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Amyloid Precursor Proteins Anchor CPEB to Membranes and Promote Polyadenylation-Induced Translation

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The cytoplasmic polyadenylation element (CPE) binding factor, CPEB, is a sequence-specific RNA binding protein that controls polyadenylation-induced translation in germ cells and at postsynaptic sites of neurons. A yeast two-hybrid screen with a mouse brain cDNA library identified the transmembrane amyloid precursor-like protein 1 (APLP1) as a CPEB-interacting factor. CPEB binds the small intracellular domain (ICD) of APLP1 and the related proteins APLP2 and APP. These proteins promote polyadenylation and translation by stimulating Aurora A catalyzed CPEB serine 174 phosphorylation. Surprisingly, CPEB, Maskin, CPSF, and several other factors involved in polyadenylation and translation and CPE-containing RNA are all detected on membranes by cell fractionation and immunoelectron microscopy. Moreover, most of the RNA that undergoes polyadenylation does so in membrane-containing fractions. These data demonstrate a link between cytoplasmic polyadenylation and membrane association and implicate APP family member proteins as anchors for localized mRNA polyadenylation and translation.

Experience-dependent biochemical and morphological changes at neuronal synapses probably underlie long-term memory storage (18, 32). Such changes may require the establishment of a tag at a synapse following stimulation; synaptic efficacy is based on the recognition of this tag (13, 27). The nature of the tag is unknown, but for at least two forms of plasticity, the long-lasting phase of long-term potentiation and long-term depression, protein synthesis in dendrites (23, 25), possibly at synapses (1, 35), is involved. Cytoplasmic polyadenylation is one mechanism that governs mRNA translation in dendrites (42). This process was previously detailed using *Xenopus* oocytes: here, nontranslating mRNAs have short poly(A) tails; in response to progesterone stimulation of meiotic maturation, the poly(A) tails are elongated and translation ensues. Two *cis*-acting 3′ untranslated region (UTR) elements control polyadenylation: the cytoplasmic polyadenylation element (CPE; UUUUAU or similar) and AAUAAA (31). Polyadenylation is initiated when the kinase Aurora A phosphorylates CPEB, the CPE-binding factor (29); this event causes an enhanced interaction between CPEB and CPSF (cleavage and polyadenylation specificity factor) (30) and between CPEB and xGLD-2, a poly(A) polymerase (6), both of which are necessary for polyadenylation. Polyadenylation, which also requires symplekin, a scaffold protein upon which the cytoplasmic polyadenylation machinery is assembled (6), stimulates translation by causing the dissociation of Maskin, a CPEB-interacting factor, and the eukaryotic translation initiation factor 4E (eIF4E) (5, 9, 39).

The synaptodendritic compartment of hippocampal and other neurons contains CPEB and several of the polyadenylation/translation factors noted above (22, 42). The cytoplasmic polyadenylation machinery is activated by *N*-methyl-D-aspartate receptors (NMDARs) and results in CPEB phosphorylation, polyadenylation, and translation of several CPE-containing mRNAs (11, 22, 41, 42). Ablation of CPEB in either knockout mice or in antisense oligonucleotide-treated *Aplysia* neurons results in defects in synaptic plasticity (2, 38).

To investigate additional CPEB activities in neurons and possibly other cells, a yeast two-hybrid screen with a mouse brain cDNA library as the prey was performed. Surprisingly, amyloid precursor-like protein 1 (APLP1) was found to be a CPEB-interacting factor. APLP1 and the related amyloid precursor protein (APP) and APLP2 are large plasma membrane-spanning proteins that have small carboxy-terminal intracellular domains (ICDs) (40). Several additional experiments including in vitro and in vivo coimmunoprecipitation, glutathione *S*-transferase (GST) pulldowns, and immunocytochemistry confirm these interactions. The ICDs, which can be cleaved from the remainder of the membrane-anchored proteins by the γ-secretase/presenilin system, are the portions that bind CPEB. The ICDs interact with a number of cytoplasmic proteins (40); in addition, they may be transported to the nucleus to activate transcription (10). In oocytes, the most efficacious system for examining CPEB activity, the APPs stimulate CPEB S174 phosphorylation, polyadenylation, and translational activation. The ICDs are necessary and sufficient for these activities.

While the ICDs are essential for the CPEB activities noted above, it is not necessary for them to be cleaved from the remainder of the protein. Indeed, sucrose gradient fractionation of oocyte extracts reveals that a substantial portion of CPEB cosediments with full-length APLP1, which also cosediments with Na/K ATPase, a marker for plasma membranes. In these fractions, not only does CPEB coimmunoprecipitate with APLP1, but immunoelectron microscopy demonstrates that both are membrane associated. Other members of the polyadenylation/translation complex (Maskin, eIF4E, Aurora A, Gld-2, and symplekin) also cofractionate with APLP1 to various extents; the proteins reside in a complex in these fractions.

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Most cytoplasmic polyadenylation occurs in the APLP1-containing fractions, where CPE-containing RNA can be found in association with membranes. Immunoelectron microscopy of sectioned oocytes shows that APLP1, CPEB, Maskin, Gld-2, and Aurora A are all associated with membranes. In cultured hippocampal neurons, CPEB and APLP1 also colocalize and transfected APLP1-ICD stimulates translation in a CPE-dependent manner. These data demonstrate that the ICDs of APP family member proteins not only anchor the cytoplasmic polyadenylation machinery to membranes but also stimulate 3′ end processing and translation. Such results have important implications for oocyte development and synaptic activity, both of which are regulated by CPEB.

MATERIALS AND METHODS

Yeast two-hybrid screen and plasmid construction. Mouse CPEB residues 1 to 290 (CPEBΔC) cloned into the NcoI-BamHI site of pGBKT7 vector (bait) and a mouse brain cDNA library cloned into the pACT2 vector (prey) were used in yeast two-hybrid assays (Clontech), which yielded several positives that encoded the carboxy-terminal region of APLP1. Full-length clones encoding APLP1, APLP2, and APP, myc-tagged at their carboxy termini, were provided by R. Homayouni (University of Tennessee Health Center, Memphis) (20). These and additional clones were used as templates for PCR (APLP amino acid residues 1 to 650, 1 to 606, 495 to 650, and 600 to 650; APLP2 residues 1 to 763; and APP residues 1 to 695, 1 to 638, and 595 to 695) for cloning into the ClaI-XhoI or ClaI-BamHI sites of pGADT7. Mouse CPEBΔC was cloned into pGEX-KG; green fluorescent protein (GFP)-CPEB has been described previously (21).

Protein-protein interaction assays. In addition to yeast two-hybrid assays, additional protein-protein interaction experiments were conducted. Cos-7 cells were transfected with DNAs encoding APLP1-myc and GFP-CPEB, using Effectene (QIAGEN). After 40 h of expression, the cells were washed three times with phosphate-buffered saline (PBS) and lysed in TX-LB (150 mM NaCl; 30 mM HEPES, pH 7.5; 5 mM $MgCl_2$; 1 mM EGTA; 1% Triton X-100; 10% glycerol; 1 mM sodium vanadate; 25 mM sodium fluoride; 2 mM phenylmethylsulfonyl fluoride; 10 μg/ml each of aprotinin, leupeptin, pepstatin, and trypsin inhibitor; and 0.1% 2-mercaptoethanol) (20). Protein A-Sepharose beads conjugated with either myc antibody or nonspecific immunoglobulin G (IgG) were then added to the lysate, which was made up to a radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mM Tris, pH 8). After 1 h of mixing at 4°C, the beads were washed extensively with 1× RIPA and the beads were then boiled in SDS sample buffer.

Plasmids encoding CPEB, CPEBΔC, APLP1, APLP-ICD, APLP2, and APP were transcribed and translated in vitro with a TNT T3/T7 coupled reticulocyte lysate system (Promega) in the presence of [^{35}S]methionine and [^{35}S]cysteine. Lysates containing various combinations of the synthesized proteins were mixed and subjected to immunoprecipitation with CPEB antibody or nonspecific IgG coupled to protein A-Sepharose. APLP1-myc mRNA was injected into *Xenopus* ooyctes (~1 ng/nl, 30 nl/oocyte), which were incubated for 12 h before lysis and immunoprecipitation with myc antibody (39). A Western blot of the immunoprecipitate was probed for CPEB.

A mouse brain extract was centrifuged to remove insoluble material and applied to the CPEBΔC-GST column or a column containing GST only. The beads were washed in RIPA buffer and eluted with SDS buffer; a Western blot was probed for APP and APLP1.

Polyadenylation and CPEB phosphorylation assays. *Xenopus* oocytes were injected with mRNAs encoding APLP1, APLP1-ICD, APLP1-ΔICD, APP, or APP-ICD (~1 ng/nl, 30 nl/oocyte) and incubated overnight. The oocytes were then injected with ^{32}P-labeled CPE-containing 3′ UTR of cyclin B1 RNA (9) and incubated for a further 0 to 6 h in the presence of progesterone (10 ng/ml or 100 ng/ml). The RNA was extracted at various times and analyzed by polyacrylamide gel electrophoresis (PAGE). Extracts from mRNA-injected oocytes were also probed on Western blots with myc antibody.

For the phosphorylation assays, oocytes were injected with mRNA encoding APLP1 and then incubated for 8 h. The oocytes were then incubated with 10 ng/ml progesterone and cultured for a further 4 or 6 h. Extracts were then prepared by homogenizing 5 oocytes each in H1 (cdk1) kinase buffer (80 mM Na β-glycerophosphate, 20 mM EGTA, 15 mM $MgCl_2$, 50 mM $NaVaO_4$) plus protease inhibitors (10 μg/ml each of pepstatin, leupeptin, and aprotinin) and then pelleting insoluble material by centrifugation for 5 min at 15,000 × *g* at 4°C. The phosphorylation reaction mixture also contained 20 μl of oocyte extract, 30 μM [γ-^{32}P]ATP (0.16 mCi/ml), and 0.5 μg of *Escherichia coli*-expressed CPEBΔC or CPEBΔC with an S174A mutation. The assays were performed in a 40-μl volume in reaction buffer containing a final concentration of 20 mM Tris, pH 7.7, 10 mM $MgCl_2$, 50 mM KCl, and 1 mM dithiothreitol. The proteins were then analyzed by SDS-PAGE and processed for two-dimensional phosphopeptide mapping following tosylphenylalanyl chloromethyl ketone (TPCK)-treated trypsin digestion (29).

CAT assays. Oocytes were injected with mRNAs encoding myc-tagged APLP1, APLP1-ICD, APLP1ΔICD, or APP (equal molar), incubated overnight, and injected with mRNAs encoding chloramphenicol acetyltransferase (CAT) with 3′ UTRs containing or lacking the CPE and AAUAAA. The oocytes were treated with 10 ng/ml progesterone and collected at various times thereafter. CAT assays were performed as described previously (9).

Sucrose gradient fractionation, coimmunoprecipitation, and immunoelectron microscopy. Fifty to 100 oocytes were homogenized in 0.5 ml of HB buffer (10% sucrose, 20 mM Tris, pH 7.6, 50 mM KCl, 10 mM $MgCl_2$, 0.3 M NaCl, 2 mM EDTA, protease inhibitor cocktail from Roche), layered onto 10 ml of 20 to 60% linear sucrose gradients (24), centrifuged in a Beckman SW41 Ti rotor at 36,000 rpm for 15 h at 4C, and collected in 0.5-ml fractions. The protein was precipitated by 7% trichloroacetic acid, the pellets were washed in cold acetone and dissolved in 8 M urea, and then one-third of the sample was analyzed by SDS-PAGE and Western blotting. For some experiments, specific fractions were combined, dialyzed against HB for 1 h at 4°C, and subjected to immunoprecipitation in the presence of 0.5% NP-40 with CPEB antibody, APLP1 antibody, or rabbit IgG linked to Dynabead M-280 sheep anti-rabbit IgG (Dynal). The beads were washed in 50 mM Tris, pH 7.5, 1 mM $MgCl_2$, 0.1 mM KCl, 2 mM EDTA, and 0.5% NP-40 and boiled in SDS sample solution.

For some experiments, the APLP1-containing fractions were pooled, diluted fivefold with HB buffer, and centrifuged in Beckman SW41 Ti rotor at 36,000 rpm for 1 h. The pellets were transferred to microcentrifuge tubes and rinsed once with PBS and pelleted at 14,000 rpm for 10 min. The pellets were incubated with CPEB or APLP1 antibody (or IgG as a control) at room temperature for 30 min and then washed with PBS. The pellets were further incubated with 15-nm-diameter gold particle-labeled goat anti-rabbit IgG at room temperature for 30 min, washed with PBS, and then fixed with 2.5% glutaraldehyde.

For other experiments, 50 oocytes were injected with ^{32}P-labeled cyclin B1 3′ UTR containing or lacking a CPE. Some oocytes were incubated with progesterone and after germinal vesicle breakdown, they were homogenized and resolved by sucrose gradient centrifugation as above. The RNA from each fraction was then extracted and analyzed by denaturing polyacrylamide gel electrophoresis.

Some oocytes were injected with 15 ng of the RNAs noted above that were labeled with digoxigenin-UTP (dig-11-UTP/UTP ratio = 1:4; Roche). Two hours after injection, the oocytes were collected and subjected to a linear sucrose gradient as above. The membrane-containing fractions, prepared as noted above, were immunostained with 25-nm-diameter gold-labeled sheep anti-digoxigenin IgG. After extensive washing, the membrane pellet was fixed by 2.5% glutaraldehyde.

Transfections. The RNAs were synthesized with mMessage mMachine and T7 Ultra kits (Ambion) and transfected into 9- to 10-day-old hippocampal neurons. Approximately 17 pmol of myc-CPEB RNA, 6.5 pmol of APLP1, APLP1-ICD, or APLP1-ICD RNA, 1.7 pmol of firefly luciferase RNA appended with a partial α-CaMKII 3′ UTR sequence containing either wild-type or mutated CPEs (42), and 1 pmol of *Renilla* luciferase RNA were cotransfected via TransMessenger transfection reagent (QIAGEN) for 3 h. Some neurons were then stimulated with 50 μM NMDA for an additional 3 h before lysis in 100 μl of buffer and analysis for dual-luciferase activity (Promega). Luciferase RNAs were quantified by real-time PCR.

RESULTS

CPEB interacts with APP family member proteins. A yeast two-hybrid screen using a mouse brain cDNA library identified APLP1 as a CPEB-interacting factor. When expressed as β-galactosidase activity relative to that observed with a p53-SV40 T antigen interaction (arbitrarily set at 100, Fig. 1B, bar 1), the amino-terminal region of CPEB strongly interacted with full-length APLP1 (bar 3), APLP2 (bar 11), and APP (bar 13).

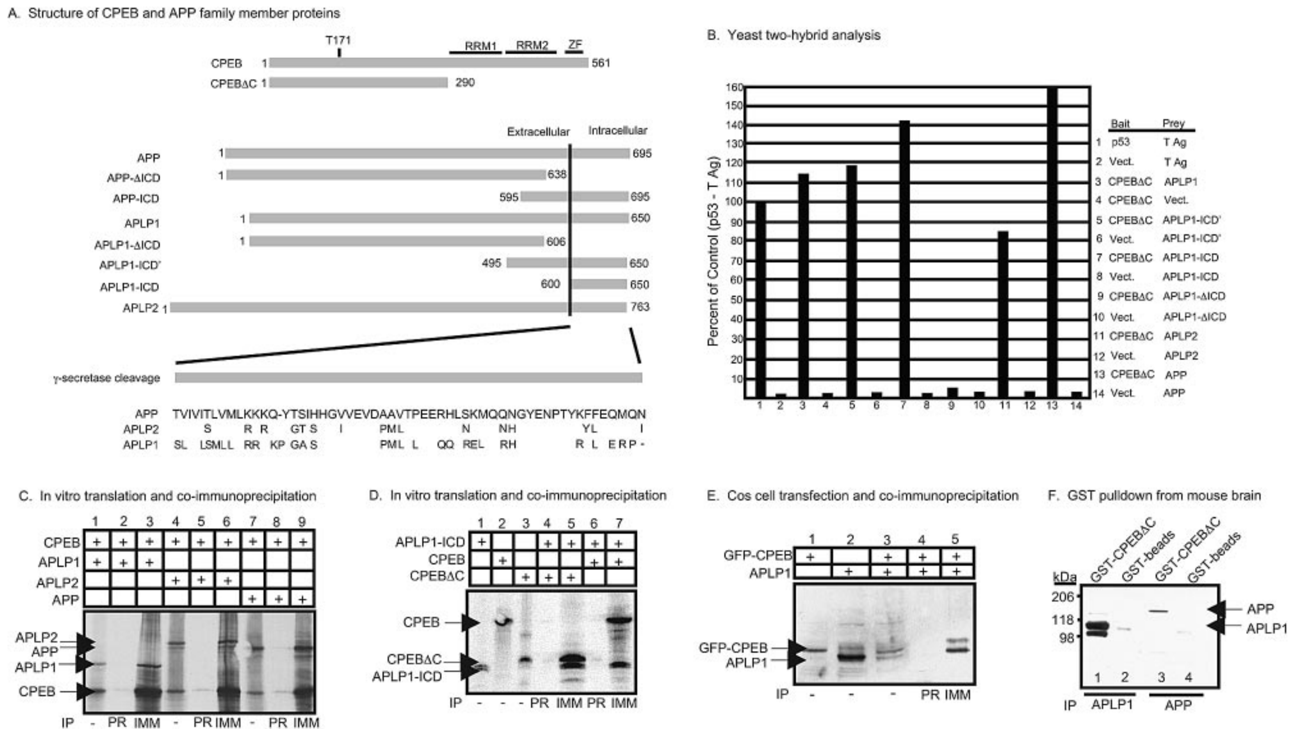

FIG. 1. CPEB interacts with APP family members. (A) Mouse CPEB contains two RNA recognition motifs (RRMs) and two zinc fingers (ZF); phosphothreonine 171 is required for polyadenylation. CPEB lacking 271 carboxy-terminal residues was used for binding assays. The transmembrane APP, APLP1, and APLP2 have small ICDs that are cleaved from the extracellular portions by γ-secretase. Wild-type and APLP1, APLP2, and APP deletion mutant proteins are depicted; the ICDs of these proteins are conserved. (B) Yeast two-hybrid interactions between CPEB and APLP1, APLP2, and APP. β-Galactosidase activity relative to that measured with p53 and SV40 T antigen (p53-T Ag) reflected the interactions between the denoted proteins. (C) Plasmids encoding APLP1, APLP2, and APP with myc epitopes were transcribed/translated in [^{35}S]methionine-primed reticulocyte lysates together with plasmid encoding CPEB. CPEB antibody (IMM) or preimmune serum (PR) was used to precipitate the proteins, which were resolved by SDS-PAGE. IP, immunoprecipitation. (D) Plasmids encoding APLP1-ICD, CPEB, and APLPL1-ΔICD were transcribed/translated in reticulocyte lysates, and the proteins were immunoprecipitated with myc antibody or preimmune serum. (E) Cos cells were transfected with GFP-CPEB and APLP1 followed by precipitation with CPEB antibody or preimmune serum. (F) Proteins from a mouse brain extract that were retained on CPEBΔC-GST or GST columns were probed for APLP1 and APP.

Moreover, CPEB interacted with just the carboxy ICD of APLP1 (compare bars 7 and 9) (Fig. 1A and B).

To confirm these observations, mRNAs encoding CPEB as well as myc-tagged APLP1, APLP2, and APP were translated in reticulocyte lysates and subjected to CPEB antibody coimmunoprecipitation. This antibody coimmunoprecipitated all APP-related proteins (Fig. 1C). Moreover, for APLP1, the ICD was necessary for the interaction with CPEB (Fig. 1D). APLP1 and CPEB were also coimmunoprecipitated from Cos cells transfected with APLP1-myc and GFP-CPEB (Fig. 1E). Finally, GST-CPEB bound both APLP1 and APP from a detergent-treated mouse brain extract (Fig. 1F). Thus, four assays demonstrate an interaction between CPEB and APP-related proteins.

Xenopus oocytes, the most efficacious system for examining the biochemistry of polyadenylation (31), were used initially to determine the physiological relevance of the APLP1-CPEB interaction. Oocytes were injected with mRNA encoding full-length APLP1-myc, or APLP1-myc with amino-terminal and carboxy-terminal truncations (cf. Fig. 1A), followed by a second injection of radiolabeled CPE-containing RNA. The oocytes were subsequently treated with a small amount of progesterone (10 ng/ml) that primes the oocytes without stimulating meiotic maturation. While progesterone alone had little effect, APLP1 and APLP1-ICD, but not APLP1-ΔICD, greatly enhanced polyadenylation (Fig. 2A). APP and APP-ICD, but not APP-ΔICD, also promoted polyadenylation irrespective of whether the oocytes were incubated with progesterone (Fig. 2B, lanes 3 to 6), although this effect was enhanced by the hormone (lanes 4 and 6). A Western blot indicates that the injected mRNAs were translated to about the same extent (Fig. 2C); the ICDs were too small to detect by this assay.

APLP1 and the APLP1-ICD, but not APLP1-ΔICD, also stimulated maturation when the oocytes were incubated with 10 ng/ml progesterone (Fig. 2D, left). However, when the oocytes were incubated with 100 ng/ml hormone, there was little difference in the rates of maturation irrespective of the agent injected, indicating that the higher steroid dose overrode the APLP1/APP effect (Fig. 2D, right). Finally, mRNAs encoding APLP1, the APLP1-ICD, and APP, but not APLP1-ΔICD, stimulated translation of a CAT reporter RNA, but only when it contained a CPE (Fig. 2E). These data show that the ICDs of APLP1 and APP stimulate cytoplasmic polyadenylation, CPE-dependent translation, and oocyte maturation.

APLP1 stimulates CPEB S174 phosphorylation. Cytoplasmic polyadenylation requires Aurora A-catalyzed CPEB serine

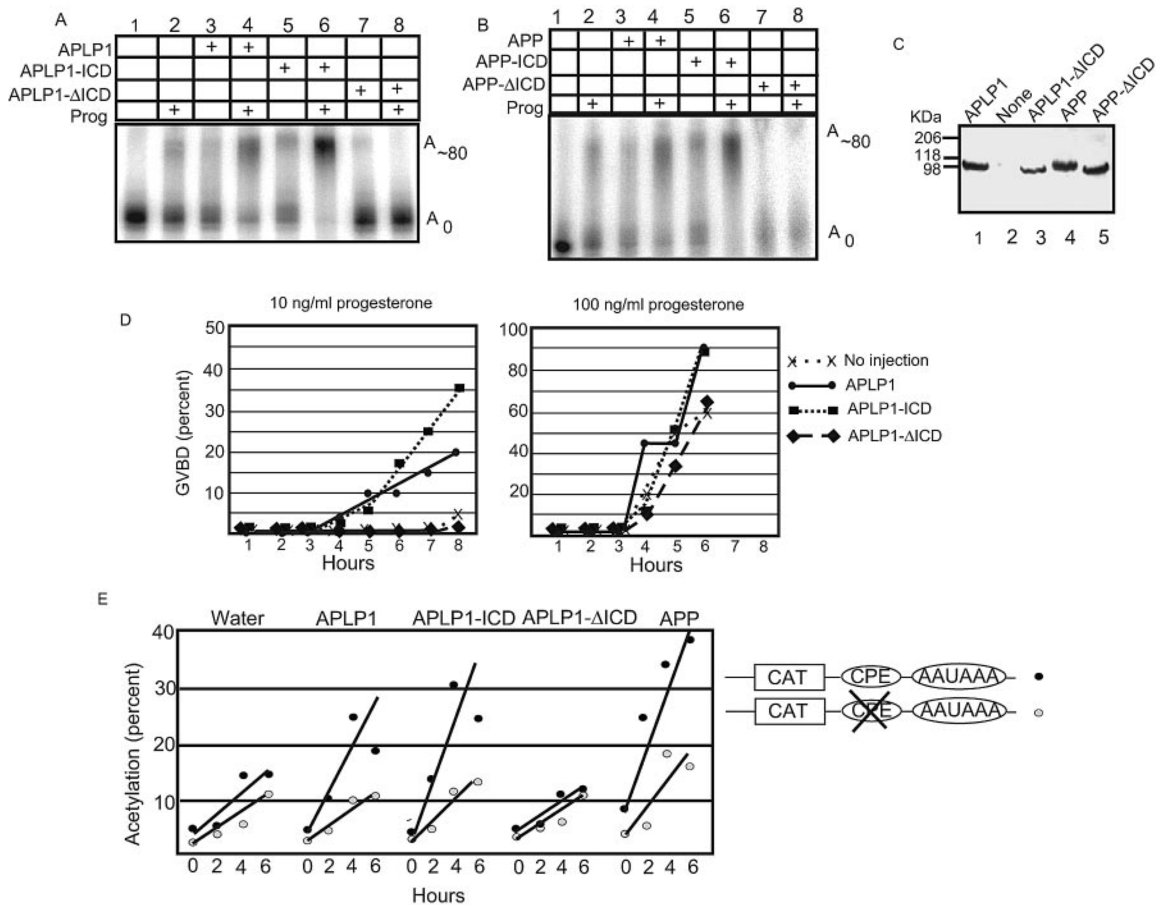

FIG. 2. APLP1 and APP stimulate polyadenylation and translation. (A) Oocytes injected with water or mRNA encoding APLP1, APLP1-ICD, or APLP1-ΔICD were cultured overnight and injected with a radioactive CPE-containing RNA; some oocytes were then incubated in buffer containing 10 ng/ml progesterone (Prog). (B) Oocytes were injected with mRNA encoding APP, APP-ICD, or APP-ΔICD; incubated overnight; and injected with a radiolabeled CPE-containing RNA. Some oocytes were further incubated with 10 ng/ml progesterone. (C) Oocytes injected with mRNA encoding fusions between the myc epitope and APLP1, APLP1-ΔICD APP, or APP-ΔICD and probed for the synthesis of these proteins with myc antibody. (D) Oocytes injected with mRNA encoding APLP1, APLP1-ICD, or APLP1-ΔICD were incubated with 10 ng/ml or 100 ng/ml progesterone. Oocyte maturation was scored by the presence of a white spot at the animal pole. (E) Oocytes injected with water or mRNA encoding APLP1, APLP1-ICD, APLP1-ΔICD, or APP were cultured overnight and then injected with CAT reporter RNAs that lacked or contained CPEs in the 3′ UTR. The oocytes were incubated with 10 ng/ml progesterone and collected every 2 h to assay for CAT activity.

174 phosphorylation (threonine 171 in the mouse) (19, 29). To assess whether APLP1 and/or APP might stimulate CPEB phosphorylation, mRNAs encoding these proteins as well as APP-ΔICD were injected into oocytes, some of which were also incubated with 10 ng/ml progesterone. A Western blot of protein from these oocytes shows that APLP1 induced an electrophoretic mobility shift of CPEB (Fig. 3A, lane 3), which indicates phosphorylation by cdk1, and presumably a preceding Aurora A phosphorylation (29; see below). Soon after this mobility shift, 70 to 80% of the CPEB was destroyed, as noted previously (28). In oocytes from a second frog, both APLP1 and APP, but not these proteins lacking their ICDs, induced a CPEB mobility shift. APLP1-myc interacted with both phosphorylated and nonphosphorylated forms of CPEB since both the fast- and slow-migrating species were coimmunoprecipitated with myc antibody (Fig. 3B, lane 2).

CPEB undergoes two rounds of phosphorylation: the first is essential for polyadenylation and is catalyzed by Aurora A on S174, but does not lead to a mobility shift (29). To determine whether APLP1 stimulates S174 phosphorylation, in vitro kinase assays and two-dimensional phosphopeptide mapping were performed. Extracts from oocytes injected with APLP1 mRNA were supplemented with [γ-^{32}P]ATP and *E. coli*-expressed wild-type or mutant CPEB containing an S174A substitution followed by SDS-PAGE (Fig. 3C, left). Compared to noninjected controls, CPEB was phosphorylated in extracts only from APLP1-injected oocytes incubated for 4 or 6 h (lanes 1 and 3). The CPEB bands from lanes 1, 2, 5, and 6 were excised, digested with trypsin, and subjected to two-dimensional phosphopeptide mapping. Figure 3C (right) shows that APLP1 stimulated the phosphorylation of a single peptide of CPEB (arrow), which was absent when the S174A mutant CPEB was used as the substrate (panels 1 and 2). Thus, the ICDs of APLP1 and APP bind CPEB and promote Aurora A-catalyzed serine 174 phosphorylation and polyadenylation-induced translation.

The cytoplasmic polyadenylation machinery is associated with membranes. Because APP family members span the

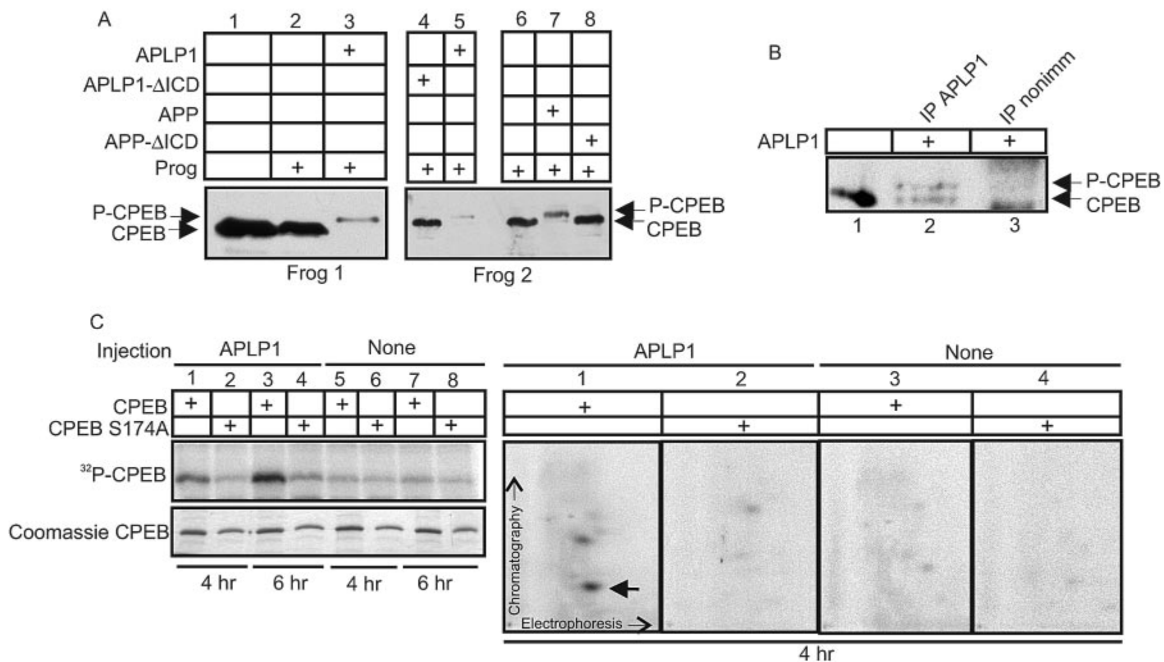

FIG. 3. APLP1 stimulates CPEB phosphorylation. (A) Oocytes from two frogs were injected with mRNA encoding APLP1, APLP1-ICD, APP, or APP-ΔICD and were cultured overnight before being treated with 10 ng/ml progesterone (Prog). Protein extracts were then prepared and probed on Western blots for CPEB. Note that the CPEB from oocytes of frog 1 underwent a mobility shift when injected with APLP1 (lane 3); this shift indicates cdk1-catalyzed phosphorylation, which takes place after the Aurora A-catalyzed phosphorylation that does not induce a mobility shift. In oocytes from frog 2, APLP1 and APP both stimulated the cdk1-catalyzed phosphorylation, and presumably the preceding Aurora A-catalyzed phosphorylation as well. Neither APLP1-ΔICD nor APP-ΔICD had any effect on CPEB phosphorylation (lanes 4 and 8). (B) Oocytes injected with mRNA encoding APLP1-myc were incubated (no progesterone) and then subjected to an immunoprecipitation with myc (IP APLP1) or control (nonimm) antibodies that were used on a Western blot probed for CPEB. Note that both the phosphorylated and nonsphosphorylated forms of CPEB coimmunoprecipitated with myc-APLP1. (C) Oocytes, some of which were injected with mRNA encoding APLP1, were incubated for 4 or 6 h (no progesterone) and then used to prepare extracts that were supplemented with [γ-^{32}P]ATP and either recombinant wild-type CPEB or recombinant S174A CPEB, which destroys the Aurora A phosphorylation site. The ^{32}P-phosphorylated recombinant CPEB was then analyzed by SDS-PAGE (left) and two-dimensional phosphopeptide mapping (right). Note that APLP1 stimulated CPEB S174 phosphorylation (arrow).

plasma membrane, we determined whether CPEB and other components of the polyadenylation/translation machinery are also associated with membranes. Immature and progesterone-stimulated oocytes were fractionated on a sucrose gradient to resolve membrane fractions (24), which were then analyzed by Western blots. During maturation, CPEB undergoes two rounds of phosphorylation: the first is Aurora A catalyzed and the second is cdc2 catalyzed. The cdc2 phosphorylations induced a gel mobility shift (29) (Fig. 4A). While CPEB sedimented throughout the gradient, APLP1 was detected only in specific fractions (6–9), which also contained Na/K ATPase, a plasma membrane marker. From these pooled fractions, CPEB and APLP1 were coimmunoprecipitated. To determine whether APLP1 and CPEB were associated with membranes, the fractions were subjected to immunocytochemistry with specific antibodies followed by gold-labeled secondary antibodies and examined by electron microscopy. Both APLP1 and CPEB were found in close proximity with membranes (Fig. 4B). (Because both antibodies were generated in rabbits, double immunogold labeling was not possible.)

In additional gradients, we probed for other components of the polyadenylation/translation machinery (Fig. 4C). While CPEB, Maskin, eIF4E, symplekin, Aurora A, and CPSF were detected in fractions containing APLP1, there was a wide variation in their prevalence; e.g., CPSF100 was strongly represented in these fractions while eIF4E was not. Nonetheless, when the APLP1-containing fractions were pooled, CPEB antibody could coimmunoprecipitate APLP1, Maskin, and CPSF (the only ones tested) (Fig. 4C).

To investigate whether RNA can also be detected in the APLP1-containing fractions, oocytes were injected with CPE-containing and CPE-lacking RNA; some of the oocytes were then treated with progesterone and were fractionated on the sucrose gradients as before. A portion of each fraction was then probed for CPEB and APLP1; RNA was extracted from the remaining portion and was analyzed by denaturing gel electrophoresis. As before, a portion of CPEB cosedimented with APLP1 (Fig. 4D). The RNA, irrespective of whether it contained a CPE, sedimented in a disperse manner throughout the gradient. However, in progesterone-stimulated oocytes, most of the CPE-containing RNA shifted to a denser part of the gradient that contained APLP1; in these fractions, it underwent polyadenylation.

Finally, digoxigenin-labeled CPE-containing and CPE-lacking RNA was injected into oocytes, which were subsequently homogenized and sedimented through sucrose gradients as before, the APLP1-containing fractions were pooled, and the injected RNA was detected by antibody against the digoxigenin moiety and immunogold labeling. While the CPE-containing RNA was detected on membranes, the CPE-lacking RNA was

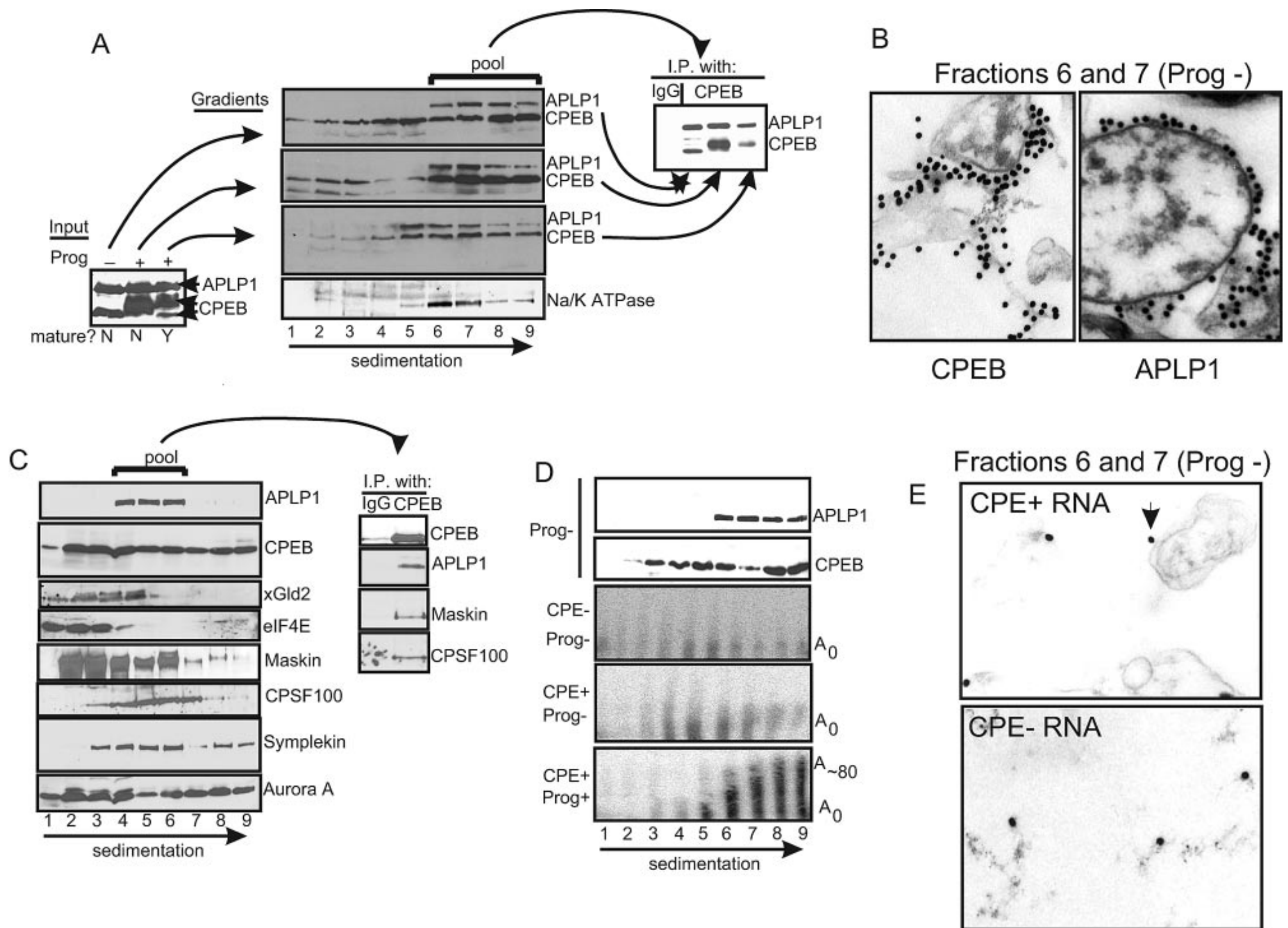

FIG. 4. The polyadenylation machinery is associated with membranes. (A) Oocytes, some of which were exposed to progesterone (Prog), were homogenized and resolved on a sucrose gradient; after fractionation, Western blots were probed for APLP1, CPEB, and Na/K ATPase. Fractions 6 to 9 were also pooled and subjected to a CPEB antibody or IgG immunoprecipitation, which was followed by Western blotting for APLP1 and CPEB. The left part of the panel shows that CPEB underwent a mobility shift as demonstrated by Mendez et al. (29, 30). The middle lane (and panel) analyzed progesterone-treated oocytes that had no obvious white spot at the animal pole, indicative of GVBD. (B) Fractions 6 and 7 from parallel gradients were pooled and analyzed by immunoelectron microscopy for APLP1 and CPEB. These oocytes were not treated with progesterone. (C) Gradients similar to those in part A (no progesterone) were probed for other components of the polyadenylation/translation machinery. The APLP1-containing fractions were pooled; subjected to CPEB antibody or IgG immunoprecipitation; and probed for CPEB, APLP1, Maskin, and CPSF100. (D) CPE-containing or CPE-lacking RNA was injected into oocytes, some of which were treated with progesterone. The oocyte homogenate was sedimented through sucrose, and a portion of these fractions was probed for APLP1 and CPEB on Western blots. RNA was extracted from the remaining portions and analyzed for polyadenylation on denatured polyacrylamide gels. (E) Oocytes injected with digoxigenin-labeled CPE-containing and CPE-lacking RNA were homogenized and sedimented through sucrose. Fractions 6 and 7, containing CPEB and APLP1, were pooled and analyzed by immunoelectron microscopy for the labeled RNA.

not; it was occasionally associated with an amorphous material (Fig. 4E). The data in Fig. 4 indicate that the polyadenylation machinery, including RNA, is associated with membranes.

Because homogenization and fractionation of cells could induce associations that do not occur in vivo, whole oocytes were fixed and embedded for immunogold detection of proteins. APLP1, Maskin, CPSF100, and Aurora A (the only ones examined) were all found in association with membranes; because the regions shown are directly beneath the plasma membrane, they could represent microvili or recycling endomembranes. (Fig. 5). Thus, at least a portion of these proteins is associated with membranes in vivo.

APLP1 and CPEB in neurons. Hippocampal neurons were transfected with DNA encoding CPEB-GFP to determine whether this protein colocalizes with APLP1. Figure 6A shows that this indeed was the case. To test the influence of APLP1 on CPE-dependent translation, neurons were transfected with mRNAs encoding *Renilla* luciferase (an internal standard) and myc-tagged CPEB (to bolster the levels of this protein), APLP1, APLP1-ΔICD, APLP1-ICD, or β-galactosidase (to equalize the amount of RNA used in each transfection) and firefly luciferase. The last mRNA was appended with a 3′ UTR-derived α-CaMKII mRNA that contained two CPEs or one in which these CPEs were mutated. Following transfection, one-half of each batch of transfected neurons was treated with NMDA. When expressed as a percentage of change in firefly luciferase activity in neurons transfected with β-galactosidase, APLP1-ΔICD had no effect irrespective of whether the firefly luciferase mRNA contained or lacked a CPE (Fig. 6B). However, APLP1 and APLP1-ICD stimulated firefly lu-

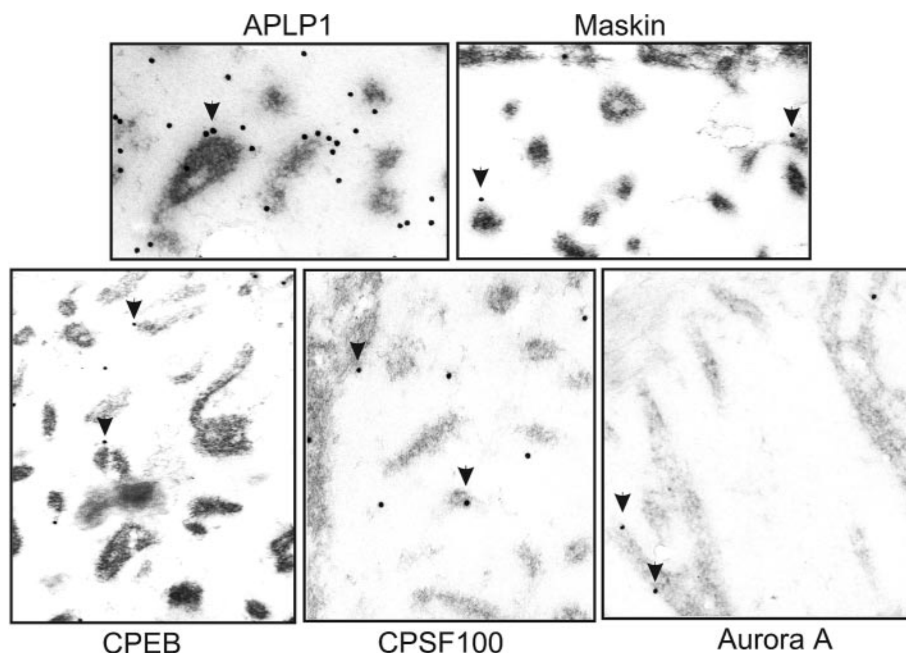

FIG. 5. Immunoelectron microscopy of oocytes. Oocytes were fixed, embedded, sectioned, and analyzed by immunoelectron microscopy for CPEB, APLP1, Maskin, Aurora A, and CPSF100. The regions shown are directly beneath the plasma membrane and may represent microvilli or recycling endomembranes.

ciferase activity in a CPE-dependent manner in response to NMDA treatment of neurons (17% for APLP1, $P = 0.002$, and 15% for APLP1-ICD, $P = 0.03$; Student's *t* test). A further quantitative reverse transcription-PCR analysis of the firefly and *Renilla* luciferase mRNAs showed that they were equally stable irrespective of whether β-galactosidase, APLP1, APLP1-ICD, or APLP1-ΔICD was cotransfected into untreated or NMDA-treated neurons (Fig. 6B). These data demonstrate that the ICD of APLP1 enhances CPE-dependent mRNA translation in neurons.

DISCUSSION

Following their cleavage from the extracellular regions of APP and APLP1, the ICDs of these proteins are both nuclear and cytoplasmic and interact with several cellular proteins to, among other functions, regulate transcription (3, 7, 10, 12). The results presented here show the ICDs bind CPEB and promote its phosphorylation on S174, an event that stimulates polyadenylation-induced translation. While the free (cleaved) ICDs can promote CPEB activation, several observations suggest that it is the membrane-associated (uncleaved) form that regulates CPEB: (i) APLP1 and other members of the polyadenylation/translation complex (CPSF, Maskin, Aurora A, etc.) are associated with membranes; (ii) APLP1, CPEB, CPSF, etc., interact in membrane-containing fractions; (iii) CPE-containing RNA is associated with membranes; and (iv) CPE-containing RNA polyadenylation occurs most strongly in fractions containing membranes and APLP1 and CPEB.

APP and APLP are found not only on the plasma membrane, but also on the endoplasmic reticulum, Golgi apparatus, and endomembranes as they cycle to lysosomes (40). Although CPEB, CPSF, etc., cosediment with Na/K ATPase, a plasma membrane marker, and appear to be associated with plasma membranes as assessed by immunoelectron microscopy, they may be associated with these other membranes as well. The localization of CPE-containing RNA on the membranes is almost certainly due to the CPEB-APLP1 complex and not the RNA per se. That is, mRNAs encoding secreted proteins associate with the endoplasmic reticulum through interactions between the signal peptide and the signal recognition particle (16). The digoxigenin-labeled RNA we injected contained no open reading frame; thus, no nascent peptide could be involved in localizing the RNA.

Why is the polyadenylation/translation machinery localized to membranes? In oocytes, progesterone stimulates M-phase progression not by activating transcription but by stimulating a membrane-associated receptor, possibly a G-protein-coupled receptor (44; but see also reference 26). If APLP1 helps concentrate CPEB and the other factors near the progesterone receptor, a wave of polyadenylation/translation could begin locally and then subsequently spread throughout the oocyte. This possibility seems attractive given the huge volume of these cells (~1 μl); an immediate and local response to progesterone stimulation could facilitate meiotic progression.

Groisman et al. (15) immunostained oocytes for CPEB and Maskin and found that although these two factors were present throughout the cytoplasm, they were somewhat concentrated in the cortex of the animal pole. At the electron microscope level, the present study also found these factors to be present throughout the oocyte, but also in cortical regions that contain membranes. After fertilization, much of the CPEB that is present in the vegetal region is destroyed and at least some of that which remains stable becomes associated with spindles and centrosomes of the early embryo that are formed in the

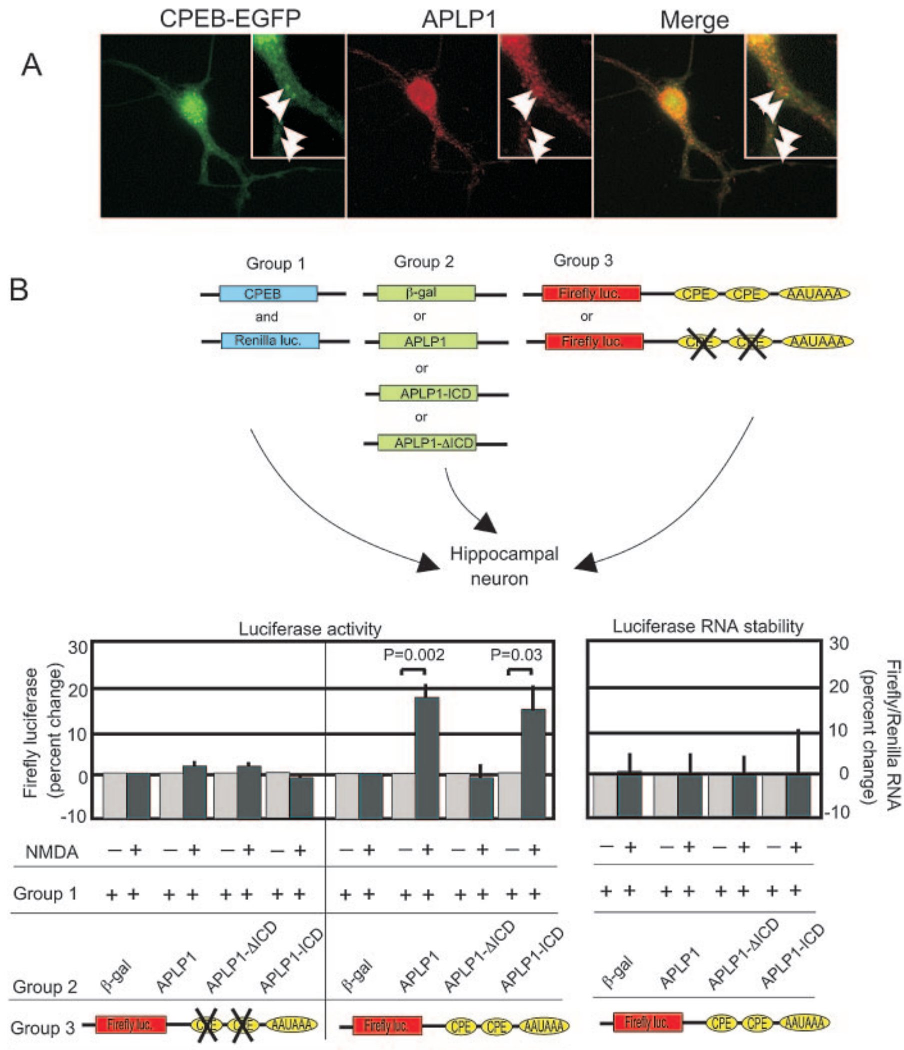

FIG. 6. APLP1 stimulates CPE-dependent translation in neurons. (A) Cultured hippocampal neurons were transfected with plasmid DNA encoding CPEB-enhanced GFP (EGFP) and cultured overnight. The neurons were then fixed and stained for endogenous APLP1. Arrows denote regions of colocalization between CPEB-EGFP and APLP1. (B) The C terminus of APLP1 enhances CPE-dependent RNA translation in cultured hippocampal neurons. Three groups of RNAs were synthesized in vitro: group 1 consisted of those encoding CPEB and *Renilla* luciferase; group 2 consisted of those encoding β-galactosidase (β-gal), APLP1, APLP1-ΔICD, or APLP1-ICD; and group 3 consisted of those encoding firefly luciferase, whose 3′ UTRs were derived from α-CaMKII mRNA containing two CPEs or a 3′ UTR where the CPEs were mutated. Both RNAs from group 1, one of the RNAs from group 2, and one of the RNAs from group 3 were transfected into 10-day-old hippocampal neurons, which were then stimulated with NMDA. Extracts were prepared and analyzed for *Renilla* and firefly luciferase activity. *Renilla* luciferase was used to normalize possible variations in transfection efficiency. All values were standardized against those obtained when β-galactosidase mRNA was transfected and are expressed as percent change as a function of NMDA stimulation. Statistically significant differences (Student's *t* test) were observed with APLP1 ($P = 0.002$) and APLP1-ICD ($P = 0.03$). Quantitative real-time reverse transcription-PCR was used to determine the relative stabilities of the two luciferase mRNAs in control and NMDA-treated neurons cotransfected with the APLP mRNAs. Based on three independent experiments, there was no statistically significant difference in the stabilities of these mRNAs under any condition.

animal pole cytoplasm (15). Not only is CPEB found on spindles and centrosomes, but Maskin, CPSF, and eIF4E are as well. While we have no evidence that the membrane-associated CPEB, Maskin, CPSF, etc., in oocytes subsequently become associated with the mitotic apparatus in embryos after fertilization, it is tempting to speculate that this could be the case. Irrespective of this possibility, these data do show a remarkable and differential localization of the polyadenylation/translation machinery in early development.

In the brain, APP undergoes a series of proteolytic cleavages by the α-, β-, and γ-secretase/presenilin system to produce not only the extracellular neurofibrillary tangles thought to be causative for Alzheimer's disease but also the liberation of the ICDs that, among other activities, stimulate transcription (8). We do not know whether oocytes contain active γ-secretase activity: we have been unable to detect free (cleaved) APLP1 (our antibody against mammalian APP can detect no clear immunoreactivity in *Xenopus* cells). On the other hand, the ICD of APLP1 is small, ~5 kDa, and thus would probably not be readily observed by Western blotting. However, the ICD has about the same polyadenylation-stimulating activity as the uncleaved membrane-bound APLP1. This result might seem

surprising since the ICD presumably would not be localized with the polyadenylation machinery. On the other hand, such a peptide would probably diffuse rather freely and, once in contact with an Aurora A/CPEB complex, could stimulate CPEB S174 phoshorylation and resulting polyadenylation. How the ICD stimulates this event is unclear; it does not enhance CPEB phosphorylation when mixed with recombinant CPEB and Aurora A in vitro (data not shown).

In neurons, CPEB-mediated polyadenylation/translation is regulated via NMDARs (22, 42), which are calcium ion channels. NMDA induces CPEB phosphorylation by Aurora A (22) and perhaps α-CaMKII as well (4). However, calcium probably does not activate Aurora A directly but may modify other upstream effectors such as those that signal to GSK3β, one of several factors that control Aurora A activity in oocytes (34) and, because it modifies synaptic plasticity, possibly neurons as well (14). APP, like CPEB, is detected in dendrites and in postsynaptic densities (36, 37) and modulates synaptic plasticity (40). We surmise that the function of the APLP1-CPEB complex in neurons, like that in oocytes, is to promote rapid local polyadenylation and translation. Indeed, neurons and oocytes seem remarkably similar in the basic molecular mechanism that regulates polyadenylation and translation (33).

Gene knockouts of the APP family members, both singly and in combination, have demonstrated at least partially redundant functions of the proteins (17). For example, while single APP family gene knockouts have relatively mild phenotypes (e.g., $APP^{-/-}$) (43), double knockouts (e.g., $APP^{-/-}/APLP2^{-/-}$ and $APLP1^{-/-}/APLP2^{-/-}$) are lethal (17). While it is not known whether the ICDs of these proteins are the portions responsible for this redundancy in animals, the fact that they are structurally similar suggests that they might contribute to it. This redundancy makes it somewhat difficult to determine whether all or some of the proteins contribute to CPEB-mediated translation in neurons. Nonetheless, future experiments will be directed toward addressing this issue.

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REFERENCES

1. **Aakalu, G., W. B. Smith, N. Nguyen, C. Jiang, and E. M. Schuman.** 2001. Dynamic visualization of local protein synthesis in hippocampal neurons. Neuron **30:**489–502.
2. **Alarcon, J. M., R. Hodgman, M. Theis, E. R. Kandel, and J. D. Richter.** 2004. Selective modulation of some forms of Schaffer collateral-CA1 synaptic plasticity in mice with a disruption of the CPEB-1 gene. Learn. Mem. **11:** 318–327.
3. **Annaert, W., and B. A. De Strooper.** 2002. Cell biological perspective on Alzheimer's disease. Annu. Rev. Cell Dev. Biol. **18:**25–51.
4. **Atkins, C. M., M. A. Davare, M. C. Oh, V. Derkach, and T. R. Soderling.** 2005. Bidirectional regulation of cytoplasmic polyadenylation element-binding protein phosphorylation by Ca2+/calmodulin-dependent protein kinase II and protein phosphatase 1 during hippocampal long-term potentiation. J. Neurosci. **25:**5604–5610.
5. **Barnard, D., Q. Cao, and J. D. Richter.** 2005. Differential phosphorylation controls Maskin association with eukaryotic translation initiation factor 4E and localization on the mitotic apparatus. Mol. Cell. Biol. **25:**7605–7615.
6. **Barnard, D., K. Ryan, J. L. Manley, and J. D. Richter.** 2004. Symplekin and xGLD-2 are required for CPEB-mediated cytoplasmic polyadenylation. Cell **119:**641–651.
7. **Biederer, T., X. Cao, T. C. Sudhof, and X. Liu.** 2002. Regulation of APP-dependent transcription complexes by Mint/X11s: differential functions of Mint isoforms. J. Neurosci. **22:**7340–7351.
8. **Brunkan, A. L., and A. M. Goate.** 2005. Presenilin function and γ-secretase activity. J. Neurochem. **93:**769–792.
9. **Cao, Q., and J. D. Richter.** 2002. Dissolution of the maskin-eIF4E complex by cytoplasmic polyadenylation and poly(A)-binding protein controls cyclin B1 mRNA translation and oocyte maturation. EMBO J. **21:**3852–3862.
10. **Cao, X., and T. C. Sudhof.** 2001. A transcriptionally active complex of APP with Fe65 and histone acetyltransferase Tip60. Science **293:**115–120.
11. **Du, L., and J. D. Richter.** 2005. Activity-dependent polyadenylation in neurons. RNA **11:**1340–1347.
12. **Fiore, F., N. Zambrano, G. Minopoli, V. Donini, A. Duilio, and T. Russo.** 1995. The regions of the Fe65 protein homologous to the phosphotyrosine interaction/phosphotyrosine binding domain of Shc bind the intracellular domain of the Alzheimer's amyloid precursor protein. J. Biol. Chem. **270:** 30853–30856.
13. **Frey, U., and R. G. Morris.** 1997. Synaptic tagging and long-term potentiation. Nature **385:**533–536.
14. **Graef, I. A., P. G. Mermelstein, K. Stankunas, J. R. Neilson, K. Deisseroth, R. W. Tsien, and G. R. Crabtree.** 1999. L-type calcium channels and GSK-3 regulate the activity of NF-ATc4 in hippocampal neurons. Nature **401:**703–708.
15. **Groisman, I., Y. S. Huang, R. Mendez, Q. Cao, W. Theurkauf, and J. D. Richter.** 2000. CPEB, maskin, and cyclin B1 mRNA at the mitotic apparatus: implications for local translational control of cell division. Cell **103:**435–447.
16. **Halic, M., and R. Beckmann.** 2005. The signal recognition particle and its interactions during protein targeting. Curr. Opin. Struct. Biol. **15:**116–125.
17. **Heber, S., J. Herms, V. Gajic, J. Hainfellner, J. Aguzzi, T. Rulicke, H. von Kretzschmar, C. von Koch, S. Sisodia, P. Tremml, H. P. Lipp, D. P. Wolfer, and U. Muller.** 2000. Mice with combined gene knock-outs reveal essential and partially redundant functions of amyloid precursor protein family members. J. Neurosci. **20:**7951–7963.
18. **Hering, H., and M. Sheng.** 2001. Dendritic spines: structure, dynamics, and regulation. Nat. Rev. Neurosci. **2:**880–888.
19. **Hodgman, R., J. Tay, and J. D. Richter.** 2001. CPEB phosphorylation and cytoplasmic polyadenylation are catalyzed by the kinase IAK1/Eg2 in maturing mouse oocytes. Development **128:**2815–2822.
20. **Homayouni, R., D. S. Rice, M. Sheldon, and T. Curran.** 1999. Disabled-1 binds to the cytoplasmic domain of amyloid precursor-like protein 1. J. Neurosci. **19:**7507–7515.
21. **Huang, Y. S., J. H. Carson, E. Barbarese, and J. D. Richter.** 2003. Facilitation of dendritic mRNA transport by CPEB. Genes Dev. **17:**638–653.
22. **Huang, Y. S., M. Y. Jung, M. Sarkissian, and J. D. Richter.** 2002. N-methyl-D-aspartate receptor signaling results in Aurora kinase-catalyzed CPEB phosphorylation and alpha CaMKII mRNA polyadenylation at synapses. EMBO J. **21:**2139–2148.
23. **Huber, K. M., M. S. Keyser, and M. F. Bear.** 2000. A role for rapid dendritic protein synthesis in hippocampal mGluR-dependent long-term depression. Science **288:**1254–1257.
24. **Hurkman, W. J., L. D. Smith, J. D. Richter, and B. A. Larkins.** 1981. Subcellular compartmentalization of maize storage proteins in *Xenopus* oocytes injected with zein messenger RNAs. J. Cell Biol. **89:**292–299.
25. **Kang, H., and E. M. Schuman.** 1996. A requirement for local protein synthesis in neurotrophin-induced hippocampal synaptic plasticity. Science **273:** 1402–1406.
26. **Liu, X. S., C. Ma, A. W. Hamam, and X. J. Liu.** 2005. Transcription-dependent and transcription-independent functions of the classical progesterone receptor in Xenopus ovaries. Dev. Biol. **283:**180–190.
27. **Martin, K., T. C. 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.
28. **Mendez, R., D. Barnard, and J. D. Richter.** 2002. Differential mRNA translation and meiotic progression require Cdc2-mediated CPEB destruction. EMBO J. **21:**1833–1844.
29. **Mendez, R., L. Hake, T. Andresson, L. Littlefield, J. V. Ruderman, and J. D. Richter.** 2000. Phosphorylation of CPE binding factor by Eg2 regulates translation of c-mos mRNA. Nature **404:**302–307.
30. **Mendez, R., K. G. K. Murthy, J. L. Manley, and J. D. Richter.** 2000. Phosphorylation of CPEB by Eg2 mediates the recruitment of CPSF into an active cytoplasmic polyadenylation complex. Mol. Cell **6:**1253–1259.
31. **Mendez, R., and J. D. Richter.** 2001. Translational control by CPEB: a means to the end. Nat. Rev. Mol. Cell Biol. **2:**521–529.
32. **Miller, S., and M. Mayford.** 1999. Cellular and molecular mechanisms of memory: the LTP connection. Curr. Opin. Genet. Dev. **9:**333–337.
33. **Richter, J. D.** 2001. Think globally, translate locally: what mitotic spindles and neuronal synapses have in common. Proc. Natl. Acad. Sci. USA **98:**7069–7071.
34. **Sarkissian, M., R. Mendez, and J. D. Richter.** 2004. Progesterone and insulin stimulation of CPEB-dependent polyadenylation is regulated by Aurora A and glycogen synthase kinase-3. Genes Dev. **18:**48–61.

35. **Scheetz, A. J., A. C. Nairn, and M. Constantine-Paton.** 2000. NMDA receptor-mediated control of protein synthesis at developing synapses. Nat. Neurosci. **3:**211–216.
36. **Schubert, W., R. Prior, A. Weidemann, H. Dircksen, G. Multhaup, C. L. Masters, and K. Beyreuther.** 1991. Localization of Alzheimer beta A4 amyloid precursor protein at central and peripheral synaptic sites. Brain Res. **563:**184–194.
37. **Shigematsu, K., P. L. McGeer, and E. G. McGeer.** 1992. Localization of amyloid precursor protein in selective postsynaptic densities of rat cortical neurons. Brain Res. **592:**353–357.
38. **Si, K., M. Giustetto, A. Etkin, R. Hsu, A. M. Janisiewicz, M. C. Miniaci, J. H. Kim, H. Zhu, and E. R. Kandel.** 2003. A neuronal isoform of CPEB regulates local protein synthesis and stabilizes synapse-specific long-term facilitation in *Aplysia*. Cell **115:**893–904.
39. **Stebbins-Boaz, B., Q. Cao, C. deMoor, R. Mendez, and J. D. Richter.** 1999. Maskin is a CPEB-associated factor that transiently interacts with eIF-4E. Mol. Cell **4:**1017–1027.
40. **Turner, P. R., K. O'Connor, W. P. Tate, and W. C. Abraham.** 2003. Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory. Prog. Neurobiol. **70:**1–32.
41. **Wells, D., X. Dong, E. Quinlan, Y. S., Huang, M. F. Bear, J. D. Richter, and J. R. Fallon.** 2002. A role for the cytoplasmic polyadenylation element in NMDA receptor-regulated mRNA translation in neurons. J. Neurosci. **21:** 9541–9548.
42. **Wu, L., D. Wells, J. Tay, D. Mendis, M. A. Abbott, A. Barnitt, E. Quinlan, A. Heynan, J. R. Fallon, and J. D. Richter.** 1998. CPEB-mediated cytoplasmic polyadenylation and the regulation of experience-dependent translation of alpha-CaMKII mRNA at synapses. Neuron **21:**1129–1139.
43. **Zheng, H., M. H. Jiang, M. E. Trumbauer, D. J. Sirinathsinghji, R. Hopkins, D. W. Smith, R. P. Heavens, G. R. Dawson, S. Boyce, M. Connerm, K. Stevens, H. Slunt, S. S. Sisodia, H. Y. Chen, and L. H. T. van der Ploeg.** 1995. Beta-amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity. Cell **81:**525–531.
44. **Zhu, Y., J. Bond, and P. Thomas.** 2003. Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progestin receptor. Proc. Natl. Acad. Sci. USA **100:**2237–2242.