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Translational Control by Neuroguidin, a Eukaryotic Initiation Factor 4E and CPEB Binding Protein

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CPEB-mediated translation is important in early development and neuronal synaptic plasticity. Here, we describe a new eukaryotic initiation factor 4E (eIF4E) binding protein, Neuroguidin (Ngd), and its interaction with CPEB. In the mammalian nervous system, Ngd is detected as puncta in axons and dendrites and in growth cones and filopodia. Ngd contains three motifs that resemble those present in eIF4G, 4EBP, Cup, and Maskin, all of which are eIF4E binding proteins. Ngd binds eIF4E directly, and all three motifs must be deleted to abrogate the interaction between these two proteins. In injected *Xenopus* oocytes, Ngd binds CPEB and, most importantly, represses translation in a cytoplasmic polyadenylation element (CPE)-dependent manner. In *Xenopus* embryos, Ngd is found in both neural tube and neural crest cells. The injection of morpholino-containing antisense oligonucleotides directed against *ngd* mRNA disrupts neural tube closure and neural crest migration; however, the wild-type phenotype is restored by the injection of a rescuing *ngd* mRNA. These data suggest that Ngd guides neural development by regulating the translation of CPE-containing mRNAs.

The regulated translation of specific mRNAs influences a large number of biological processes, including meiotic progression (39, 60), the mitotic cell cycle (25, 46), and body axis specification (44, 50). In the adult nervous system, translational control modifies synaptic efficacy (29, 32), which probably underlies long-term memory storage (40). In developing neurons, translation is important for axon guidance (9, 10, 68). While several regulatory proteins probably control translation during these cellular processes, at least one, CPEB, appears to be involved in them all. CPEB was identified as the cytoplasmic polyadenylation element (CPE) binding factor in *Xenopus* oocytes (27, 36). When these cells are stimulated to reenter the meiotic divisions, several dormant CPE-containing mRNAs that have short poly(A) tails undergo poly(A) elongation; translational activation is the result of this elongation (6, 60).

The mechanism by which cytoplasmic polyadenylation induces translation was elucidated in *Xenopus* oocytes. Here, a CPEB-associated factor, Maskin, was shown to be a key regulatory molecule: it binds not only CPEB, but also the cap binding factor eukaryotic initiation factor 4E (eIF4E) (59). The site on eIF4E that interacts with Maskin is the same as that normally occupied by eIF4G, a scaffold protein that is necessary for translation because it (indirectly) recruits the 40S ribosomal subunit to the 5′ untranslated region (UTR) of the mRNA. Thus, Maskin is an inhibitor of translation because it precludes the interaction of eIF4G and eIF4E. In this sense, Maskin is functionally similar to the 4EBPs (47, 49), factors that inhibit translation by interfering with the eIF4E-eIF4G association. However, Maskin is an unusual 4EBP because its tethering to CPEB makes it a message-specific translation-inhibitory protein.

The inhibition of translation by the 4EBPs is reversible and is dictated by their state of phosphorylation (23). Maskin inhibition is also reversible, but in this case, both cytoplasmic polyadenylation and phosphorylation are the important regulatory events. During oocyte maturation, progesterone induces the inactivation of glycogen synthase kinase 3, which in turn is necessary for the activation of Aurora A (also known as Eg2 [2]), a serine/threonine kinase (52). Aurora A phosphorylates CPEB serine 174, an event that enhances the interaction between CPEB and cleavage and polyadenylation specificity factor; such an interaction may help cleavage and polyadenylation specificity factor stably associate with a second important *cis* element, AAUAAA (37, 38). In a mechanism that is as yet unknown, CPEB phosphorylation stimulates the activity of Gld2, an unusual poly(A) polymerase that catalyzes poly(A) addition (6). The newly elongated poly(A) associates with poly(A) binding protein (PABP); PABP also binds eIF4G. PABP-bound eIF4G then out-competes Maskin for binding to eIF4E, the result of which is the initiation of translation (11). During maturation, Maskin also undergoes a number of phosphorylation events that also help it to dissociate from eIF4E (5).

In addition to oocytes, CPEB has been detected in the mammalian central nervous system, where it has been found to reside at postsynaptic sites (67). The stimulation of synapses by visual experience or *N*-methyl-D-aspartate receptor activation results in the polyadenylation and translation of the CPE-containing α*CaMKII* and other mRNAs (19, 28, 54, 64, 67). In addition, CPEB knockout mice have deficits in long-term potentiation and hippocampus-dependent memories (1, 7). At synapses, many of the factors involved in polyadenylation-induced translation have been detected (28); however, other proteins, particularly those with a possible Maskin-like activity, also may function in the nervous system. In this regard, the product of another gene, which we call *Neuroguidin* (*ngd*), functions in the *Drosophila* embryonic nervous system (L. Lorenz et al., unpublished data). Here, we show that Ngd resembles Maskin in that it interacts with eIF4E through a

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discrete binding motif. The protein is widely expressed in the mammalian nervous system and is often detected as puncta that resemble RNP particles in axons and dendrites, as well as in growth cones and lamellipodia. In addition to eIF4E, Ngd also interacts with CPEB, and in injected *Xenopus* oocytes, it inhibits mRNA translation in a CPE-dependent manner. In *Xenopus* embryos, Ngd is found in the embryonic nervous system and neural crest. The injection of morpholino-containing antisense oligonucleotides against *Ngd* RNA results in a failure of neural tube closure and inhibited neural crest cell migration. These data indicate that Ngd functions as an important translational regulatory protein during development of the vertebrate nervous system.

MATERIALS AND METHODS

Plasmid construction and Northern blotting. Based on homology with *Drosophila ngd*, a mouse *ngd* cDNA was obtained and cloned into EcoRI/BamHI-digested pBluescriptII SK and BglII/BamHI-digested pET-30a. *Xenopus ngd* was amplified by reverse transcription-PCR from total RNA of stage V and VI stage oocytes and cloned into BamHI/SalI-digested pBluescriptII SK. Twenty micrograms of total RNA was resolved by formaldehyde/agarose gel electrophoresis and probed with ^{32}P-labeled random-primed Ngd DNA.

Generation of Ngd antibody and Western blotting. The mouse *ngd* open reading frame was cloned behind a histidine tag in pET-30a, expressed in *Escherichia coli*, and isolated on an Ni column, which was used to generate antisera. For Western blotting, tissue or cell cultures were homogenized in 10 volumes of HB buffer (0.1 M KCl, 1 mM $MgCl_2$, 50 mM Tris-HCl [pH 7.5], 0.5% Triton X-100, and 10 μg/ml each of the protease inhibitors pepstatin, chymostatin, and leupeptin), and 50 μg of protein was probed with antibodies against Ngd, CPEB, Maskin, dynein (Chemicon), eIF4E (Transduction Laboratories), extracellular signal-regulated kinase 2 (ERK2) (Santa Cruz), or tubulin (Sigma).

Coimmunoprecipitation and cap column assays. Groups of 15 oocytes were homogenized in 150 μl of HB buffer containing RNase A (0.1 mg/ml) and centrifuged, and the postmitochondrial supernatant was preabsorbed to protein A-Sepharose for 1 h at 4°C. The solution was then added to antibody-bound protein A-Sepharose beads, which were prepared by mixing 25 μl of protein A-Sepharose and 10 μl of preimmune or immune serum at 4°C overnight and then washing with HB buffer. Immunoprecipitation was performed for 4 h at 4°C, after which the beads were washed with HB buffer and then boiled in sodium dodecyl sulfate (SDS) sample buffer.

Whole mouse brain or cerebellum was homogenized in HB buffer containing RNase A and centrifuged to pellet insoluble material, and the supernatant was subjected to immunoprecipitation as described above or to cap column chromatography as described previously (59).

Ngd-eIF4E binding assay. The mouse Ngd sequence containing or lacking the putative eIF4E binding motifs was cloned into pRSET (Invitrogen). These clones and DNA encoding human eIF4E (59) were expressed in *E. coli*. Protein G-Sepharose 4B was bound with eIF4E antibody and eIF4E and then mixed with the Ngd proteins at 4°C for 2 h. Bacterial extracts were prepared in 100 mM KCl, 1 mM $MgCl_2$, 50 mM Tris-Cl (pH 8.0), 1 mM dithiothreitol, 0.4% NP-40, 50 μg of RNase A, and protease inhibitor cocktail (Roche); the whole extracts were used for binding assay. The protein G beads were boiled in SDS sample buffer, and the bound proteins were analyzed by Western blotting.

CAT assays. CAT-CPE$^+$ and CAT-CPE$^-$ mRNAs have been described previously (62). Stage VI oocytes were coinjected with mRNAs (8 ng) and Ngd (16 ng) or buffer. Two hours after injection, the oocytes were collected for chloramphenicol acetyltransferase (CAT) assays as described previously (17). Some oocytes were injected with [^{32}P]UTP-labeled CAT-CPE$^+$ or CAT-CPE$^-$ mRNA and Ngd protein and then assessed for RNA stability on a denaturing polyacrylamide gel.

Immunohistochemistry. Brain and spinal cord sections were obtained from Novagen Co. and from C. Jung (30). Rehydrated paraffin sections were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min; blocked with 10% goat serum, 5% bovine serum albumin, and 0.1% Triton X-100 in PBS at 4°C overnight; and probed with primary antibody (Ngd or neurofilament) and then secondary antibody (Alexa 546- or 488-labeled goat anti-rabbit immunoglobulin G [IgG] or Alexa 546- or 488-labeled goat anti-mouse IgG [Molecular Probes]). Some sections were incubated with biotinylated horse anti-rabbit IgG (Vectastain ABC kit; Vector Laboratories) for 90 min at room temperature after primary antibody incubation with Ngd antibody, followed by diaminobenzidine staining as described previously (31).

Hippocampal neuron cultures and immunocytochemistry. Embryonic rat hippocampal neurons were cocultured with glial cells at a density of 5,000 to 20,000 cells/cm^2 (4, 16). The coverslips were rinsed with PBS, fixed with 4% paraformaldehyde in PBS for 15 min, and permeabilized with 0.1% Triton X-100 in PBS for 3 min. They were blocked with 10% goat serum and 5% bovine serum albumin in PBS for 1 h at 37°C, incubated at 37°C for 2 h with primary antibodies, and then incubated again at 37°C for 90 min with secondary antibody. Some neurons were labeled for filamentous actin with Alexa 488-conjugated phalloidin at 0.2 unit per coverslip in PBS for 1 h at 37°C.

Analysis of *Xenopus* embryos. In situ hybridization to *Xenopus* material in whole mount and on sections (10 μm) was carried out as described previously (55; http://www.hhmi.ucla.edu/derobertis/). Probes for *slug* and *nrp-1*, which have been described elsewhere (35, 48), were labeled with digoxigenin-UTP according to standard methods (Roche) and reacted with alkaline phosphatase-conjugated digoxigenin antibody. A standard control morpholino oligonucleotide (CM) and *Xenopus ngd* antisense morpholino oligonucleotide (AM) directed against the 5′ UTR and the first 4 bases of the open reading frame consisting of 5′-CCATTG TACCCCACGTCTGTGTTAC were purchased from Gene Tools. Phenotype rescue experiments entailed the coinjection of oligonucleotides (3 ng) with in vitro-synthesized *ngd* mRNA (2 ng).

RESULTS

Ngd in the vertebrate nervous system. The distribution of *ngd* mRNA in mouse tissues was determined by Northern blotting. With the exception of muscle, *ngd* mRNA was detected in all tissues examined, including several parts of the brain, kidney, spleen, ovary, and testis (Fig. 1A). To assess Ngd protein, a mouse *ngd* cDNA was expressed in *E. coli*, purified, and used to immunize rabbits. The antibody was affinity purified (Fig. 1B) and used to probe a Western blot of protein from several different mouse tissues (Fig. 1C). As with the RNA gel blot described above, Ngd protein was detected in all tissues except muscle; it was also detected in cultured hippocampal neurons and glia.

To obtain more precise information on Ngd expression in the central nervous system, immunohistochemistry was performed on sagittal sections of adult mouse brain. High immunoreactivity was detected in all parts of the hippocampus, particularly the dentate gyrus, and the cerebellum (Fig. 1D and E, top panels) (note the heavy brown diaminobenzidine stain used to detect Ngd). Ngd expression was further examined using double-antibody-labeled immunofluorescence microscopy (Fig. 1D and E, lower panels). Unphosphorylated neurofilament in green served as a specific marker for neurons; its immunoreactivity was found only in the perinuclear space as well as in dendrites. Ngd (red) was detected in hippocampal neurons (Fig. 1D) and in the granule cell layer and Purkinje cells of the cerebellum (Fig. 1E). The Ngd immunoreactivity was punctate in appearance and was evident in the nucleus and the cytoplasm. Ngd particles were also found in the adult spinal cord, where the highest immunoreactivity was detected in ventral motor neurons (Fig. 1F, top and bottom panels).

Localization of Ngd in cultured neurons. Hippocampal neurons undergo characteristic morphological changes as they mature both in vivo and in vitro (4). To determine whether Ngd expression correlated with any of these changes, neurons were cultured for various times and immunostained with Ngd antibody; Alexa-labeled phalloidin, which binds to filamentous actin, served as a counterstain. After 4 hours of culture, most of the Ngd immunoreactivity was detected in the soma, in both nuclear and cytoplasmic compartments. In neurites, Ngd particles were clearly

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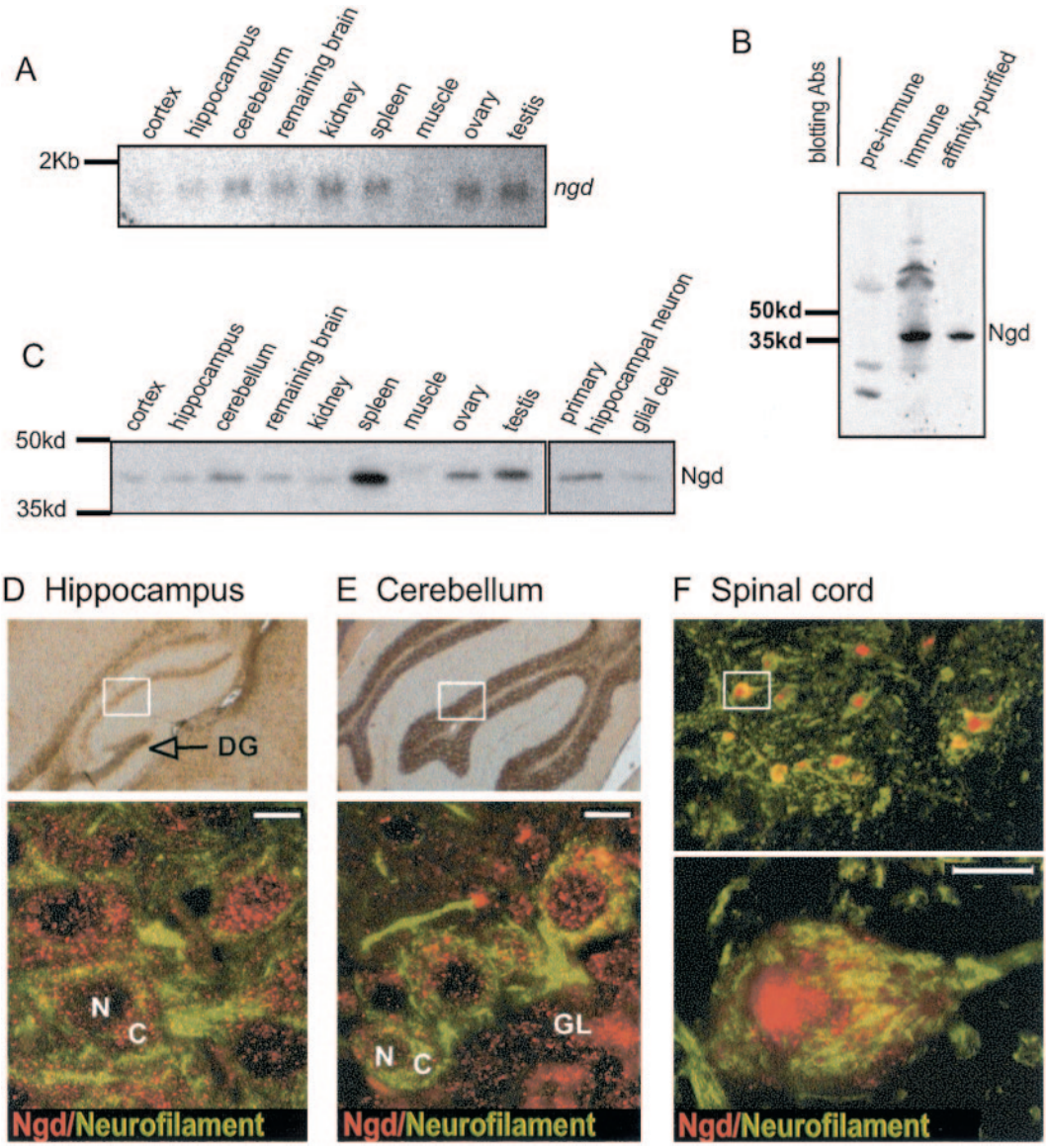


FIG. 1. Tissue distribution of mouse Ngd. (A) Northern blotting analysis of total RNA (20 μg) from several adult mouse tissues probed for *ngd*. (B) Western analysis of mouse brain extract (50 μg), using preimmune and Ngd immune serum, and affinity-purified Ngd antibody. Ab, antibody. (C) Western analysis of protein (50 μg) from several adult mouse tissues as well as cultured rat hippocampal neurons (14-day cultures) or cultured rat cortical glial cells probed with affinity-purified Ngd antibody. (D and E) Immunohistochemistry of Ngd in the hippocampus and cerebellum of adult mouse brain. Sagittal sections of paraffin-embedded tissue were probed with Ngd antibody followed by horseradish peroxidase-conjugated anti-rabbit IgG. Similar sections were also doubly immunostained with antibodies for Ngd (red) and unphosphorylated neurofilament (green); the boxes denote approximate corresponding areas between the two sections. N, nucleus; C, cytoplasm; GL granule cell layer; scale bars, 10 μm. (F) Immunofluorescent images of sagittal sections of an adult mouse spinal cord. To better demonstrate high Ngd immunoreactivity in motor neurons, an image taken of the ventral part of a spinal cord section (top panel) was magnified (lower panel).

detected along the edges of the motile lamellipodia of growth cones (Fig. 2A). At 24 h of culture, Ngd again was predominantly in the soma, but Ngd particles were also observed in growth cones, often at the tips of filopodia (Fig. 2B). After 14 days of culture, when the neurons are fully differentiated, Ngd particles were observed in neurites, particularly near the base and in the shaft and tips of filipodia (Fig. 2C, panels 1 to 4). To more clearly differentiate between axon and dendritic Ngd staining, 14-day cultures were costained with antibody against Map2, a dendritic marker, or with phosphoneurofilament (P-NP), an axonal marker. Figure 2D shows Ngd particles on or near Map2-postive dendrites. However, this staining was consistent not only with dendritic staining but also with a situation where Ngd-containing axons possibly formed a sheath around the dendrites. Figure 2D (panel 6) shows clear Ngd immunostaining in a neurite where there was no Map2 immunostaining, pointing to a likely presence of Ngd in axons. This possibility was confirmed in Fig. 2E (panel 7), where Ngd immunostaining was coincident with P-NP immunostaining, an axon marker. However, Fig. 2E (panel 8) shows Ngd particles in large neurites that did not immunostain with

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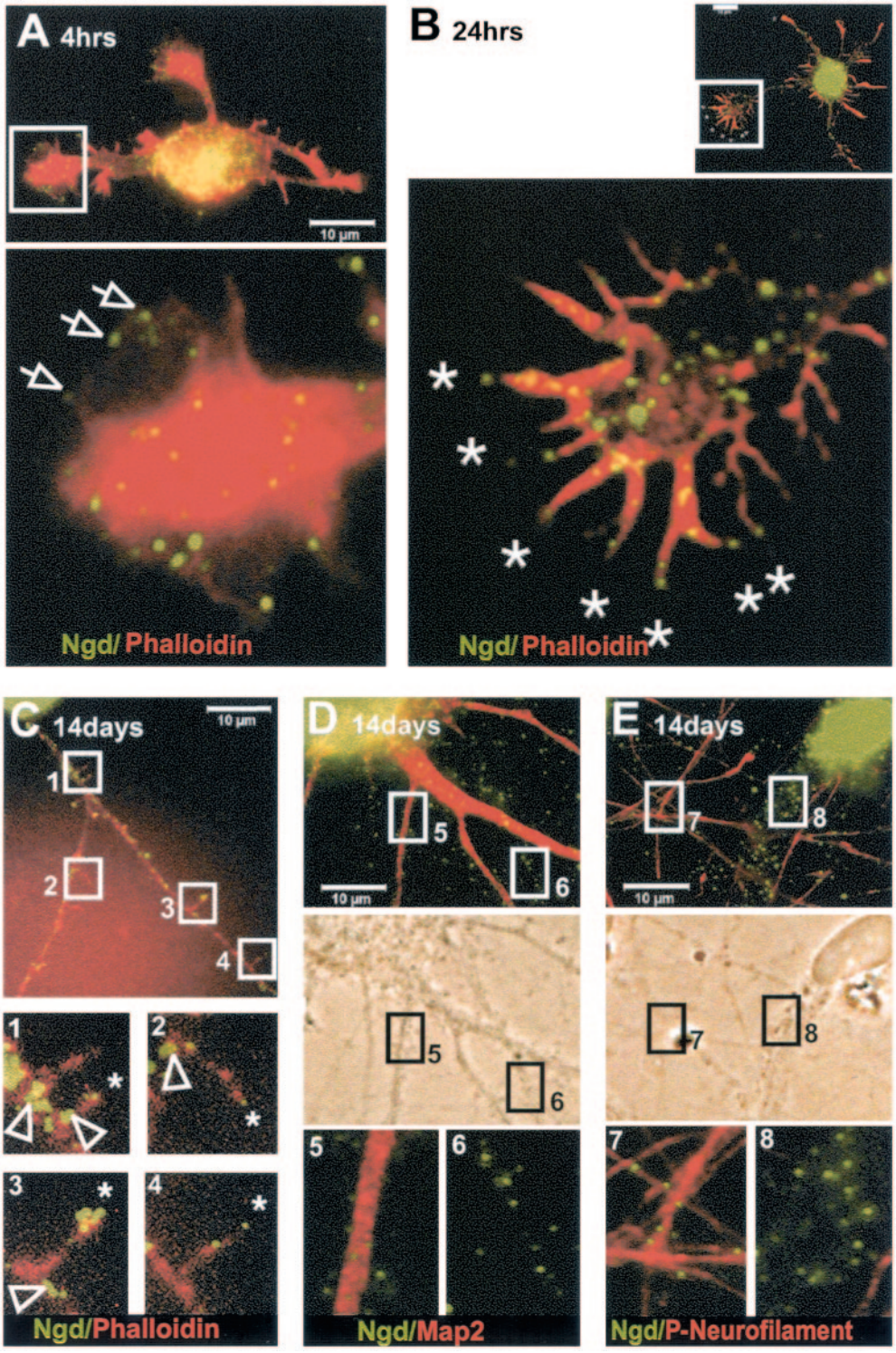


FIG. 2. Ngd localization in cultured rat hippocampal neurons. (A to C) Images of Ngd immunofluorescence (green) in primary hippocampal neurons cultured for 4 h (A), 24 h (B), or 14 days (C). Phalloidin (red), which binds to F-actin, helps to define the morphology of cells and growth cones. Ngd immunoreactivity was observed at the edges (open arrows) and tips (asterisks) of growth cones and filopodia; Ngd was also strongly detected in the cell body. Open arrowheads denote Ngd expression at the base and in the shaft of filopodia (C). (D and E) Immunofluorescent images of Ngd (green) and Map2 (red in panel D), which identifies dendrites, or phosphorylated neurofilament (red in panel E), which identifies axons in 14-day-old hippocampal neurons. Ngd-containing particles were detected in both axons and dendrites. The middle panels show neuron morphology by bright-field imaging. Scale bars, 10 μm.

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P-NP and hence are dendrites. In summary, Ngd resides in particles in neurons of all stages; the particles are present in axons and dendrites and are detected in filopodia. Also, Ngd is not particularly colocalized with PSD95, a synaptic marker.

Ngd interacts with eIF4E. The mouse, human, and *Xenopus* Ngd protein sequences are shown in Fig. 3A. The mouse and human sequences are 89% identical, whereas the mouse and *Xenopus* sequences are 61% identical. Residues 103 to 116 are particularly well conserved among these three animals; these residues are also very highly conserved in *Drosophila* Ngd (93% relative to the mouse), which has only a 38% overall identity with the mouse protein. These residues resemble a motif that is present in proteins that bind the cap binding factor eIF4E (Fig. 3A, lower panel) (33, 34, 49, 58). These proteins include *Xenopus* Maskin (which alone among eIF4E binding proteins contains a threonine at position 6 of this motif), eIF4G, and 4EBP1. Of these factors, eIF4G binding to eIF4E is the only one that is required for translation; the other factors inhibit translation by competing with eIF4G for binding to eIF4E. Ngd contains two additional putative eIF4E binding regions that are similarly conserved.

To determine whether Ngd resides in a complex with eIF4E, an extract prepared from adult mouse brain was supplemented with GTP (0.2 mM) and RNase A (0.1 mg/ml) and applied to a column containing ^{7m}GTP affixed to Sepharose (cap column). In some cases, the extract was supplemented with 0.2 mM free cap (^{7m}GTP) in place of GTP. Following extensive washing, the Sepharose beads were boiled in SDS sample buffer and the retained protein was subjected to Western blotting. Figure 3B (left panel) shows that in the presence of free GTP, both eIF4E and Ngd were retained on the cap column. When the extract contained free ^{7m}GTP, most of the eIF4E and all of the Ngd were out-competed for binding to the cap beads. These data suggest that Ngd was retained on the cap column through an interaction with eIF4E. To assess this further, an extract from mouse cerebellum was supplemented with RNase A and subjected to an immunoprecipitation procedure with eIF4E antibody; Western analysis demonstrates that eIF4E was indeed immunoprecipitated, as was Ngd. When preimmune serum was used in the initial immunoprecipitation, neither eIF4E nor Ngd was detected on the Western blot (Fig. 3B, second panel). In a reciprocal experiment, Ngd antibody precipitated not only Ngd but eIF4E as well (Fig. 3B, third panel). ERK2 was not coimmunoprecipitated with Ngd antibody and hence served as a negative control. These data indicate that eIF4E and Ngd reside in a multiprotein complex in the mouse brain.

We next determined which Ngd residues are necessary for interacting with eIF4E. The putative eIF4E biding motifs highlighted in Fig. 3A were sequentially deleted, and the proteins were expressed in bacteria and mixed with *E. coli*-expressed eIF4E (in the presence of 50 μg/ml RNase A), which in turn was bound to eIF4E antibody tethered to protein G beads. The bound proteins were then analyzed by Western blotting (Fig. 3B, fourth panel). Compared to wild-type Ngd, deletion of motif 2 or motifs 1 and 2 had little effect on binding to eIF4E. Deletion of motifs 2 and 3 lowered binding to eIF4E by about 50%, while deletion of all three motifs completely abolished Ngd binding to eIF4E. These results indicate that all three motifs can contribute to binding to eIF4E and that they can compensate for one another.

If Ngd acts as a negative regulator of translation by binding eIF4E, these two proteins should colocalize. On the other hand, the interaction between Ngd and eIF4E may be regulated, and thus their binding may only be transient. In spite of this caveat, we performed double immunostaining for Ngd and eIF4E on neurites and growth cones of cultured hippocampal neurons cultured for 3 days (Fig. 3C). Both Ngd and eIF4E were readily detected, and surprisingly, as much as 21% of the eIF4E was colocalized with Ngd. Thus, four different assays, i.e., cap column chromatography, antibody coimmunoprecipitation, mutagenesis followed by protein-protein interaction in vitro, and immunocytochemistry, all consistently point to an interaction between Ngd and eIF4E.

Ngd inhibits translation in a CPE-dependent manner. Ngd, like Maskin, might interact with a sequence-specific RNA binding protein. This possibility, together with its expression in neurons, prompted us to determine whether it might associate with CPEB and, if so, whether it controls translation in a CPE-dependent manner (11, 60). We examined Ngd activity in the injected *Xenopus* oocyte, which is a well-characterized system to study CPEB-mediated translation. Initially, we tested whether Ngd interacts with CPEB by coimmunoprecipitation analysis. Although *Xenopus* oocytes contain *ngd* mRNA, they do not contain detectable Ngd protein (see Fig. 5A). Consequently, we injected recombinant NgD (Fig. 4A, left panel) into stage VI oocytes; 2 hours later, extracts were subjected to coimmunoprecipitation with Ngd and CPEB antibodies. As shown by Western blotting (Fig. 4A, right panel), Ngd antibody precipitated not only Ngd but also CPEB (lane1); preimmune serum precipitated neither CPEB nor Ngd (lane 2). Reciprocally, CPEB antibody coimmunoprecipitated Ngd along with CPEB (lane 3), demonstrating that Ngd and CPEB were in the same complex.

Next, we addressed whether Ngd could inhibit translation in vivo. mRNAs encoding CAT were fused to the cyclin B1 3′ UTR, which contains three CPEs (CAT-CPE$^+$), or to one in which the CPEs have been mutated (CAT-CPE$^-$) (62). They were mixed with recombinant Ngd or buffer and injected into oocytes; 2 hours later, extracts were prepared for CAT assays. The CPE is a repressor as well as an activator element (18) and serves to anchor Maskin indirectly through CPEB (4, 59). In the absence of Ngd, the CPE inhibited translation of the CAT reporter by ~40% (data not shown). A further 51% reduction of CAT activity was obtained when the CAT-CPE$^+$ mRNA was injected together with Ngd. Ngd did not affect the translation of CAT-CPE$^-$ mRNA (Fig. 4B). The Ngd-mediated reduction of CAT activity derived from CAT-CPE$^+$ RNA was statistically significant ($P = 0.01$, Student's t test). To ensure that the two mRNAs were equally stable, oocytes were injected with [^{32}P]UTP-labeled CAT-CPE$^+$ and CAT-CPE$^-$ mRNAs together with Ngd or buffer and cultured for 2 hours; the RNA was then extracted and analyzed on a denaturing gel. Figure 4B (bottom) shows that the RNAs were stable. These results demonstrate that Ngd inhibits the translation of CPE-containing mRNAs.

Control of neural tube closure and neural crest migration by Ngd. To assess the possible function of Ngd in the vertebrate nervous system, we examined developing *Xenopus* embryos

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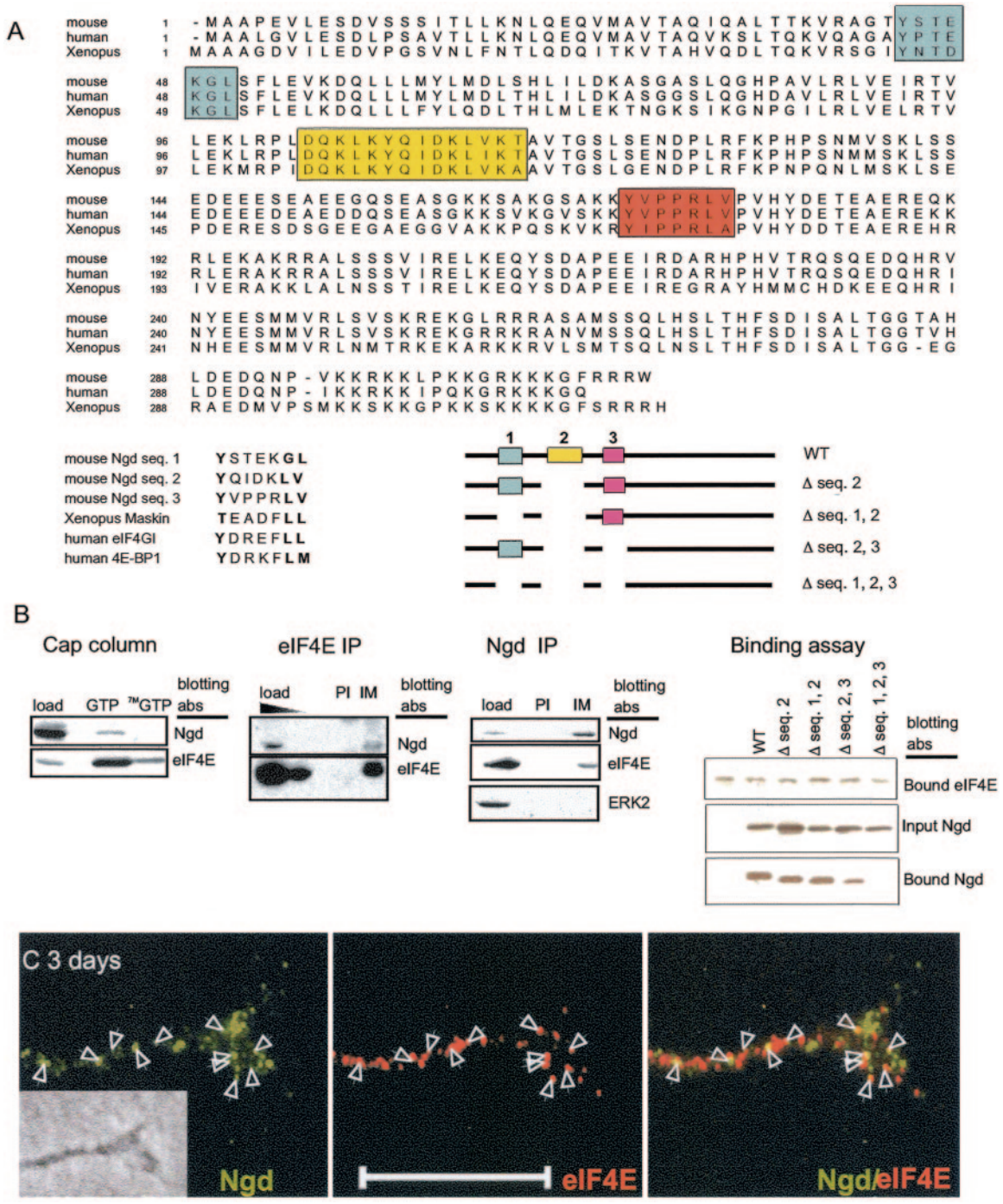


FIG. 3. Ngd is an eIF4E binding protein. (A) Alignment of mouse, human, and *Xenopus* Ngd sequences. The residues (boxed) correspond to putative eIF4E binding domains. The lower part of the panel shows a limited alignment of several eIF4E binding proteins; those residues that are particularly well conserved are in boldface. The boxed areas denote putative eIF4E binding regions. (B) Interaction between Ngd and eIF4E. The first panel shows a cap column assay in which an adult mouse brain extract was supplemented with GTP (0.2 mM) and RNase A (0.1 mg/ml) and applied to a ^{7m}GTP-Sepharose column. In one case, the extract also contained free ^{7m}GTP (0.2 mM). Proteins that were retained on the column were probed for the presence of Ngd and eIF4E. abs, antibodies. The second panel shows an eIF4E coimmunoprecipitation assay in which a mouse cerebellum extract was immunoprecipitated (IP) with preimmune serum (PI) or eIF4E immune serum (IM) and the material probed for the presence of Ngd and eIF4E. The third panel shows a similar coimmunoprecipitation assay using Ngd antibody; the precipitated proteins were probed for the presence of Ngd, eIF4E, and ERK2, a negative control. The fourth panel shows binding assays between mutant Ngd proteins and eIF4E. The top blot shows that equal amounts of eIF4E were bound to eIF4E antibody beads, the second blot demonstrates that similar amounts of Ngd proteins were incubated with the eIF4E-containing antibody beads, and the third blot shows the amount of Ngd bound to the eIF4E beads. (C) Images of Ngd (green) and eIF4E (red) immunofluorescence in a hippocampal neuron cultured for 3 days. The arrows show regions where these two proteins colocalized. Scale bar, 10 μm.

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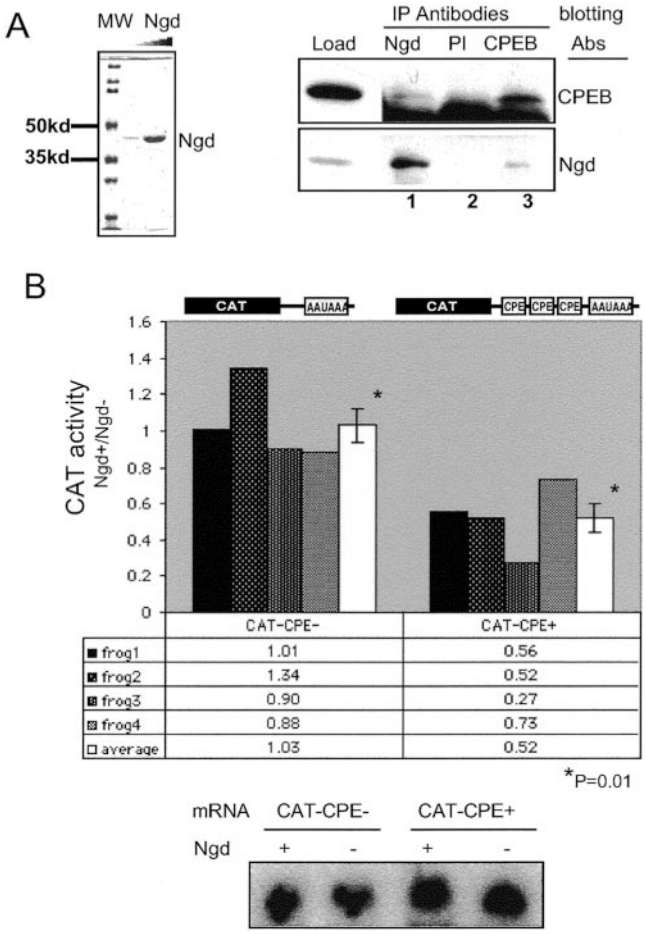


FIG. 4. Ngd inhibits translation in a CPE-dependent manner. (A) CPEB and Ngd can be coimmunoprecipitated. Mouse Ngd, overexpressed in and purified from *E. coli* (left panel shows a Coomassie blue-stained gel) was injected into *Xenopus* oocytes. After 2 h of culture, Ngd and CPEB antibodies (Abs), as well as preimmune serum (PI), were used for coimmunoprecipitation assays; the resulting Western blots were probed for CPEB and Ngd. The arrow denotes the heavy chain of IgG. MW, molecular weight markers. (B) A schematic diagram of the CAT reporter mRNAs, containing or lacking CPEs, that were injected into oocytes is illustrated. The RNAs (8 ng) were mixed with buffer or with purified Ngd protein (16 ng), and injected into stage VI oocytes (Ngd^+ or Ngd^-). Two hours after injection, the oocytes were collected and used for CAT assays. The graph shows the ratio of CAT activity from oocytes of four different frogs injected with Ngd or buffer (Ngd^+/Ngd^-) for both CPE-lacking and CPE-containing mRNAs. The asterisk denotes statistical difference between the groups ($P = 0.01$, Student's t test). Error bars indicate standard deviations. The bottom part shows the relative stability of ^{32}P-labeled mRNAs ($CAT\text{-}CPE^-$ and $CAT\text{-}CPE^+$) injected in the presence or absence of Ngd; the oocytes were collected 2 hours after injection.

because they are amenable to loss-of-function experiments by injection of antisense oligonucleotides. As noted previously, oocytes contain no detectable Ngd protein, nor do early embryos; by Western analysis, Ngd was detected only from the early tailbud stage (Fig. 5A). Interestingly, steady-state Maskin levels inversely correlated with Ngd; Maskin was present in oocytes and early embryos but diminished in tailbud stage embryos (Fig. 5A, left and right panels). Coupled with the functional data presented in Fig. 4, such an inverse correlation suggests that Ngd is the main regulator of CPE-dependent translation in later development.

We examined *ngd* mRNA expression in embryos by whole-mount in situ hybridization. *ngd* mRNA was weakly detected in animal pole blastomeres of the four-cell embryo and in the neural crest and neural folds of the neurula stage embryo (Fig. 5B, upper panel), specifically in the lateral cranial neural folds and in the center of the neural plate. As a control, a sense probe for Ngd never resulted in any detectable signal (data not shown). Additional in situ hybridizations with the neural crest marker *slug* and the pan-neural marker *nrp-1* (Fig. 5B, bottom panels) demonstrated that *ngd* mRNA was present not only in the spinal cord but also in the brain and eyes and in trunk cells and branchial arches, both of which are derived from the neural crest.

The injection of a morpholino-containing antisense oligonucleotide (AM) inhibits the translation of a target mRNA (61), allowing one to score for a loss-of-function phenotype. Using this approach, we first tested the efficacy of an *ngd* AM directed against the *Xenopus ngd* 5′ UTR to inhibit the translation of *ngd* mRNA in a rabbit reticulocyte lysate. As shown in Fig. 6A, the *ngd* AM completely blocked translation of full-length *ngd* mRNA in a dose-dependent manner; however, a control morpholino oligonucleotide (CM) had no effect on translation even at a relatively high concentration of 10 μM. Based on these results, embryos were injected with AM or CM and cultured until the CM-injected embryos reached the tadpole stage. The injection of *ngd* AM resulted in abnormally patterned embryos, particularly along the medial region normally occupied by the neural tube (Fig. 6B). In situ hybridization with the pan-neural marker *nrp-1* shows what appears to be a bifurcated neural tube or an unclosed neural tube in stage 25 tailbud embryos (Fig. 6B, right panel). The injected embryos were further analyzed by performing *nrp-1* in situ hybridization on sectioned material derived from paraffin-embedded samples (Fig. 6C). Compared to a CM-injected embryo that had a normal neural tube (left panel), an *ngd* AM-injected embryo displayed a neural tube that failed to close Fig. (6D). This phenotype occurred in 36% of injected embryos; CM-injected embryos showed a similar phenotype only about 2% of the time, a difference that is very highly statistically significant ($P < 0.001$, Student's t test) (Fig. 6E). To rescue the morpholino-induced phenotype, *ngd* AM was mixed with in vitro-synthesized *ngd* mRNA that lacked the region in the 5′ UTR that would anneal with the AM (to allow initiation on the proper AUG codon). Embryos injected with this mixture displayed abnormal phenotypes similar to those mentioned above only 20% of the time (Fig. 6F). This amount of rescue was statistically significant ($P < 0.05$, Student's t test).

Additional *ngd* AM-injected embryos were analyzed by in situ hybridization with *slug*, a neural crest marker. Figure 6F (CM) shows a normal pattern of *slug* RNA expression in stage 25 embryos, where hybridization was evident in the branchial arches and the trunk neural crest (35). In AM-injected embryos, the branchial arches were reduced and the migration of neural crest cells to the midline was inhibited (injected side). The percentage of AM-injected embryos that exhibited this phenotype (56%, 20 of 36 injected embryos) was much greater than that of CM-injected embryos (5%, 2 of 36 injected embryos). Moreover, when the AM was mixed with in vitro-synthesized *ngd* mRNA lacking the 5′ UTR region corresponding

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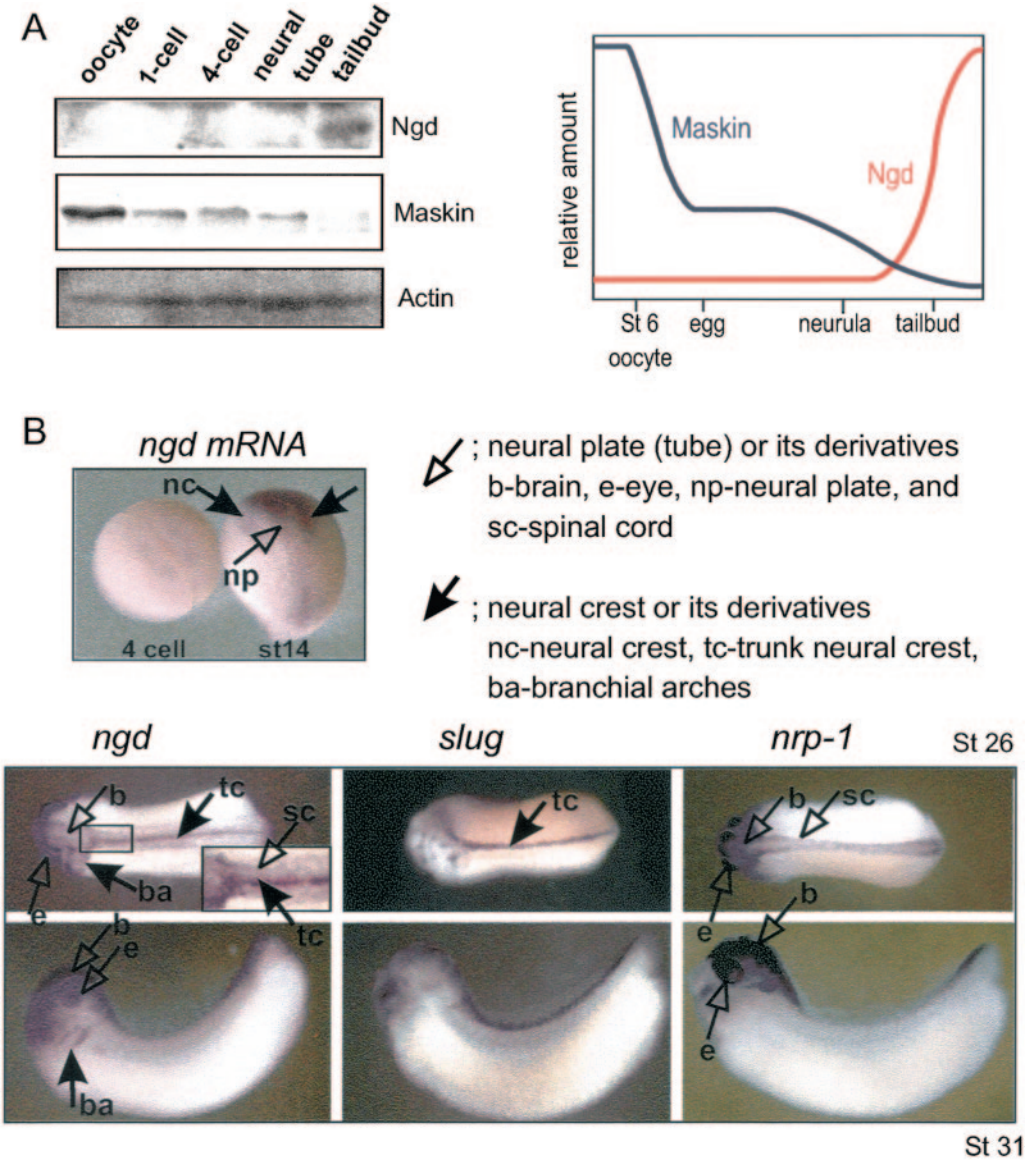


FIG. 5. Expression of Ngd in *Xenopus* embryos. (A) Western blots of protein from several stages of *Xenopus* development probed for Ngd, Maskin, and actin. The graph on the right shows the relative steady-state levels of Maskin and Ngd. (B) Whole-mount in situ hybridization for *Ngd*, *slug* (a neural crest marker), and *nrp-1* (a pan-neural marker) RNAs during *Xenopus* development.

to the annealing morpholino prior to injection, only 24% exhibited a phenotype (8 of 33 injected embryos). Taken together, these results suggest that Ngd is important not only for neural tube closure but also for the neural crest cell migration.

DISCUSSION

Short sequence elements that reside in the 3′ untranslated regions of many mRNAs modulate translational repression and activation. Although specific proteins have often been shown to bind these sequences, with few exceptions, the mechanism by which they exert their presumed translational regulatory effects is unknown. In the case of CPEB, a factor with which it associates, Maskin, is the proximal regulator of translation; its control of initiation is dependent upon a reversible association with eIF4E, the cap binding factor (4, 18, 59). Ngd is also a CPEB and eIF4E binding protein that can repress translation in a CPE-dependent manner; whether Ngd inhibition of translation is reversible, however, is not known.

Ngd is a CPE-dependent eIF4E binding protein. eIF4G and the 4EBPs (also known as PHAS I) contain a consensus motif, Y/TXXXXLΦ, where Φ denotes a hydrophobic amino acid, that allows them to compete for binding to eIF4E, thereby controlling initiation (26, 34, 24). Maskin contains this motif, as does Ngd. While the 4EBPs can repress the translation of probably most mRNAs by indiscriminately binding eIF4E, Maskin and Ngd, through tethering to CPEB, control the translation of only specific mRNAs.

Two additional proteins that contain an eIF4E binding motif have been reported to resemble Maskin and Ngd by

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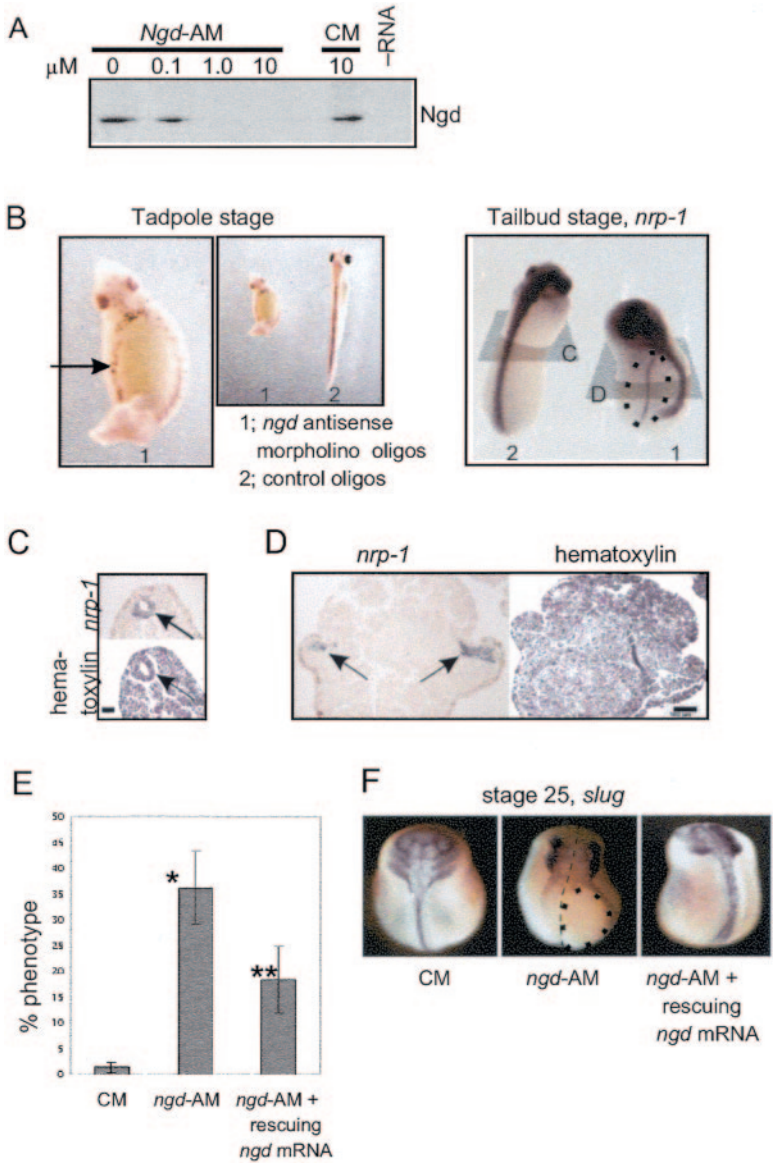


FIG. 6. Inhibition of *ngd* mRNA translation results in defects in neuronal and neural crest structures. (A) A rabbit reticuloyte translation system was primed with *Xenopus ngd* mRNA as well as a *Xenopus ngd* morpholino antisense oligonucleotide (Ngd-AM) or a control oligonucleotide (CM); the amount of Ngd synthesis was monitored by [^{35}S]methionine incorporation and SDS gel electrophoresis. (B) Dorsal views of albino embryos following injection of *ngd* antisense or control morpholino oligonucleotides; the left panels show embryo morphology, while the right panels show the pattern of *nrp-1* mRNA. A dotted circle denotes abnormally developed neural tissue. (C and D) Tailbud stage embryos were paraffin embedded, sectioned, and probed for *nrp-1* RNA. Adjacent sections were stained with hematoxylin for general morphology. Arrows denote neural tube or neural tissue. Scale bars, 50 μm in panel C and 100 μm in panel D. (E) Quantification of the *ngd* AM-induced phenotype and rescue with *ngd* mRNA. Phenotypes similar to those presented in panels B and C were induced by the Ngd AS in 36% of injected embryos, whereas the CM induced a phenotype in only 2% of injected embryos; this difference is very highly statistically significant ($P < 0.001$, Student's *t* test). Only 18% of embryos had a similar phenotype when *ngd* RNA was coinjected with AM; the difference between these samples (i.e., AM plus *ngd* RNA) is statistically significant ($P < 0.05$, Student's *t* test). Error bars indicate standard deviations. (F) AM- and CM-injected embryos were probed for *slug* RNA by whole-mount in situ hybridization. The broken line denotes the dorsal Ngd of the embryo, and the broken circle denotes an area that has an aberrant morphology and lacks *slug* RNA-positive tissue. In this assay, 56% of AM-injected embryos, 5% of CM-injected embryos, and 24% of AM- and *ngd* RNA-injected embryos (i.e., rescue) had this phenotype.

repressing specific mRNA translation through an interaction with eIF4E. One of these, *Drosophila* Bicoid, represses the translation of uniformly distributed *caudal* mRNA only in the anterior region of the embryo. Bicoid, which is itself localized to the anterior portion of the embryo, accomplishes this task not only by binding to a specific sequence in the 3′ UTR of *caudal* mRNA, the Bicoid response element (20, 51), but also by interacting with eIF4E, presumably preventing the eIF4E-eIF4G interaction (44). Because *caudal* mRNA is never (properly) translated in the anterior part of the embryo, Bicoid-mediated repression is probably not reversible. It should be noted, however, that a recent study indicates that

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Bicoid interacts not with eIF4E but with another cap binding factor called 4EHP (12).

In another example in *Drosophila*, *nanos* mRNA is distributed throughout the embryo but is more concentrated at the anterior pole (21, 22, 63), where Nanos protein represses the translation of *hunchback* mRNA (21, 65). Unlocalized *nanos* mRNA is translationally repressed; this repression requires 3′ UTR stem-loop structures known as Smaug response elements (13, 14, 56, 57). Smaug not only binds these elements but also associates with Cup, a protein that in turn interacts with eIF4E and represses translation (43). Cup is thus functionally similar to Maskin and Ngd. Along these same lines, Cup interacts with Barentz, a protein that indirectly associates with and represses *oskar* mRNA translation in the anterior region of the embryo (66). Finally, Cup association with the RNA binding protein Bruno represses *oskar* mRNA translation during oogenesis (42). It is not known whether Cup-mediated translational repression is reversible.

Ngd in neurons. In mammalian neurons, Ngd is detected as puncta in both axons and dendrites but also, perhaps most interestingly, in growth cones and filopodia. Moreover, in growth cones from neurons cultured for 3 days, Ngd is colocalized with eIF4E (Fig. 3), suggesting that the translation of one or more mRNAs is regulated at that time in that location. While neurites at this stage have not yet differentiated into axons or dendrites, there is little doubt that young growth cones have the capacity to synthesize protein. Indeed, the detection of polysomes by electron microscopy (16) and the incorporation of radiolabeled amino acids into protein into isolated growth cones (15) demonstrate that translation does occur in this structure. Moreover, axon turning in vitro in response to exogenous cures also requires protein synthesis, although it is not known if this takes place in the growth cone, the axon shaft, or both (69). Finally, growth cone motility is reduced if β-actin mRNA is prevented from localizing to this region (69), implying that local actin synthesis is necessary growth cone extension. Such results lead us to suggest that this protein might mediate mammalian growth cone movement by regulating local mRNA translation.

Filopodia are thin projections emanating from either dendritic shafts or growth cones. While the function of either of these structures is not known, the former have been suggested to constitute precursors to synapses or synaptic spines, while the latter may be involved in activity-dependent growth and/or branching (45). Ngd immunoreactivity is often associated with shaft filopodia (Fig. 2), suggesting that regulated mRNA translation may occur in these structures. Given this result, it is perhaps surprising that Ngd is not detected in mature synapses (Fig. 2F), which do support protein synthesis (3, 53). On the other hand, Maskin immunoreactivity is detected at synapses (28), perhaps suggesting the replacement of one of these proteins with the other.

Ngd control of neural development. In contrast to the case for Maskin, no Ngd is detected in *Xenopus* oocytes and embryos, although they do contain *ngd* mRNA. In the mouse, Ngd is first detected at E9 at the roof of the hindbrain and near the somites (data not shown); in *Xenopus*, *ngd* RNA is clearly observed at stage 25 in the embryonic nervous system, including the brain, spinal cord, and dorsal root ganglia. Because Ngd protein does not appear to be maternally inherited and because the embryonic expression profile suggests that *ngd* mRNA is prevalent in the nervous system (central nervous system and neural crest), we have injected morpholino-containing antisense oligonucleotides in an attempt to generate an Ngd loss-of-function mutant embryo. The inhibition of Ngd expression resulted in an unclosed neural tube, as shown by using the pan-neural marker *nrp-1* (Fig. 6). Importantly, the mutant phenotype was often rescued when an *ngd* mRNA that cannot anneal to the oligonucleotide was coinjected. In a similar vein, neural crest cells, identified by the marker *slug*, did not properly migrate in embryos injected with the Ngd antisense oligonucleotide. As before, most embryos were rescued to the wild-type phenotype when an *ngd* RNA that cannot anneal to the oligonucleotide was coinjected.

Our challenge is to identify mRNA targets whose translation is misregulated in the *ngd* antisense oligonucleotide-injected embryos. Possible targets include those that are involved in anterior-posterior patterning of the developing nervous system, such as fibroblast growth factor, Wnts, and mesodermal retinoids (8, 41). Determining which mRNA(s) is regulated by Ngd in embryos is a difficult task, since it will be essential to differentiate primary targets versus those that might only secondarily be affected. We are therefore exploring several approaches to identify mRNAs that reside in a complex together with Ngd.

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