor simultaneously cap-dependent translation (enhanced cyan fluorescent protein expression) and internal ribosome entry site (IRES)-dependent translation (enhanced yellow fluorescent protein expression) from the IRES in *Aplysia* egg-laying hormone as previously described (19). Wild-type and mutant 4EBPs and/or eIF4E was inserted into the pNEX3 vector and coinjected into neurons along with the bicistronic reporter to determine their effects on translation. Fluorescence was measured 2 days after injection, using a Nikon TE200 fluorescence microscope equipped with cyan GFP-BP v2, FGP(Y)-BP (yellow), and Y-2E/C (red) filters. Digital images were captured using Metavalue (Universal Imaging Co.).

	RAIP site	
<i>Aplysia</i> 4EBP	MAG QDQSPKGREIPAI RRVVLNDVSQLP	28
Human 4EBP-1	M SGGSSCSQTP SRAIPAT RRVVLGDGVQLP	30
Human 4EBP-2	MS SSAGSGHQPSQSRAPT RTVAISDAAQLP	31
Human 4EBP-3	MSTSTS CIPGGR DQLP	17
<i>Drosophila</i> 4EBP	MSASPTARQAIT QALPMITKVVISDPIQME	31
<i>Dicty</i> 4EBP	MST TRAIIVSL KDRSDI	17
	eIF4E binding	
<i>Aplysia</i> 4EBP	S DYSSTPGGTLFSTTPGGTRIVYDRSLMQLRN	62
Human 4EBP-1	PGDYSTTPGGTLFSTTPGGTRIIYDRKFLMECRN	65
Human 4EBP-2	H DYCTTPGGTLFSTTPGGTRIIYDRKFLLDNRN	65
Human 4EBP-3	D CYSTTPGGTLATTPGGTRIIYDRKFLLECKN	51
<i>Drosophila</i> 4EBP	E VYSSTPGGTLYSTTPGGTKLIYERAFMKNLRG	65
<i>Dicty</i> 4EBP	D FSTSLGGTLTYCTTPGGTKIYDRNALLQYRN	50
<i>Aplysia</i> 4EBP	SPLARSPFPNMAKIPGITDFEPESSEKSEAKDKE	96
Human 4EBP-1	SPVTKTPPRDLPTIPGVTS SPDEPPMEASQSH	98
Human 4EBP-2	SPMAQTTPCHLPNIPGVTS GTLIEDSKVEVNN	98
Human 4EBP-3	SPIARTPPCCLPQIPGVTS PTAPL SKLEELK	83
<i>Drosophila</i> 4EBP	SPLSQTPPSNVPSCLLGRTPRTFRKCVFVPTL	99
<i>Dicty</i> 4EBP	SPLSKTTPPPQLAHITNTLNNKVEKSTTT PTTT	83
	TOS site	
<i>Aplysia</i> 4EBP	NGPKEDSKDESKFGHDEHPQFEMDI.	121
Human 4EBP-1	LRNSPE DKR AGGEESQFEMDI.	119
Human 4EBP-2	LNNLNNHDKR HAVGDDAQFEMDI.	121
Human 4EBP-3	EQETEE IPDDAQFEMDI.	101
<i>Drosophila</i> 4EBP	IKQTKSLK IEDQEQLDL.	118
<i>Dicty</i> 4EBP	TPPTTTAK PKPTNDDIFTME.	104

FIG. 1. Amino acid sequence for *Aplysia* 4EBP. The eIF4E binding motif, RAIP motif (R-E-I-P in *Aplysia*), and TOS site (each denoted with a line above the sequence) are well conserved. The four serine/threonine phosphorylation sites most linked to the regulation of eIF4E binding are also conserved (*).

Quantitation of immunofluorescence. Experiments using injected cells included a vector control (cells injected with only the pNEX3 vector). Both control and experimental conditions used sensory cells from the same animal(s). All staining was done simultaneously with aliquots of one antibody dilution. All pictures were taken under identical imaging parameters (brightness, fluorescence duration, etc.). Fluorescence was then quantified blindly to the experimental condition by using the NIH imaging program. Pixel intensities were quantitated by measuring the average pixel intensity of the cytoplasm (for immunofluorescence) or the maximal pixel intensity of a line profile drawn through the most intensely fluorescent part of the cell body (for fluorescent proteins). The extracellular background was subtracted from this maximum. For fluorescent protein measurement, the maximal pixel intensity (after extracellular background subtraction) from noninjected cells was also subtracted to remove background from endogenous cell fluorescence. For each experiment, the average pixel intensity of vector-injected cells was set to 1, and all individual cells (including the vector-injected cells) were normalized to that average. For experiments on isolated neurites, 3 to 10 representative line scans through the processes were taken for each cell. The pixel density (process background) was calculated. The average of these values was used for the pixel density of that cell or that group of processes. In general, standard errors for line scans from the same group of processes did not vary by more than 25% of the mean.

RESULTS

Characterization of *Aplysia* 4EBP. A sequence for *Aplysia* 4EBP from an *Aplysia* expressed sequence tag collection was obtained courtesy of Eric Kandel (Columbia University). We designed primers to the 5' and 3' terminals of this sequence and cloned a 363-bp molecule from an *Aplysia* cDNA library by using PCR and confirmed this sequence in three independent clones. Notably, *Aplysia* 4EBP conserves a strong eIF4E binding motif and two motifs important for protein-protein interaction, the RAIP motif (59) and the TOS site (Fig. 1) (54). Of

FIG. 2. Characterization of *Aplysia* 4EBP and 4EBP antibodies. (A) Combined P2/P3 fractions (see below [panel C]) from the synaptosome preparation were loaded next to purified, bacterially expressed GST-4EBP. The anti-4EBP antibody recognized a single band at 21 kDa in P2/P3 and a band at 45 kDa in adjacent lanes in which GST-4EBP was loaded (0.5 to 5 ng). Both anti-PS62/67 and anti-PT34/43 antibodies recognized a strong band in P2/P3 which overlaid the band recognized by the anti-4EBP antibody. Neither phospho-specific antibody reacted with the nonphosphorylated GST-4EBP. (B) Immunoprecipitation (IP) of 4EBP from the ovotestis and nervous system (NS) with anti-4EBP or rabbit IgG (R-IgG). Immunoblotting (IB) was subsequently performed with anti-4EBP, anti-PT34/43, and anti-PS62/67 antibodies. The pellet contained material brought down by the IP antibody while the void was the supernatant from which the protein was immunoprecipitated. A fraction of the starting material (SM) from the ovotestis was loaded for comparison. The large arrow points to the light-chain IgG bands from the anti-4EBP and rabbit IgG pellets in order to highlight equal loading between the two conditions. Small arrows denote the two differentially migrating 4EBP species detected. A second representation of the anti-PS62/67 immunoblot (using a shorter enhanced chemiluminescence film exposure time) is shown at the bottom in order to display enrichment of the slow-migrating band in the anti-4EBP lane within the pellet. (C) Coomassie stain of GST-tagged wild-type 4EBP (WT) and 4EBP with S/T-E mutations at T34/43 (T34/43E), S62/67 (S62/67E), and T34/43 and S62/67 (4xE) run out on a 13% SDS-PAGE gel. (D) Fractions from the synaptosome preparation ($\sim 10 \mu\text{g}/\text{lane}$) are shown. P2 and P3 are the synaptosome fractions, with P3 being more enriched for synaptosomes and P2 containing more protein (17). S2 and S3 are soluble fractions, whereas P1 contains nuclei and debris. A single band of approximately 21 kDa recognized by the 4EBP antibody (anti-4EBP) is evident in all fractions. This band is also recognized by both phospho-specific antibodies. (E) Quantitation of anti-PT34/43 immunoreactivity shown in panel D. Values are means $\pm$ standard errors of the means standardized to the P1 fraction ($n = 3$). [#], $P < 0.05$; ^{*}, $P < 0.001$ (by a paired Student t test versus P1).

the seven phosphorylation sites identified in vertebrate 4EBPs, only four are conserved (Fig. 1); however, these are the four sites most linked to regulating 4EBP binding to eIF4E (25, 27, 43, 44).

To detect 4EBP in *Aplysia*, we generated an antibody directed against a peptide derived from the carboxy-terminal end of 4EBP. Anti-4EBP recognized a single band migrating at

approximately 21 kDa (Fig. 2A). A commercial antibody raised against mammalian isoforms of 4EBP phosphorylated at T37 and/or T46, T34/43 in *Aplysia*, was used to recognize phosphorylated 4EBP. The sequence adjacent to these two sites is identical, TTPGGT, and is conserved between *Aplysia* and vertebrate 4EBPs (Fig. 1). Since these two sites are identical, the antibody does not distinguish between 4EBP phosphorylated

at T34 or T43 or both residues. We also raised an antibody to a peptide containing phosphorylated serines at both positions 62 and 67. Again, we do not know whether this antibody recognizes singly phosphorylated S62 or S67 or a conjunction of both sites. Both of these antibodies recognize a band migrating at approximately 21 kDa that directly overlaid the band recognized by the anti-4EBP antibody (Fig. 2A). The anti-PT34/43 and anti-PS62/67 antibodies were phospho specific since they did not recognize nonphosphorylated GST-4EBP produced in bacteria (Fig. 2A).

To confirm that all three 4EBP antibodies recognize the same protein, we used the anti-4EBP antibody for immunoprecipitation. Immunoprecipitation using the anti-4EBP antibody, but not rabbit immunoglobulin G (IgG), greatly enriched the band at 21 kDa (Fig. 2B). The antigens recognized by both phospho-specific antibodies were removed from the void and enriched in the pellet after immunoprecipitation with the anti-4EBP antibody (Fig. 2B). Thus, the phospho-specific antibodies recognize the same antigen as does the antibody to 4EBP. The recognition of the same bands by three independent antibodies generated against the 4EBP sequence is strong confirmation that this band represents *Aplysia* 4EBP. Consistent with vertebrate 4EBPs, the slower migration of bands recognized by both phospho-4EBP antibodies could be detected when large, high-percentage SDS-PAGE gels were used (Fig. 2B). Importantly, the total 4EBP antibody recognized both fast- and slow-migrating bands (Fig. 2B). No difference in the migration of the bands recognized by the two phospho-4EBP antibodies was detected. This suggests either that phosphorylation at these sites is coupled such that most phosphorylated 4EBP is phosphorylated at all of the sites or that the shift in migration requires only some of the phosphorylation events. Consistent with a requirement for all sites to be phosphorylated, a shift in migration of bacterially expressed *Aplysia* GST-coupled 4EBPs occurred only after all four phosphorylation sites were converted to glutamic acid to mimic phosphorylation (Fig. 2C).

As we are interested in the regulation of translation at synapses, we first determined whether 4EBP is present at synapses by using a previously characterized synaptosome preparation (14, 17, 37). Using this fractionation, synaptosomes are present in P2 and P3 fractions, with P2 containing the most synaptosomes but P3 containing the highest enrichment of synaptosomes. Using the antibody to total 4EBP, we observed that *Aplysia* 4EBP is distributed in roughly equal amounts throughout the different fractions, suggesting its presence, but not enrichment, in synaptosomes (Fig. 2D). Interestingly, there was significantly less phosphorylation of 4EBP in the synaptosome-enriched fractions than in soluble fractions that are derived mainly from neuronal cell soma cytosol. This hypophosphorylation is particularly dramatic in the most enriched synaptosome fraction (Fig. 2D and E).

5HT increases the phosphorylation of both T34/43 and S62/65 in a rapamycin-sensitive manner. In order to determine whether phosphorylation of 4EBP may be an important target of TOR at the synapse in *Aplysia*, we incubated synaptosomes with 5HT and/or rapamycin and measured the level of 4EBP phosphorylation using the phosphopeptide antibodies (gel shifts were difficult to measure in these experiments due to the use of mini gels, because of the small amount of starting material). A 10-min incubation period with 5HT (20 μ M)

significantly increased both anti-PT34/43 and anti-PS62/67 immunoreactivity, and this was blocked when synaptosomes were incubated with 5HT in the presence of rapamycin (Fig. 3A and B). Rapamycin did not significantly decrease basal levels of 4EBP phosphorylation (Fig. 3A and B), suggesting that basal phosphorylation of 4EBP is TOR independent. It should be noted that the increase measured is probably an underestimate since not every synapse has receptors for 5HT and contaminants in the synaptosome preparation will dilute the changes observed.

Calyculin causes a large increase in the phosphorylation of 4EBP, and phosphatidylinositol 3-kinase inhibitor blocks the effect of 5HT. The activation of 4EBP in vertebrate and invertebrate systems has previously been shown to depend on phosphatidylinositol 3-kinase (26, 42, 63). Indeed, 5HT did not induce a significant change in 4EBP phosphorylation in the presence of LY at either set of phosphorylation sites (Fig. 3C and D). Like rapamycin, LY did not affect basal levels of 4EBP phosphorylation.

While some evidence suggests that TOR directly phosphorylates downstream effector molecules (11, 13), other evidence suggests that this step involves the repression of a calyculin-sensitive phosphatase (14, 37, 47, 66). In order to test this model, we incubated synaptosomes with calyculin (100 nM) in the presence of 5HT and/or rapamycin. Calyculin caused a large increase in the phosphorylation of 4EBP at T34/43 (Fig. 3C and D). Calyculin also increased phosphorylation of S62/67, albeit significantly less than 4EBP phosphorylation at T34/43 (Fig. 3C and D). The increase in phosphorylation by calyculin occluded the effects of 5HT, consistent with 5HT effects being mediated by phosphatase inhibition or 5HT and calyculin converging on a common target (Fig. 3C and D). However, if TOR acted through repression of a phosphatase, calyculin's action should be insensitive to rapamycin. Instead, rapamycin blocked the increase in phosphorylation induced by calyculin (Fig. 3C and D). It is possible that calyculin acts by inhibiting a 4EBP phosphatase not regulated by TOR, but this would not explain the occlusion of the effects of 5HT or the ability of rapamycin to block calyculin-induced, but not basal, 4EBP phosphorylation. The most consistent interpretation of these data is that calyculin activates TOR, presumably increasing some phosphorylation involved in the pathway towards TOR activation. The larger effect on T34/43 phosphorylation than on S62/67 (the inverse of the effect of 5HT) can be explained by the concurrent inhibition of a TOR-independent phosphatase that is more active on the T34/43 site than on the S62/67 site. It should be pointed out that these results are quite distinct from our earlier studies examining TOR-dependent phosphorylation of S6 kinase in synaptosomes (37), and this will be addressed in Discussion.

Time course of 4EBP phosphorylation in synaptosomes. Treatment of *Aplysia* ganglia with 5HT has been shown to decrease the phosphorylation of eIF4E, consistent with a decrease in cap-dependent translation (21, 50). This seems in contradiction with the ability of 5HT to increase 4EBP phosphorylation, which should increase cap-dependent translation. To examine this issue more closely, we examined the time course of 4EBP and eIF4E phosphorylation in synaptosomes during the continued presence of 5HT. The phosphorylation of 4EBP in synaptosomes was highest at both T34/43 and S62/67

FIG. 3. Phosphorylation of 4EBP and eIF4E in synaptosomes. (A) Synaptosomes were treated with 5HT (20 μ M) and/or rapamycin (rap) (20 nM) for 10 min. Samples were immediately put in Laemmli's buffer, boiled and separated on SDS-PAGE gels, transferred to nitrocellulose, and blotted with the phospho-specific antibodies (anti-PT34/43 and anti-PS62/67). Equal amounts of protein ($\sim$ 25 to 30 μ g) were loaded into each lane in all synaptosome experiments. (B) Quantitation of data shown in panel A. Values are means $\pm$ standard errors of the means (SEM) standardized to the average of the synaptosomes treated with control solution. The results of a paired Student *t* test versus the control, using nonstandardized values and a Bonferroni correction for multiple tests, are shown: 5HT, anti-PT34/43 (*n* = 27) and anti-PS62/67 (*n* = 12); rap, anti-PT34/43 (*n* = 24) and anti-PS62/67 (*n* = 7); 5HT plus rap, anti-PT34/43 (*n* = 19) and anti-PS62/67 (*n* = 7). #, *P* < 0.05. (C) Synaptosomes were incubated with calyculin (calyc) (100 nM) or LY (20 μ M) either alone for 10 min or throughout a 10-min treatment with 5HT or rapamycin. (D) Quantitation of data shown in panel C. Values are means $\pm$ SEM standardized to the average of the synaptosomes treated with control solution. The results of a paired Student *t* test using nonstandardized values (PT34/43, *n* = 12; PS62/67, *n* = 4) between either group and the control or between two groups joined by a connector, with a Bonferroni correction for multiple tests (comparisons are made between two groups stained with the same antibody, except for the calyculin group, where anti-PT34/43 and anti-PS62/67 were compared), are shown. NS, not significant; #, *P* < 0.05; *, *P* < 0.01. (E) Time course of 4EBP and eIF4E phosphorylation in synaptosomes following treatment with 5HT. (F) Quantitation of data shown in panel E. Values are means $\pm$ SEM standardized to the average of the synaptosomes treated with control solution. The results of a paired Student *t* test using nonstandardized values (anti-PT34/43, *n* = 4; anti-PS62/67, *n* = 12; anti-PS207-eIF4E, *n* = 4) between groups and the control, with a Bonferroni correction for multiple tests, are shown. #, *P* < 0.05.

at 10 min but had returned to baseline by 35 min, even in the continued presence of 5HT (Fig. 3E and F). In contrast, eIF4E phosphorylation gradually decreased but did not reach significance until the 35-min time point. This suggests that there is a biphasic regulation of translation by 5HT: initially, there is activation of cap-dependent translation through 4EBP phosphorylation, but in the continued presence of 5HT, the TOR pathway is inactivated and a separate pathway is stimulated, leading to a decrease in cap-dependent translation. The two are not dependent on one another since 5HT still decreases eIF4E phosphorylation in the presence of rapamycin (21).

5HT increases the phosphorylation of 4EBP in isolated neurites. In order to further investigate the local activation of 4EBP phosphorylation, we performed immunocytochemical experiments with sensory cell neurites, isolated by removing cell bodies with a glass electrode tip. Neurites were subsequently left to recover from incision for 2 h. This process allowed us to use the healthiest neurites. Processes badly dam-

aged by axotomy decay over time and are easily identifiable; these processes were excluded from our analysis. We found that bathing neurites in 5HT (20 μ M) for 10 min caused a significant increase in staining for anti-PT34/43 (Fig. 4A and B). This antibody was used because, in contrast to anti-PS62/67, it produced only a single band on Western blots. Consistent with our results from synaptosomal preparations, the 5HT-induced increase in phosphorylation of 4EBP was blocked by rapamycin (20 nM) (Fig. 4A and B). Again, rapamycin had no effect on basal phosphorylation at T34/43 (Fig. 4A and B).

4EBP is not phosphorylated at either T34/43 or S62/67 when bound to eIF4E. To determine whether phosphorylation regulates eIF4E binding to 4EBP in *Aplysia*, we examined the phosphorylation state of eIF4E-bound 4EBP by extracting eIF4E from nervous system extracts with 7-methyl-GTP beads in the presence of phosphatase inhibitors. A large amount of 4EBP remained associated with eIF4E after washing, but there was no detectable immunoreactivity to eIF4E-bound 4EBP

FIG. 4. 5HT increases phosphorylation of 4EBP in isolated neurites. (A) Sensory neurons from pleural ganglia were cultured in hemolymph-enriched L15 (see Materials and Methods). Cell bodies were removed or neurites were incised, and neurites were left to recover for 2 h. Neurites were treated for 10 min with 5HT (20 μ M) and/or rapamycin (rap) (20 nM) and fixed and stained with anti-PT34/43. (B) Quantitation of data shown in panel A. Values are means $\pm$ standard errors of the means standardized to the mean of the control-treated processes. The numbers of cells and dishes, respectively, for the groups are as follows: control, 48 and 5; 5HT, 92 and 7; rapamycin, 48 and 4; and rapamycin plus 5HT, 38 and 5. F was 8.2 by analysis of variance (222 cells and 3 degrees of freedom) ($P < 0.001$). *, $P < 0.05$ according to Tukey's post hoc tests between groups and the control.

with either phospho-specific antibody (Fig. 5A). This was not due to dephosphorylation of 4EBP during the extraction procedure because 4EBP that was present in the wash fractions after incubation showed considerable immunoreactivity to the

phosphopeptide antibodies (Fig. 5A). It was somewhat surprising to see that no T34/43-phosphorylated 4EBP was associated with eIF4E, as it has previously been shown in vertebrates that 4EBP is removed only after phosphorylation of the second two sites (S62/67) (25, 27, 44).

In order to more clearly identify which residues must be phosphorylated before eIF4E can be released, we performed an in vitro binding assay using bacterially expressed S/T-E mutants of GST-4EBP and a His-tagged eIF4E construct. GST-4EBP constructs were incubated for 1 h with a stoichiometric amount of His-eIF4E (~ 100 nM) and precipitated using 7-methyl-GTP beads. Serine/threonine-to-glutamic acid mutations at either T34/43 or S62/67 alone did not significantly decrease coprecipitation with eIF4E (Fig. 5B and C). Rather, S/T-E mutations were required at all four residues on 4EBP before its binding to eIF4E was significantly attenuated (Fig. 5B and C), consistent with the model in which all sites need to be phosphorylated to remove eIF4E binding (27). The lack of bound 4EBP phosphorylated at T34/43 from nervous system extracts could be due to the fact that most 4EBP phosphorylated at T34/43 is also phosphorylated at S62/67; this is also consistent with the comigration of the antigen recognized by the two phospho-specific antibodies (Fig. 2B). It is also possible that phosphorylation at these residues has a stronger effect than the S/T-E mutation or that the other eIF4E binding proteins competing with 4EBP in *Aplysia* extracts exert a more stringent test of eIF4E binding than the in vitro experiments. During these experiments, we noted that the amount of eIF4E bound to 7-methyl-GTP caps was increased by the presence of 4EBP but not the quadruple S/T-E construct (Fig. 5B and D). To examine this more carefully, we titrated the levels of 4EBP while keeping levels of eIF4E constant (~ 100 nM) (Fig. 5E). Interestingly, the increased binding of eIF4E to the 7-methyl-GTP caps was seen only at approximately equimolar amounts of 4EBP (up to ~ 100 nM) and disappeared as levels of 4EBP rose (Fig. 5E). At these concentrations of 4EBP, a significant amount of nonspecific binding of 4EBP to the beads was observed (seen as the amount bound in the absence of eIF4E) (Fig. 5E). The increase of eIF4E bound to caps in the presence of 4EBP is consistent with previous work showing that 4EBP binding stabilizes the conformation of eIF4E bound to the cap (61). The absence of this effect at higher concentrations of 4EBP may be due to aggregation of the purified 4EBP or a lower-affinity binding conformation in which 4EBP no longer stabilizes the conformation of eIF4E bound to the cap.

In order for 4EBP phosphorylation to significantly influence the level of free eIF4E and translation rates in *Aplysia* neurons, nervous system levels of total 4EBP must be comparable to those of eIF4E. The anti-4EBP antibody was indeed able to coimmunoprecipitate eIF4E effectively from the nervous system (Fig. 5F). While eIF4E was significantly depleted, only a fraction of the starting material was found in the pellet. This is likely due to release of eIF4E during washing of the pellet. The substantial depletion of eIF4E in these experiments suggests that levels of 4EBP in the nervous system are in the range where they could regulate the availability of eIF4E.

Overexpression of 4EBP decreases cap-dependent translation in the cell soma. If the level of free eIF4E is rate limiting for cap-dependent translation, then overexpressing 4EBP should decrease somatic cap-dependent translation. We in-

FIG. 5. Characterization of eIF4E-4EBP interaction. (A) Coprecipitation of 4EBP with eIF4E by using 7-methyl-GTP beads. Starting material (SM), postincubation flowthrough (FT), LCB wash fractions (LCB wash), and the 7-methyl-GTP bead pellets (7m GTP beads) were loaded for comparison. (B) Coprecipitation of purified, bacterially expressed His-eIF4E (4E) and 4EBP constructs by using 7-methyl-GTP beads. Beads were incubated with eIF4E and GST, wild-type 4EBP (WT), or 4EBP with S/T-E mutations at T34/43 (T34/43E), S62/67 (S62/67E), or T34/43 and S62/67 (4xE). Washed pellets were treated with Laemmli's buffer, boiled and separated on SDS-PAGE gels, transferred to nitrocellulose, and blotted with the 4EBP and eIF4E antibodies. An equal fraction of each 4EBP construct used in the binding assay was also loaded to display equal starting conditions (SM). (C and D) Quantitation of 4EBP and eIF4E levels, respectively, from panel B. Values are means $\pm$ standard errors of the means standardized to WT (C) or GST (D) ($n = 5$). The results of a paired Student t test using nonstandardized values between groups and WT (C) or GST (D), with a Bonferroni correction for multiple tests, are shown. *, $P < 0.05$. (E) Titration of 4EBP keeping constant levels of eIF4E. Various amounts of bacterially expressed wild-type 4EBP (4EBP) were incubated with 7-methyl-GTP beads in the presence (+4E) or absence (−4E) of eIF4E. (F) Coimmunoprecipitation of 4EBP and eIF4E from the soluble (cytosolic) fractions of the synaptosome preparation. Anti-4EBP or rabbit IgG (R-IgG) was used for immunoprecipitation (IP), and immunoblotting (IB) was subsequently performed with anti-4EBP, anti-PT34/43, and anti-eIF4E antibodies. The pellet contained material brought down by the IP antibody, while the void was the supernatant from which the protein was immunoprecipitated.

jected either a plasmid encoding 4EBP or a control plasmid together with a previously characterized bicistronic construct that measures the relative ratio of IRES-dependent to cap-dependent translation (I/C ratio) (19) into *Aplysia* sensory neu-

rons. Indeed, expression of 4EBP caused a decrease in cap-dependent translation, an increase in IRES-dependent translation, and an increase in the I/C ratio (Fig. 6A). While absolute changes in cap-dependent and IRES-dependent

FIG. 6. Overexpressing 4EBP inhibits cap-dependent translation. (A) Isolated, cultured *Aplysia* sensory neurons were injected with a fluorescent bicistronic reporter and either an empty vector (Control) or wild-type 4EBP (BP-WT). After 2 days, cap-dependent (Cap) and IRES-dependent (IRES) translation was estimated from the levels of the two fluorescent proteins encoded by the bicistronic reporter. The ratio of IRES- to cap-dependent translation was calculated. Representative cells and quantitation of all cells are shown. Values are means standardized to the control $\pm$ standard errors of the means (SEM) from 12 experiments (control, $n = 129$; BP-WT, $n = 127$). The results of a t test versus the control, with Bonferroni's correction for multiple tests, are shown. *, $P < 0.0001$. (B) Cells from experiments whose results are shown in panel A were fixed and stained with antibodies to 4EBP (BP Total), 4EBP-PT34/43 (BP-P), eIF4E (4E Total), or eIF4E-PS207 (4E-P). Representative cells and quantitation of all cells are shown. Values are means standardized to the control $\pm$ SEM. The numbers of experiments, control cells, and BP-WT cells, respectively, for the groups are as follows: BP-Total, 3, 27, and 24; BP-P, 3, 26, and 25; 4E Total, 2, 19, and 17; and 4E-P, 4, 37, and 30. The results of a t test versus the control, with Bonferroni's correction for multiple tests, are shown. #, $P < 0.05$; *, $P < 0.0001$.

translation between neurons could be affected by the amount of plasmid injected or effects on RNA stability, the I/C ratio is independent of these variables as both fluorescent proteins are derived from the same mRNA. Immunostaining of injected neurons indicated that levels of 4EBP increased approximately fourfold during these experiments compared to immunostaining of neurons injected with control plasmid (Fig. 6B). It should be noted that the change (n -fold) is likely an underestimate, since we do not know what degree of control staining could be due to background immunofluorescence and changes in immunofluorescence are not necessarily linear. We also saw an equivalent increase in 4EBP phosphorylation in these neurons, consistent with the high constitutive phosphorylation of 4EBP seen on Western blots from the soma cytoplasm (Fig. 2D). However, even if an equivalent percentage of overexpressed 4EBP is phosphorylated, this will still lead to a large increase in the total level of nonphosphorylated 4EBP and it is this level that should be critical for regulating the amount of free eIF4E.

Earlier, we had predicted that dissociation of eIF4E from eIF4G should decrease eIF4E phosphorylation (50). Indeed, cells injected with the plasmid encoding 4EBP showed signif-

icant decreases in eIF4E phosphorylation (Fig. 6B), consistent with expressed 4EBP sequestering eIF4E from eIF4G and the eIF4G-associated kinase, ApMnk. The decrease in eIF4E phosphorylation was not due to decreases in eIF4E, as there was actually a small but significant increase in levels of eIF4E in cells overexpressing 4EBP (Fig. 6B).

Another possible explanation for the decrease in cap-dependent translation observed after overexpression of 4EBP is the sequestering of TOR through the TOS site on expressed 4EBP. To determine whether this contributed to the effects of 4EBP, we examined the effect of injecting a plasmid encoding 4EBP where the TOS site at the carboxy terminus was removed (4EBP-dTOS). There was a significant decrease in the amount of phosphorylated 4EBP compared to that in cells injected with plasmid encoding wild-type 4EBP, suggesting, as expected, that removing the TOS site decreases 4EBP phosphorylation (Fig. 7A). However, there were no differences in the ability of 4EBP-dTOS to inhibit cap-dependent translation compared to that of 4EBP, suggesting that the inhibition by 4EBP expression is not due to sequestering TOR (Fig. 7A). One caveat in this experiment is that removing the TOS site also removed the antigen recognized by our anti-4EBP anti-

FIG. 7. Overexpressing eIF4E can prevent the inhibition of cap-dependent translation by a 4EBP mutant lacking the TOS site. (A) Sensory neurons were injected as described for Fig. 6A and fixed and stained for phospho-4EBP as described for Fig. 6B, except a third group received 4EBP lacking the TOS site (dTOS). Representative cells and quantitation of all cells are shown. Values are means standardized to the control $\pm$ standard errors of the means (SEM) from four experiments (control, $n = 34$; BP-WT, $n = 29$; dTOS, $n = 32$). Differences between groups by analysis of variance (ANOVA) are as follows (with numbers of cells and dishes, respectively, in parentheses): Cap (92, 2), $F = 5.97$, $P < 0.01$; IRES (92, 2), $F = 1.01$, $P > 0.5$; I/C (92, 2), $F = 12.5$, $P < 0.0001$; 4EBP (11, 2), $F = 9.9$, $P < 0.001$. The results of Tukey's post hoc test between either group and the control or between two groups joined by a connector are shown. #, $P < 0.01$; *, $P < 0.001$; NS = $P > 0.1$. (B) Sensory neurons were injected as described for Fig. 6A, except groups received the bicistronic reporter and empty vector (Con), eIF4E (4E), 4EBP-dTOS (dTOS), or eIF4E plus 4EBP-dTOS (4E + dTOS), and were stained for eIF4E (4E Total). Representative cells and quantitation of all cells are shown. Values are means standardized to the control $\pm$ SEM from two experiments (control, $n = 24$; 4E, $n = 23$; dTOS, $n = 25$; 4E + dTOS, $n = 25$). Differences between groups by ANOVA are as follows (with numbers of cells and dishes, respectively, in parentheses): Cap (93, 3), $F = 6.6$, $P < 0.001$; IRES (93, 3), $F = 0.3$, $P > 0.5$; I/C (93, 3), $F = 19.4$, $P < 0.00001$; total eIF4E (88, 3), $F = 35.6$, $P < 1 \times 10^{-13}$. #, $P < 0.05$; *, $P < 0.001$ (according to Tukey's post hoc test between either group and the control or between two groups joined by a connector).

body. Thus, we could not conclusively determine whether 4EBP-dTOS was more potent than 4EBP at inhibiting cap-dependent translation.

Our results thus show that in the cell body, the level of 4EBP is rate limiting, as increasing 4EBP expression decreases cap-dependent translation. Since 4EBP is less phosphorylated at the synapse, it is not clear whether these results would be the same for synaptic translation. However, this could not be determined since this reporter is not sensitive enough to measure translation occurring specifically at synapses.

Overexpression of eIF4E does not lead to an increase in cap-dependent translation but does rescue cap-dependent translation in 4EBP-injected cells. Whereas the preceding results suggest that decreasing the level of free eIF4E in the soma can decrease cap-dependent translation, they do not address whether the level of free eIF4E is normally rate limiting. Previously, we showed that overexpression of eIF4E did not increase cap-dependent translation (19). We repeated these experiments and observed the same results; overexpression of eIF4E alone had no effect on the I/C ratio despite a significant increase in total levels of eIF4E (Fig. 7B). However, coexpression of eIF4E with 4EBP-dTOS completely rescued the decrease in cap-dependent translation caused by increased 4EBP levels in these cells, demonstrating that the expressed eIF4E was functional and that the mechanism of 4EBP-dTOS action is through sequestration of eIF4E (Fig. 7B). Therefore, in the cell soma, the levels of eIF4E are not normally rate limiting for cap-dependent translation, and thus, increasing eIF4E levels has no effect on general cap-dependent translation. Only after overexpression of 4EBP are eIF4E levels rate limiting, so that in cells overexpressing 4EBP, concurrent overexpression of eIF4E significantly increases cap-dependent translation. Surprisingly, in cells coexpressing 4EBP-dTOS and eIF4E, levels of eIF4E were much higher than in cells expressing eIF4E alone (Fig. 7B). This suggests that there is a restriction on eIF4E levels in sensory neurons, perhaps through degradation of free eIF4E, and suggests that 4EBP can protect eIF4E from degradation by acting as an eIF4E chaperone. This is consistent with the increase in endogenous eIF4E levels seen after overexpression of 4EBP (Fig. 6), although the small effect seen in the earlier experiment suggests that there is relatively little free eIF4E to stabilize. It should also be noted that overexpression of 4EBP-dTOS did not lead to a significant increase in free eIF4E levels (Fig. 7B), as was seen for overexpression of wild-type 4EBP in Fig. 6. The reason for this discrepancy is not clear but could be due to lower levels of expression of 4EBP-dTOS.

DISCUSSION

To date, there have been few studies of 4EBPs outside mammals. In *Drosophila melanogaster*, 4EBP binds eIF4E poorly due to a nonconsensus eIF4E binding site in 4EBP and overexpression of *Drosophila* 4EBP was ineffective at reducing cell size and number (42). The removal of *Drosophila* 4EBP had some effects on immune regulation and survival during starvation, suggesting that in *Drosophila*, 4EBP may be required only in special circumstances (7, 60). In contrast, we have shown that *Aplysia* 4EBP regulates translation in a manner more similar to that of vertebrate 4EBPs. *Aplysia* 4EBP

contains a consensus eIF4E binding site, and overexpression of *Aplysia* 4EBP was sufficient to decrease cap-dependent translation in the soma. Additionally, the regulation of 4EBP through TOR was also conserved in *Aplysia*. TOR regulation of 4EBP has been observed in *Drosophila*, but only modulation of the amino-terminal sites was observed (41), whereas in *Aplysia*, both amino- and carboxy-terminal phosphorylation sites were modulated by 5HT in a rapamycin-sensitive manner. These results are consistent with findings from sea urchin, the only other invertebrate system in which 4EBP activity has been characterized. In sea urchin, 4EBP phosphorylation and dissociation from eIF4E occur in a rapamycin-sensitive manner following fertilization of sea urchin eggs (18, 52, 53), an event that triggers a large increase in cap-dependent translation (8).

Comparison with S6 kinase regulation. There is controversy over how TOR regulates the activities of 4EBP and S6 kinase. While the majority of evidence suggests that TOR directly phosphorylates both T37/46 in 4EBP and T389 in S6 kinase (11, 13, 25, 65), there are large differences between the sequences of these sites. It is also unclear whether rapamycin blocks kinase activity or other regulatory aspects of the TOR complex (10, 29, 43, 47). In synaptosomes, we found that while 5HT increased both S6 kinase (37) and 4EBP phosphorylation in a rapamycin-sensitive manner, other pharmacological agents reveal differences in their regulation. For instance, calyculin-mediated increases in S6 kinase phosphorylation were insensitive to rapamycin (37), whereas calyculin-mediated increases in 4EBP phosphorylation were blocked by rapamycin. The simplest explanation for this result is that modulation of S6 kinase by TOR is mainly through a calyculin-sensitive phosphatase, with the kinase activity being insensitive to rapamycin, whereas 4EBP is regulated mainly through a TOR-sensitive kinase that is activated by calyculin, likely TOR itself. Indeed, S6 kinase in vertebrates can also be phosphorylated by the rapamycin-insensitive rictor complex under certain conditions (1). Another difference in the regulation of 4EBP and S6 kinase is that in the presence of calyculin, 5HT caused a large decrease in S6 kinase phosphorylation (37) but had no effect on 4EBP phosphorylation (Fig. 3).

A third major difference in the regulation of 4EBP and S6 kinase in *Aplysia* is the action that LY has on their phosphorylation. LY blocked basal phosphorylation of S6 kinase, but 5HT still increased phosphorylation in the presence of LY (37). In contrast, LY did not block basal phosphorylation of 4EBP but did block 5HT-mediated increases in 4EBP phosphorylation. LY could act through inhibition of the pathway between 5HT and TOR or may directly inhibit the kinase activity of TOR (12). Finally, rapamycin by itself had little or no effect on basal 4EBP phosphorylation but strongly decreased basal S6 kinase phosphorylation. While there is no clear model to explain all of these differences, our results are consistent with previous findings that suggest the outputs from mTOR to 4EBP1 and S6 kinase are distinct (64).

Phosphorylation of 4EBP. In vertebrate 4EBP, phosphorylation of N-terminal sites, T37 and T46, appears to prime for phosphorylation of downstream sites, S65 and T70 (25, 27, 43). Although T37/T46 phosphorylation appears to be sufficient for eIF4E release in some cases, in both mammals (13, 67) and *Drosophila* (41), the bulk of evidence suggests that a hierarchical phosphorylation event terminating in S65 phosphorylation

is critical for the release of eIF4E (25, 27, 43, 44). Indeed, in *Aplysia*, T-E mutations at T34/43 alone did not abrogate eIF4E-4EBP binding. Instead, S/T-E mutations were required at all four phospho-specific residues before binding was significantly inhibited. It is therefore somewhat surprising that when the same assay was performed with endogenous protein, none of the eIF4E-bound 4EBP from nervous system extracts was phosphorylated at T34/43. One plausible explanation is that in *Aplysia*, most phosphorylated 4EBP is phosphorylated at all four sites. Consistent with this, there are only two differentially migrating 4EBP bands: one recognized by all three 4EBP antibodies (slower-migrating isoform) and the other recognized only by the total 4EBP antibody (lower band) (Fig. 2B and 5A). If any of the 4EBP here had in fact been singly phosphorylated at T34/43, then anti-PT34/43 should have detected two different bands, since T34/43 or S62/67 phosphorylation alone does not appear to slow 4EBP migration (Fig. 2C).

4EBP regulation of translation. There are a growing number of eIF4E binding partners (48). The degree of control of 4EBP over free eIF4E depends on the stoichiometry of the two partners and other eIF4E binding proteins. In the soma of *Aplysia* sensory neurons, levels of nonphosphorylated 4EBP are not saturating since increasing levels of 4EBP substantially reduced cap-dependent translation and this could be rescued by adding back eIF4E. For 4EBP regulation to be important, the increase in free eIF4E liberated by phosphorylation must be sufficient to alter the balance of translation. This is probably not true in the soma of sensory neurons since overexpression of eIF4E increased its level substantially but did not increase cap-dependent translation. It is possible that the lack of an effect of overexpressing eIF4E is due to a feedback mechanism; however, since we are measuring cumulative translation after expression of the constructs, the initial rise in cap-dependent translation that would be required to activate the feedback controls should have been detected. We also did not detect increased levels of 4EBP in eIF4E-expressing neurons (data not shown). It should be noted though that in this case, cap-dependent translation is monitored using a short nonstructured 5' untranslated region and increasing free eIF4E may be important for the translation of a subset of messages with complicated 5' untranslated regions, such as Myc in vertebrate neurons (28, 38, 48, 56). Moreover, these results may be quite different at synapses, where 4EBP is normally hypophosphorylated and the relative level of eIF4E liberated may be more substantial.

Indeed, the results presented here may partially address a discrepancy in the literature between the regulation of translation rates by 5HT in isolated neurites and pleural sensory cells and ganglia. In sensory cells and ganglia, during the first 30 min of 5HT treatment, there is a decrease in the rate of translation (4, 68). This decrease was not seen in isolated neurites (16). We have previously proposed one explanation for this discrepancy: in isolated neurites, phosphorylation of eEF2 is enhanced due to the increase in calcium that occurs during axotomy. The subsequent addition of 5HT leads to dephosphorylation of eEF2, thereby activating translation through a process not present in whole cells or ganglia (14). Additionally, if 4EBP phosphorylation plays a larger role at the synapse than in the cell body, then 5HT may enhance synaptic translation rates through TOR, whereas the activation of TOR

in the cell body may be less important than the actions of 5HT on decreasing cap-dependent translation.

It is important to note that for synaptic 4EBP regulation to be physiologically important, one might expect to see a larger change in phosphorylation than reported here. However, in *Aplysia*, the synaptosomal preparation is not pure in synaptic compartments, yielding roughly 25% purity in P3 and 7% purity in P2 (17). Therefore, a large increase in phosphorylation at the synapse could be masked by contamination from other cellular components where 5HT induces little or no change in 4EBP phosphorylation. Moreover, we do not know what percentage of synapses contain 5HT receptors that are coupled to activation of TOR.

4EBP as an eIF4E chaperone. It is quite striking that when 4EBP and eIF4E were coexpressed, levels of eIF4E were significantly higher than when eIF4E was expressed alone. This would be consistent with 4EBP acting as an eIF4E chaperone and free eIF4E having a short half-life (61, 62). Alternatively, overexpressing eIF4E may activate feedback mechanisms that downregulate eIF4E, but in the presence of 4EBP, these mechanisms would not be turned on. Finally, overexpression of 4EBP by itself led only to a small increase in endogenous eIF4E levels, suggesting that in the absence of overexpression, there is little eIF4E to stabilize and most eIF4E is probably bound to other binding proteins, such as eIF4G and eIF4E-T (23).

Summary. We have characterized 4EBP in the *Aplysia* nervous system. Phosphorylation of 4EBP by TOR is induced by the facilitating transmitter, 5HT, suggesting that regulation of translation by 4EBP may be important in different phases of memory formation. 4EBP regulation is similar to that of vertebrates. Overexpressing 4EBP was sufficient to specifically decrease cap-dependent translation. Overexpressing eIF4E was not sufficient, however, to increase cap-dependent translation in the soma, suggesting that eIF4E levels are not rate limiting for such translation under normal circumstances. These results should lead to further studies to determine the role of 4EBP phosphorylation in memory formation in *Aplysia*.

REFERENCES

1. Ali, S. M., and D. M. Sabatini. 2005. Structure of S6 kinase 1 determines whether raptor-mTOR or rictor-mTOR phosphorylates its hydrophobic motif site. *J. Biol. Chem.* **280**:19445–19448.
2. Banko, J. L., L. Hou, F. Poulin, N. Sonenberg, and E. Klann. 2006. Regulation of eukaryotic initiation factor 4E by converging signaling pathways during metabotropic glutamate receptor-dependent long-term depression. *J. Neurosci.* **26**:2167–2173.
3. Banko, J. L., F. Poulin, L. Hou, C. T. DeMaria, N. Sonenberg, and E. Klann. 2005. The translation repressor 4E-BP2 is critical for eIF4F complex formation, synaptic plasticity, and memory in the hippocampus. *J. Neurosci.* **25**:9581–9590.
4. Barzilai, A., T. E. Kennedy, J. D. Sweatt, and E. R. Kandel. 1989. 5-HT modulates protein synthesis and the expression of specific proteins during long-term facilitation in *Aplysia* sensory neurons. *Neuron* **2**:1577–1586.
5. Beaumont, V., N. Zhong, R. Fletcher, R. C. Froemke, and R. S. Zucker. 2001. Phosphorylation and local presynaptic protein synthesis in calcium- and calcineurin-dependent induction of crayfish long-term facilitation. *Neuron* **32**:489–501.
6. Beretta, L., A. C. Gingras, Y. V. Svitkin, M. N. Hall, and N. Sonenberg. 1996. Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. *EMBO J.* **15**:658–664.
7. Bernal, A., and D. A. Kimbrell. 2000. *Drosophila* Thor participates in host immune defense and connects a translational regulator with innate immunity. *Proc. Natl. Acad. Sci. USA* **97**:6019–6024.
8. Brandhorst, B. P. 1976. Two-dimensional gel patterns of protein synthesis before and after fertilization of sea urchin eggs. *Dev. Biol.* **52**:310–317.
9. Brown, E. J., M. W. Albers, T. B. Shin, K. Ichikawa, C. T. Keith, W. S. Lane, and S. L. Schreiber. 1994. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature* **369**:756–758.
10. Brunn, G. J., P. Fadden, T. A. Haystead, and J. C. Lawrence, Jr. 1997. The mammalian target of rapamycin phosphorylates sites having a (Ser/Thr)-Pro motif and is activated by antibodies to a region near its COOH terminus. *J. Biol. Chem.* **272**:32547–32550.
11. Brunn, G. J., C. C. Hudson, A. Sekulic, J. M. Williams, H. Hosoi, P. J. Houghton, J. C. Lawrence, Jr., and R. T. Abraham. 1997. Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. *Science* **277**:99–101.
12. Brunn, G. J., J. Williams, C. Sabers, G. Wiederrecht, J. C. Lawrence, Jr., and R. T. Abraham. 1996. Direct inhibition of the signaling functions of the mammalian target of rapamycin by the phosphoinositide 3-kinase inhibitors, wortmannin and LY294002. *EMBO J.* **15**:5256–5267.
13. Burnett, P. E., R. K. Barrow, N. A. Cohen, S. H. Snyder, and D. M. Sabatini. 1998. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc. Natl. Acad. Sci. USA* **95**:1432–1437.
14. Carroll, M., O. Warren, X. Fan, and W. S. Sossin. 2004. 5-HT stimulates eEF2 dephosphorylation in a rapamycin-sensitive manner in *Aplysia* neurites. *J. Neurochem.* **90**:1464–1476.
15. Carter, P. S., M. Jarquin-Pardo, and A. De Benedetti. 1999. Differential expression of Myc1 and Myc2 isoforms in cells transformed by eIF4E: evidence for internal ribosome repositioning in the human c-myc 5'UTR. *Oncogene* **18**:4326–4335.
16. Casadio, A., K. C. Martin, M. Giustetto, H. Zhu, M. Chen, D. Bartsch, C. H. Bailey, and E. R. Kandel. 1999. A transient, neuron-wide form of CREB-mediated long-term facilitation can be stabilized at specific synapses by local protein synthesis. *Cell* **99**:221–237.
17. Chin, G. J., E. Shapiro, S. S. Vogel, and J. H. Schwartz. 1989. *Aplysia* synaptosomes. I. Preparation and biochemical and morphological characterization of subcellular membrane fractions. *J. Neurosci.* **9**:38–48.
18. Cormier, P., S. Pyronnet, J. Morales, O. Mulner-Lorillon, N. Sonenberg, and R. Belle. 2001. eIF4E association with 4E-BP decreases rapidly following fertilization in sea urchin. *Dev. Biol.* **232**:275–283.
19. Dyer, J. R., S. Michel, W. Lee, V. F. Castellucci, N. L. Wayne, and W. S. Sossin. 2003. An activity-dependent switch to cap-independent translation triggered by eIF4E dephosphorylation. *Nat. Neurosci.* **6**:219–220.
20. Dyer, J. R., A. M. Pepio, S. K. Yanow, and W. S. Sossin. 1998. Phosphorylation of eIF4e at a conserved serine in *Aplysia*. *J. Biol. Chem.* **273**:29469–29474.
21. Dyer, J. R., and W. S. Sossin. 2000. Regulation of eukaryotic initiation factor 4E phosphorylated in the nervous system of *Aplysia californica*. *J. Neurochem.* **75**:872–881.
22. Dyer, J. R., W. S. Sossin, and M. Klein. 1996. Cloning and characterization of apocalcin and *Aplysia* neurocalcin, two new members of the calmodulin superfamily of small calcium-binding proteins. *J. Neurochem.* **67**:932–942.
23. Ferraiuolo, M. A., S. Basak, J. Dostie, E. L. Murray, D. R. Schoenberg, and N. Sonenberg. 2005. A role for the eIF4E-binding protein 4E-T in P-body formation and mRNA decay. *J. Cell Biol.* **170**:913–924.
24. Fingar, D. C., and J. Blenis. 2004. Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene* **23**:3151–3171.
25. Gingras, A. C., S. P. Gygi, B. Raught, R. D. Polakiewicz, R. T. Abraham, M. F. Hoekstra, R. Aebersold, and N. Sonenberg. 1999. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes Dev.* **13**:1422–1437.
26. Gingras, A. C., S. G. Kennedy, M. A. O'Leary, N. Sonenberg, and N. Hay. 1998. 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the Akt(PKB) signaling pathway. *Genes Dev.* **12**:502–513.
27. Gingras, A. C., B. Raught, S. P. Gygi, A. Niedzwiecka, M. Miron, S. K. Burley, R. D. Polakiewicz, A. Wyslouch-Cieszyńska, R. Aebersold, and N. Sonenberg. 2001. Hierarchical phosphorylation of the translation inhibitor 4E-BP1. *Genes Dev.* **15**:2852–2864.
28. Graff, J. R., and S. G. Zimmer. 2003. Translational control and metastatic progression: enhanced activity of the mRNA cap-binding protein eIF4E selectively enhances translation of metastasis-related mRNAs. *Clin. Exp. Metastasis* **20**:265–273.
29. Heesom, K. J., and R. M. Denton. 1999. Dissociation of the eukaryotic initiation factor-4E/4E-BP1 complex involves phosphorylation of 4E-BP1 by an mTOR-associated kinase. *FEBS Lett.* **457**:489–493.
30. Heitman, J., N. R. Movva, and M. N. Hall. 1991. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science* **253**:905–909.
31. Hou, L., and E. Klann. 2004. Activation of the phosphoinositide 3-kinase-Akt-mammalian target of rapamycin signaling pathway is required for metabotropic glutamate receptor-dependent long-term depression. *J. Neurosci.* **24**:6352–6361.
32. Hu, J. Y., F. Wu, and S. Schacher. 2006. Two signaling pathways regulate the expression and secretion of a neuropeptide required for long-term facilitation in *Aplysia*. *J. Neurosci.* **26**:1026–1035.
33. Huber, K. M., M. S. Kayser, and M. F. Bear. 2000. Role for rapid dendritic protein synthesis in hippocampal mGluR-dependent long-term depression. *Science* **288**:1254–1257.

34. Kandel, E. R. 2001. The molecular biology of memory storage: a dialogue between genes and synapses. *Science* **294**:1030–1038.
35. Kelleher, R. J., III, A. Govindarajan, H. Y. Jung, H. Kang, and S. Tonegawa. 2004. Translational control by MAPK signaling in long-term synaptic plasticity and memory. *Cell* **116**:467–479.
36. Kelleher, R. J., III, A. Govindarajan, and S. Tonegawa. 2004. Translational regulatory mechanisms in persistent forms of synaptic plasticity. *Neuron* **44**:59–73.
37. Khan, A., A. M. Pepio, and W. S. Sossin. 2001. Serotonin activates S6 kinase in a rapamycin-sensitive manner in Aplysia synaptosomes. *J. Neurosci.* **21**:382–391.
38. Koromilas, A. E., A. Lazaris-Karatzas, and N. Sonenberg. 1992. mRNAs containing extensive secondary structure in their 5' non-coding region translate efficiently in cells overexpressing initiation factor eIF-4E. *EMBO J.* **11**:4153–4158.
39. Manseau, F., X. Fan, T. Hueftlein, W. Sossin, and V. F. Castellucci. 2001. Ca²⁺-independent protein kinase C α 1 II mediates the serotonin-induced facilitation at depressed Aplysia sensorimotor synapses. *J. Neurosci.* **21**:1247–1256.
40. Martin, K. C., A. Casadio, H. Zhu, E. Yaping, J. C. Rose, M. Chen, C. H. Bailey, and E. R. Kandel. 1997. Synapse-specific, long-term facilitation of Aplysia sensory to motor synapses: a function for local protein synthesis in memory storage. *Cell* **91**:927–938.
41. Miron, M., P. Lasko, and N. Sonenberg. 2003. Signaling from Akt to FRAP/TOR targets both 4E-BP and S6K in *Drosophila melanogaster*. *Mol. Cell. Biol.* **23**:9117–9126.
42. Miron, M., J. Verdu, P. E. Lachance, M. J. Birnbaum, P. F. Lasko, and N. Sonenberg. 2001. The translational inhibitor 4E-BP is an effector of PI(3)K/Akt signalling and cell growth in *Drosophila*. *Nat. Cell Biol.* **3**:596–601.
43. Mothe-Satney, I., G. J. Brunn, L. P. McMahon, C. T. Capaldo, R. T. Abraham, and J. C. Lawrence, Jr. 2000. Mammalian target of rapamycin-dependent phosphorylation of PHAS-I in four (S/T)P sites detected by phospho-specific antibodies. *J. Biol. Chem.* **275**:33836–33843.
44. Mothe-Satney, I., D. Yang, P. Fadden, T. A. Haystead, and J. C. Lawrence, Jr. 2000. Multiple mechanisms control phosphorylation of PHAS-I in five (S/T)P sites that govern translational repression. *Mol. Cell. Biol.* **20**:3558–3567.
45. Nakhost, A., J. R. Dyer, A. M. Pepio, X. Fan, and W. S. Sossin. 1999. Protein kinase C phosphorylated at a conserved threonine is retained in the cytoplasm. *J. Biol. Chem.* **274**:28944–28949.
46. Pause, A., G. J. Belsham, A. C. Gingras, O. Donze, T. A. Lin, J. C. Lawrence, Jr., and N. Sonenberg. 1994. Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. *Nature* **371**:762–767.
47. Peterson, R. T., B. N. Desai, J. S. Hardwick, and S. L. Schreiber. 1999. Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycin-associated protein. *Proc. Natl. Acad. Sci. USA* **96**:4438–4442.
48. Richter, J. D., and N. Sonenberg. 2005. Regulation of cap-dependent translation by eIF4E inhibitory proteins. *Nature* **433**:477–480.
49. Rosenwald, I. B., A. Lazaris-Karatzas, N. Sonenberg, and E. V. Schmidt. 1993. Elevated levels of cyclin D1 protein in response to increased expression of eukaryotic initiation factor 4E. *Mol. Cell. Biol.* **13**:7358–7363.
50. Ross, G., J. R. Dyer, V. F. Castellucci, and W. S. Sossin. 2006. Mnk is a negative regulator of cap-dependent translation in Aplysia neurons. *J. Neurochem.* **97**:79–91.
51. Rousseau, D., R. Kaspar, I. Rosenwald, L. Gehrke, and N. Sonenberg. 1996. Translation initiation of ornithine decarboxylase and nucleocytoplasmic transport of cyclin D1 mRNA are increased in cells overexpressing eukaryotic initiation factor 4E. *Proc. Natl. Acad. Sci. USA* **93**:1065–1070.
52. Salaun, P., S. Boulben, O. Mulner-Lorillon, R. Belle, N. Sonenberg, J. Morales, and P. Cormier. 2005. Embryonic-stage-dependent changes in the level of eIF4E-binding proteins during early development of sea urchin embryos. *J. Cell Sci.* **118**:1385–1394.
53. Salaun, P., S. Pyronnet, J. Morales, O. Mulner-Lorillon, R. Belle, N. Sonenberg, and P. Cormier. 2003. eIF4E/4E-BP dissociation and 4E-BP degradation in the first mitotic division of the sea urchin embryo. *Dev. Biol.* **255**:428–439.
54. Schalm, S. S., and J. Blenis. 2002. Identification of a conserved motif required for mTOR signaling. *Curr. Biol.* **12**:632–639.
55. Shantz, L. M., and A. E. Pegg. 1994. Overproduction of ornithine decarboxylase caused by relief of translational repression is associated with neoplastic transformation. *Cancer Res.* **54**:2313–2316.
56. Sonenberg, N. 1993. Translation factors as effectors of cell growth and tumorigenesis. *Curr. Opin. Cell Biol.* **5**:955–960.
57. Takei, N., N. Inamura, M. Kawamura, H. Namba, K. Hara, K. Yonezawa, and H. Nawa. 2004. Brain-derived neurotrophic factor induces mammalian target of rapamycin-dependent local activation of translation machinery and protein synthesis in neuronal dendrites. *J. Neurosci.* **24**:9760–9769.
58. Tang, S. J., G. Reis, H. Kang, A. C. Gingras, N. Sonenberg, and E. M. Schuman. 2002. A rapamycin-sensitive signaling pathway contributes to long-term synaptic plasticity in the hippocampus. *Proc. Natl. Acad. Sci. USA* **99**:467–472.
59. Tee, A. R., and C. G. Proud. 2002. Caspase cleavage of initiation factor 4E-binding protein 1 yields a dominant inhibitor of cap-dependent translation and reveals a novel regulatory motif. *Mol. Cell. Biol.* **22**:1674–1683.
60. Teleman, A. A., Y. W. Chen, and S. M. Cohen. 2005. 4E-BP functions as a metabolic brake used under stress conditions but not during normal growth. *Genes Dev.* **19**:1844–1848.
61. Tomoo, K., Y. Matsushita, H. Fujisaki, F. Abiko, X. Shen, T. Taniguchi, H. Miyagawa, K. Kitamura, K. Miura, and T. Ishida. 2005. Structural basis for mRNA cap-binding regulation of eukaryotic initiation factor 4E by 4E-binding protein, studied by spectroscopic, X-ray crystal structural, and molecular dynamics simulation methods. *Biochim. Biophys. Acta* **1753**:191–208.
62. Tomoo, K., X. Shen, K. Okabe, Y. Nozoe, S. Fukuhara, S. Morino, M. Sasaki, T. Taniguchi, H. Miyagawa, K. Kitamura, K. Miura, and T. Ishida. 2003. Structural features of human initiation factor 4E, studied by X-ray crystal analyses and molecular dynamics simulations. *J. Mol. Biol.* **328**:365–383.
63. von Manteuffel, S. R., A. C. Gingras, X. F. Ming, N. Sonenberg, and G. Thomas. 1996. 4E-BP1 phosphorylation is mediated by the FRAP-p70s6k pathway and is independent of mitogen-activated protein kinase. *Proc. Natl. Acad. Sci. USA* **93**:4076–4080.
64. Wang, X., A. Beugnet, M. Murakami, S. Yamanaka, and C. G. Proud. 2005. Distinct signaling events downstream of mTOR cooperate to mediate the effects of amino acids and insulin on initiation factor 4E-binding proteins. *Mol. Cell. Biol.* **25**:2558–2572.
65. Weng, Q. P., M. Kozlowski, C. Belham, A. Zhang, M. J. Comb, and J. Avruch. 1998. Regulation of the p70 S6 kinase by phosphorylation in vivo. Analysis using site-specific anti-phosphopeptide antibodies. *J. Biol. Chem.* **273**:16621–16629.
66. Westphal, R. S., R. L. Coffee, Jr., A. Marotta, S. L. Pelech, and B. E. Wadzinski. 1999. Identification of kinase-phosphatase signaling modules composed of p70 S6 kinase-protein phosphatase 2A (PP2A) and p21-activated kinase-PP2A. *J. Biol. Chem.* **274**:687–692.
67. Yang, D., G. J. Brunn, and J. C. Lawrence, Jr. 1999. Mutational analysis of sites in the translational regulator, PHAS-I, that are selectively phosphorylated by mTOR. *FEBS Lett.* **453**:387–390.
68. Yanow, S. K., F. Manceau, J. Hislop, V. F. Castellucci, and W. S. Sossin. 1998. The biochemical pathways by which serotonin regulates translation in the nervous system of Aplysia. *J. Neurochem.* **70**:572–583.