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Neurabin is a synaptic protein linking p70 S6 kinase and the neuronal cytoskeleton

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ABSTRACT **p70 S6 kinase ($p70^{S6k}$) is a mitogen-activated protein kinase that plays a central role in the control of mRNA translation. It physiologically phosphorylates the S6 protein of the 40s ribosomal subunit in response to mitogenic stimuli and is a downstream component of the rapamycin-sensitive pathway, which includes the 12-kDa FK506 binding protein and includes rapamycin and the 12-kDa FK506 binding protein target 1. Here, we report the identification of neurabin (neural tissue-specific F-actin binding protein), a neuronally enriched protein of 1,095 amino acids that contains a PDZ domain and binds $p70^{S6k}$. We demonstrate the neurabin-$p70^{S6k}$ interaction by yeast two-hybrid analysis and biochemical techniques. $p70^{S6k}$ and neurabin coimmunoprecipitate from transfected HEK293 cells. Site-directed mutagenesis of neurabin implicates its PDZ domain in the interaction with $p70^{S6k}$, and deletion of the carboxyl-terminal five amino acids of $p70^{S6k}$ abrogates the interaction. Cotransfection of neurabin in HEK293 cells activates $p70^{S6k}$ kinase activity. The mRNA of neurabin and $p70^{S6k}$ show striking colocalization in brain sections by *in situ* hybridization. Subcellular fractionation of rat brain demonstrates that neurabin and $p70^{S6k}$ both localize to the soluble fraction of synaptosomes. By way of its PDZ domain, the neuronal-specific neurabin may target $p70^{S6k}$ to nerve terminals.**

A common characteristic of mitogenic signals is an expedient up-regulation of translation to support the concomitant increase in transcriptional activity (1). The precise nature and mechanism of this translational activation has not been established. However, one important aspect is the dramatic increase in ribosomal phosphorylation, particularly on the S6 protein of the 40s subunit, and the resulting alterations in the ribosome's affinity for certain abundant mRNAs with a polypyrimidine tract in their 5′-untranslated region (2). The kinase that physiologically performs this phosphorylation is the 70-kDa S6 kinase ($p70^{S6k}$), a member of the protein kinase C family of serine/threonine kinases (3).

Regulation of $p70^{S6k}$ is complex, and many signal transduction molecules such as phosphoinositide 3-kinase, phosphoinositide-dependent kinase 1, cdc2, and rapamycin and 12-kDa FK506 binding protein (FKBP12) target 1 (RAFT1) are implicated in its control (4). However, activation of $p70^{S6k}$ by all stimuli can be inhibited by rapamycin, an immunosuppressant macrolide antibiotic related to FK506 (3). Recent work has characterized the rapamycin-sensitive signaling process that influences crucial components of the translational machinery, especially eukaryotic initiation factor-4E binding proteins 1 and 2 (5), elongation factor 2 (6), and $p70^{S6k}$. Pharmacologic intervention in this signaling system is initiated by the immunophilin FKBP12, which is a cytosolic receptor protein for FK506 and rapamycin. In the presence of rapamycin, FKBP12 binds to and perturbs the function of RAFT1 (7), also referred to as FKBP12 rapamycin associated-protein (FRAP) (8) or mammalian target of rapamycin (mTOR). Recently, we found that RAFT1 directly phosphorylates $p70^{S6k}$ on Thr-389 (9).

Seeking novel proteins that might regulate $p70^{S6k}$, we performed yeast two-hybrid analysis. We now report a neuronally enriched protein whose PDZ domain interacts with $p70^{S6k}$. This protein has been identified independently as an actin-binding protein and designated neurabin (neural tissue-specific F-actin binding protein) (10).

MATERIALS AND METHODS

Plasmids and Fusion Proteins. cDNA for the full-length rat $p70^{S6k}$ and the segment coding for amino acids 332–502 were amplified from p85 in Pmt2 by using PCR with appropriate primers. cDNA for rat AKT was amplified from AKT pCDNA by using PCR with appropriate primers. The amplified products were cloned into the *Sal*I and *Not*I sites of hemagglutinin (HA)-pRK5, myc-pRK5, pPC86, pPC97 (11), or pGEX-4T2 (Pharmacia). All cDNAs prepared with PCR were verified by DNA sequencing. Mutations in $p70^{S6k}$ and neurabin cDNAs were generated via PCR mutagenesis (12).

Yeast Two-Hybrid Screen. The cDNA corresponding to amino acids 332–502 in $p70^{S6k}$ was cloned into yeast expression vector pPC97 containing the GAL4 DNA binding domain and was used to screen a rat hippocampal cDNA library cloned into pPC86 (13) containing the GAL4 transactivation domain. The plasmids were introduced sequentially by LiAc-mediated transformation (11) into the HF7C yeast strain (CLONTECH). A total of 1.0×10^7 independent clones were screened, and positive interacting proteins were identified by selecting for histidine prototrophy. Positive clones were further evaluated for β-galactosidase expression by nitrocellulose filter lift assays as described (11).

Transient Transfections and Cell Treatments. HEK293 cells plated on 10-cm dishes and grown in media A (DMEM supplemented with 2 mM L-glutamine, 100 units/ml penicillin G, 100 mg/ml streptomycin sulfate, and 10% fetal bovine serum) were transfected with the calcium phosphate precipitate method (14). Unless otherwise noted, 10 μg of each cDNA was transfected. Cells were lysed 24 h after transfection or made quiescent by rinsing the plate once with PBS and then incubating for 24 h in media A lacking fetal bovine serum. Where indicated, cells were treated with 10 nM rapamycin (Calbiochem) or ethanol vehicle for 30 min before stimulation with 10% fetal bovine serum for 1 h. Cells were washed once

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Abbreviations: FKBP12, 12-kDa FK506 binding protein; RAFT1, rapamycin and FKBP12 target 1; GST, glutathione *S*-transferase; HA, hemagglutinin.

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in PBS before lysis in 1 ml of lysis buffer (50 mM Tris·KOH, pH 7.4/40 mM NaCl/1 mM EDTA/0.5% Triton X-100/1.5 mM Na_3VO_4/50 mM NaF/10 mM sodium pyrophosphate/10 mM sodium β-glycerophosphate/1 mM phenylmethylsulfonyl fluoride/5 mg/ml aprotinin/1 mg/ml antipain/1 mg/ml leupeptin/1 mg/ml chymostatin/0.7 mg/ml pepstatin A).

Antibodies and Immunoblots. Anti-hemagglutinin (HA) antibody (Babco, Richmond, CA) and anti-myc 9E-10 antibody (Calbiochem) were used to detect and immunoprecipitate epitope-tagged proteins. A rabbit polyclonal antibody was raised against glutathione *S*-transferase (GST)-neurabin amino acids 486–751. The antiserum was affinity-purified as described (15). Protein samples were diluted in SDS sample buffer, were boiled for 3 min, were resolved on SDS/PAGE, were transferred in 3-[cyclohexylamino]-L-propane-sulfonic acid (CAPS)-methanol buffer (pH 11) to poly(vinylidene difluoride), and were probed with primary antibodies. Western blots were developed with anti-mouse or anti-rabbit antibodies (Amersham) and chemiluminescence (NEN).

Rat Brain cDNA Library Screen and Probe Generation. A random primed rat brain cDNA library in λZAPII (Stratagene) was screened per manufacturer's protocol. The probe was generated from the neurabin two-hybrid clone and was nick translated (GIBCO) in the presence of [^{32}P]α dATP and dCTP (NEN).

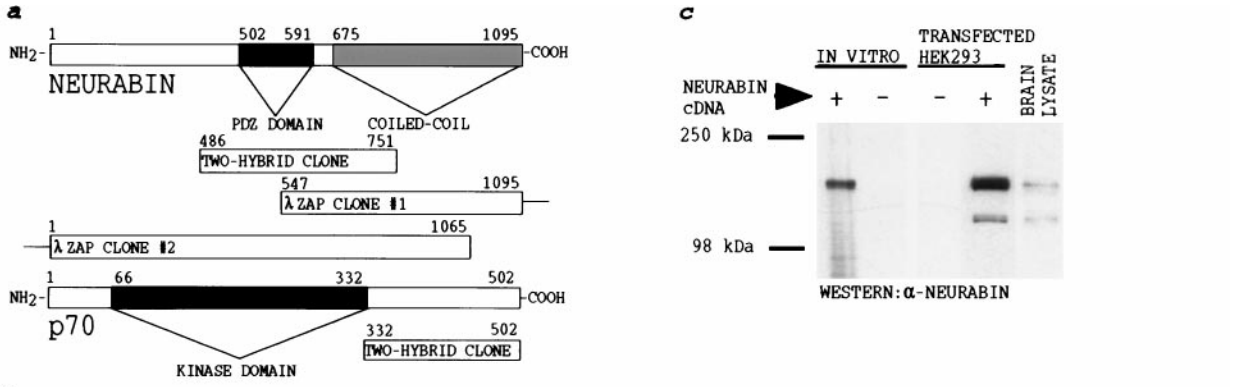
***In Vitro* Binding Experiments.** Purified GST and GST-neurabin (amino acids 486–751) glutathione-conjugated agarose (Sigma) were prepared. Twenty-four hours after transfection with 10 μg of HA-p70^{S6k} cDNA, a 10-cm plate of HEK293 cells was washed once in PBS, was lysed in 1 ml lysis buffer, and was centrifuged for 10 min at 14,000 × *g*. From this, 500 μl of supernatant were added to 20 μg of GST or GST-neurabin agarose, were incubated with slow rotation for 1 h, and were washed three times with lysis buffer containing the indicated concentration of NaCl. The agarose then was resuspended in 25 μl of concentrated sample buffer separated by SDS/PAGE followed by immunoblot using the anti-HA antibody. Equal loading of GST fusion proteins was confirmed with Coomassie blue staining.

Coimmunoprecipitation of p70^{S6k} and Neurabin. A 10-cm dish of HEK293 cells was cotransfected with 5 μg each of myc-neurabin (amino acids 486–751) cDNA and either HA-p70^{S6k} or AKT cDNA. The supernatant, prepared as above, was combined with 2 μl of anti-HA antiserum and 40 μl of 50% slurry protein G agarose (Calbiochem) and was incubated with rotation at 4°C for 3 h. The agarose pellet was washed three times with lysis buffer containing the indicated concentration of NaCl. The pellet then was diluted in 25 μl of SDS sample buffer and bound protein separated by SDS/PAGE followed by immunoblot using the anti-myc antibody.

Northern Blot. A tissue mRNA blot membrane (CLONTECH) was hybridized with the neurabin probe and was washed according to the manufacturer's protocol.

Tissue Western Blot. Homogenates were prepared by glass/teflon homogenization of adult rat tissues in lysis buffer. A portion (20 μg) of protein from each tissue was separated by SDS/PAGE followed by immunoblot.

***In Vitro* Coupled Transcription/Translation.** A rabbit reticulocyte lysate coupled *in vitro* transcription and translation system (Promega) was used per manufacturer's protocol to express and translate full-length neurabin mRNA from pRK5 by using the SP6 RNA polymerase. A portion (20 μl) of the reaction was separated by SDS/PAGE followed by immunoblot.



b

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                                                                      100
MLKAESSGER TTLRSASPHR NAYRTEFQAL KSTFDKPKPD GEQKTKEGEG SQQSRGRKYG SNVNRIKNLF MQMGMEPNEN AAIIAKTRGK GRPSSPQKRM
                                                                      200
KPKEFVEKTD GSVVKLESSV SERISRFDTM HDGPSYAKFT ETRKMFERSG HESGQNNRHS PKKEKAGEAE PQDEWGGSKS NRGSSDSLDS LSPRTEAVSP
                                                                      300
TVSQLSAVFE NSESPGAITP GKAENSNYSV TGHYPLNLPS VTVTNLDTFG RLKDSNSRPS SNKQATDTEE PEKSEAVPVP EVAQKGTSLA SLPSEERQLS
                                                                      400
TEAEDVTAQP DTPDSTDKDS PGEPSAESQA MPKSNTLSRP KEPLEDAEAN VVGSEAEQPQ RRDLTGGGDL TSPDASASSC GKEVPEDSNS FEGSHVYMHS
                                                                      500
DYNVYRVRSR YNSDWGETGT EQDEGDDSDE NNYYQPDMEY SEIVGLPQEE EIPANRKIKF SCAPIKVFNT YSNEDYDRRN DDVDPVAASA EYELEKRVEK
                                                                      600
LELFPVELEK DEDGLGISII GMGVGADAGL EKLGIFVKTV TEGGAAQRDG RIQVNDQIVE VDGISLVGVT QNFAATVLRN TKGNVRFVIG REKPGQVSEV
                                                                      700
AQLISQTLEQ ERRQRELLER HYAQYDADDD ETGEYATDEE EDEVGPILPG GDMAIEVFEL PENEDMFSPS DLDTSKLSHK FKELQIKHAV TEAEIQKLKT
                                                                      800
KLQAAENEKV RWELEKNQLQ QNIEENKERM VKLESYWIEA QTLCHTVNEH LKETQSQYQA LEKKYNKAKK LIKDFQQKEL DFIRRQEVER KKLEEVEKAH
                                                                      900
LVEVQGLQVR IRDLEAEVFR LLKQNGTQVN NNNNIFERRP SPGEVSKGDT MENVEVKQTS CQDGLSQDLN EAVPETERLD SKALKTRAQL SVKNRRQRPT
                                                                     1000
RTRLYDSVSS TDGEDSLERK NFTFNDDFSP SSTSSADLSG LGAEPKTPGL SQSLALSSDE SLDMIDDEIL DDGQSPKHTQ SQSRAVHEWS VQQVSHWLVG
                                                                     1095
LSLDQYVSEF SAQNISGEQL LQLDGNKLKA LGMTSSQDRA LVKKKLKEMK MSLEKARKAQ EKMEKQREKL RRKEQEQMQR KSKKSEKMTS TTEQP
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FIG. 1. Cloning and expression of neurabin cDNA. (*a*) Structure of neurabin and p70^{S6k}. The region of p70^{S6k} used to screen the two-hybrid library and the region of neurabin identified in the two-hybrid screen are indicated. Also shown are the two neurabin clones (λ ZAP #1 and #2) recovered from a rat brain library. These contain the entire ORF of the neurabin protein. (*b*) Deduced amino acid sequence from the neurabin cDNA. The PDZ domain is underlined, and the conserved amino acids Gly-Leu-Gly-Ile, which were mutated to Gly-Leu-Ala-Ala, are in bold print. (*c*) Expression of neurabin cDNA. Neurabin cDNA in pRK5 either was transcribed and translated *in vitro* or was transfected into HEK293 cells. Both generated protein that comigrates with the neurabin protein from brain lysate.

Table 1. Two-hybrid analysis of p70 and neurabin

Binding domain fusion protein (pPC97)	Activation domain fusion protein (pPC86)	Interaction assessed by β-gal filter assay	Interaction assessed by histidine prototrophy
p70 (amino acids 332–502)	neurabin (amino acids 486–751)	+	+
neurabin (amino acids 486–751)	p70 (amino acids 332–502)	+	+
p70 (amino acids 332–502)	cyclophilin	−	−
cyclophilin	neurabin (amino acids 486–751)	−	−
p70 (amino acids 332–502)	neurabin-GLAA (amino acids 486–751)	−	−
p70 Δ5 (amino acids 332–497)	neurabin (amino acids 486–751)	−	−

p70^{S6k} *In Vitro* Kinase Assay. A 10-cm dish of HEK293 cells was transfected with 100 ng of the HA-p70^{S6k} cDNA, either wild type or mutant, and supernatant prepared as above. HA-p70^{S6k} was immunoprecipitated with 1 μl of anti-HA antiserum and 40 μl of a 50% slurry of protein G agarose, washed as described (9). Kinase assays were performed on immunoprecipitates as described (16).

***In Situ* Hybridization.** The digoxigenin cRNA probes corresponding to amino acids 332–502 of p70^{S6k} and 486–751 of neurabin were generated and hybridized to 20-μm sections as described (17).

Subcellular Fractionation. Brains from five male adult rats were homogenized in 100 ml of 0.32 M sucrose with a glass/teflon homogenizer. Homogenate was centrifuged for 10 min at 800 × *g* to give pellet (P1) and supernatant (S1). S1 was centrifuged for 15 min at 9,200 × *g* to give pellet (P2) and supernatant (S2). S2 was centrifuged for 90 min at 100,000 × *g* to give pellet (P3) and supernatant (S3). The P2 fraction was resuspended in 3 ml of 0.32 M sucrose and hypotonically lysed in 27 ml of ice-cold water. Lysate was homogenized with a glass/teflon homogenizer. Hepes (2 M; pH 7.4) was added to a final concentration of 50 mM and was centrifuged for 20 min at 25,000 × *g* to give pellet (LP1) and supernatant (LS1). LS1 was centrifuged for 90 min at 165,000 × *g* to give pellet (LP2) and supernatant (LS2). Protein concentration of the fractions was determined, and 20 μg of protein from each fraction was separated by SDS/PAGE followed by immunoblot.

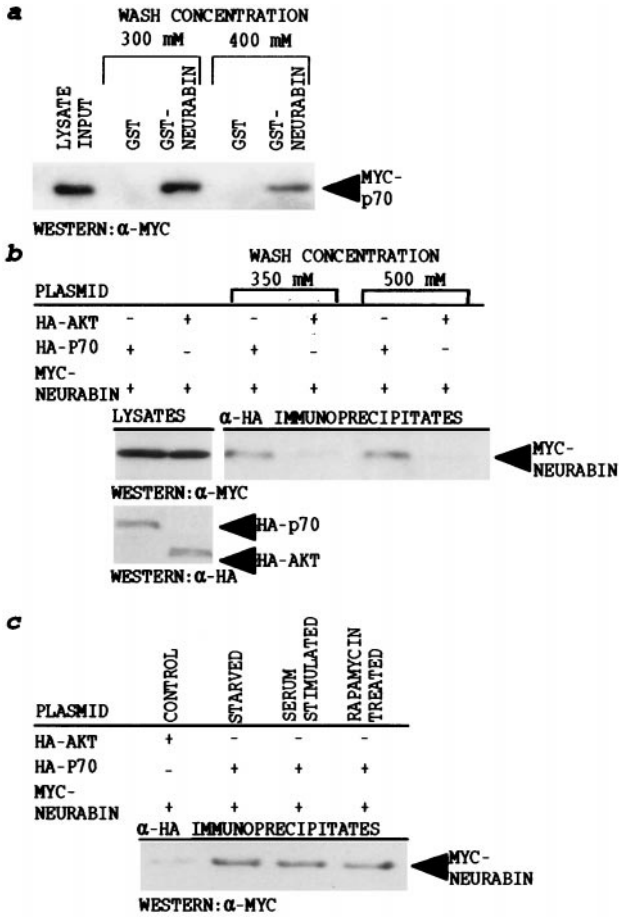

FIG. 2. Neurabin interacts with p70^{S6k} both *in vitro* and *in vivo*. (*a*) *In vitro* binding of myc- p70^{S6k} from transfected HEK293 cells to a GST or GST-neurabin (amino acids 486–751) column. Salt concentrations of washes are indicated. (*b*) Coimmunoprecipitation of myc-neurabin (amino acids 486–751) with HA- p70^{S6k} but not HA-AKT (*Upper*). Equal transfection of HA- p70^{S6k} and HA-AKT is confirmed by Western blot (*Lower*). Salt concentrations of washes are indicated. (*c*) Interaction of p70^{S6k} with neurabin is phosphorylation-independent. Coimmunoprecipitation of neurabin and p70^{S6k} is not affected by 24-h serum starvation, serum stimulation, or rapamycin treatment.

RESULTS

Identification of Neurabin as a p70^{S6k} Interacting Protein. Although p70^{S6k} is known to phosphorylate the S6 protein of ribosomes, its full role in protein translation and the regulatory mechanisms that modulate its activity are not understood fully. The phosphotransferase activity of p70^{S6k} depends on phosphorylation events on serine and threonine residues, a number of which are located in the C terminus (4). Structure/function studies of p70^{S6k} demonstrate the C terminus to be required for physiologic regulation of kinase activity through a mechanism that has not been defined (18). To examine a possible regulatory role of protein–protein interactions mediated by the densely phosphorylated region of p70^{S6k} distal to the kinase domain (amino acids 332–502) (Fig. 1*a*), we performed a yeast two-hybrid analysis using a rat hippocampal cDNA library and found an interacting protein (Table 1). The 800-bp sequence initially identified contains a PDZ domain (Fig. 1*a*). A rat brain cDNA library was screened by using the neurabin two-hybrid clone as a probe. Eighteen clones were recovered, but the full-length cDNA of neurabin was assembled from two overlapping clones containing 4,450 nucleotides. The starting methionine is assigned to the first in-frame AUG codon preceded by an upstream stop codon. The 3.3-kilobase ORF codes for a protein of 1,095 amino acids with a predicted molecular mass of 120 kDa, which, outside of the PDZ domain, shows no sequence homology to any known protein (Fig. 1*b*). Independently, Nakanishi *et al.* (10) identified the same protein based on its actin-binding properties and called it neurabin.

Using a rabbit reticulocyte system, we demonstrated *in vitro* transcription and translation of neurabin that appears as a

Table 2. Neurabin transfection enhances p70 kinase activity

p70 construct	Neurabin construct	Kinase activity (arbitrary units)
HA-p70	Neurabin	147 ± 13.3*
HA-p70	Neurabin–Gly-Leu-Ala-Ala	100
HA-p70 Δ5	Neurabin	97 ± 5.3
HA-p70 Δ5	Neurabin–Gly-Leu-Ala-Ala	100

Kinase activity normalized to neurabin–Gly-Leu-Ala-Ala mutant control.
*Significant value $P < 0.015$, paired Student's *t* test.

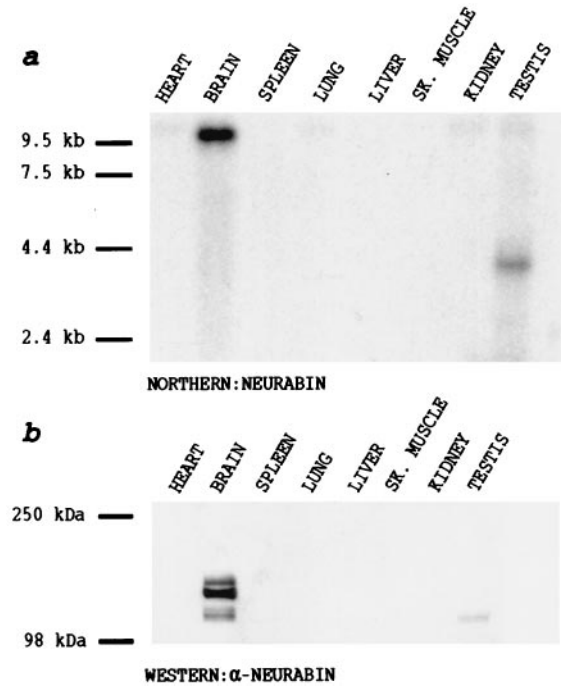

FIG. 3. Tissue distribution of neurabin mRNA and protein in rat. (*a*) Northern blot of poly-A mRNA hybridized with radiolabeled neurabin probe. (*b*) Western blot probed with anti-neurabin antibody.

180-kDa single band in Western analysis with an anti-neurabin antibody (Fig. 1*c*). Transfection of HEK293 cells with neurabin cDNA reveals the same 180-kDa band as well as a smaller 140-kDa band whose relative abundance varies in different experiments. Brain lysates also display the same two bands with variable expression of the 140-kDa protein. Because of the variable appearance of the 140-kDa band and its absence in the *in vitro* protein synthesis study, we suspect that the 140-kDa band is a degradation product of the 180-kDa protein. Nakanishi *et al.* (10) also observed the 180- and 140-kDa bands for neurabin. Based on amino acid sequence, the predicted molecular mass for neurabin should be ≈120 kDa. Reasons for the larger size in SDS/PAGE analysis are not clear.

The association of neurabin and p70^{S6k} is confirmed in a number of different systems. We demonstrate selective binding of myc-p70^{S6k} to a GST-neurabin column (Fig. 2*a*). To assess whether this interaction occurs in intact cells, we immunoprecipitated HA-tagged p70^{S6k} from HEK293 cells cotransfected with myc-tagged neurabin. Western blotting with anti-myc antibody shows coimmunoprecipitation of myc-neurabin with HA-p70^{S6k} but not HA-AKT, a protein kinase of similar size (Fig. 2*b*). The C-terminal region of p70^{S6k} is phosphorylated by a number of kinases subsequent to serum stimulation of cells, and many of these phosphorylation events can be reversed with rapamycin treatment. To ascertain whether these events influence the neurabin-p70^{S6k} interaction, we examined the effects of serum deprivation, serum stimulation, and rapamycin treatment on coimmunoprecipitation and found no difference among these conditions (Fig. 2*c*).

The PDZ Domain of Neurabin Binds the C Terminus of p70^{S6k}. The PDZ domains of proteins typically bind to the C terminus of partner proteins through a broadly defined consensus sequence. Initial studies of PDZ domain containing proteins such as PSD-95 and related proteins revealed an apparent consensus of Thr/Ser-X-Val-COOH (with X indicating any amino acid) for the PDZ binding substrate. On examination of a more representative subset of PDZ domains, Cantley and associates (19) have described a broader range of permissible C terminal amino acids. The Leu-Arg-Met-Asn-Leu-COOH terminus of p70^{S6k} is consistent with this more inclusive concept of a PDZ binding partner, and deletion of these amino acids prevents p70^{S6k}'s binding to neurabin in yeast two-hybrid analysis (Table 1).

Although PDZ domains are ≈100 amino acids in length, homology between the multitude of recently identified proteins that contain this motif has identified amino acids that are conspicuously conserved: e.g., the Gly-Leu-Gly-Phe residues in the carboxylate binding loop (20). This portion of the PDZ domain of neurabin contains the four amino acids Gly-Leu-Gly-Ile (Fig. 1*c*), and mutation to Gly-Leu-Ala-Ala abolishes binding to p70^{S6k} (Table 1). Thus, this region identified in neurabin by ourselves and others (10) appears to be a functional PDZ domain that mediates the neurabin-p70^{S6k} interaction.

The Kinase Activity of p70^{S6k} is Regulated by Interactions with Neurabin. To determine whether binding to neurabin is important for phosphotransferase activity of p70^{S6k}, we assayed the ability of p70^{S6k} to phosphorylate synthetic substrate peptides under various conditions (Table 2). In HEK293 cells transfected with HA-p70^{S6k} cDNA, deletion of the C-terminal five amino acids of p70^{S6k} reduces kinase activity by 75% but

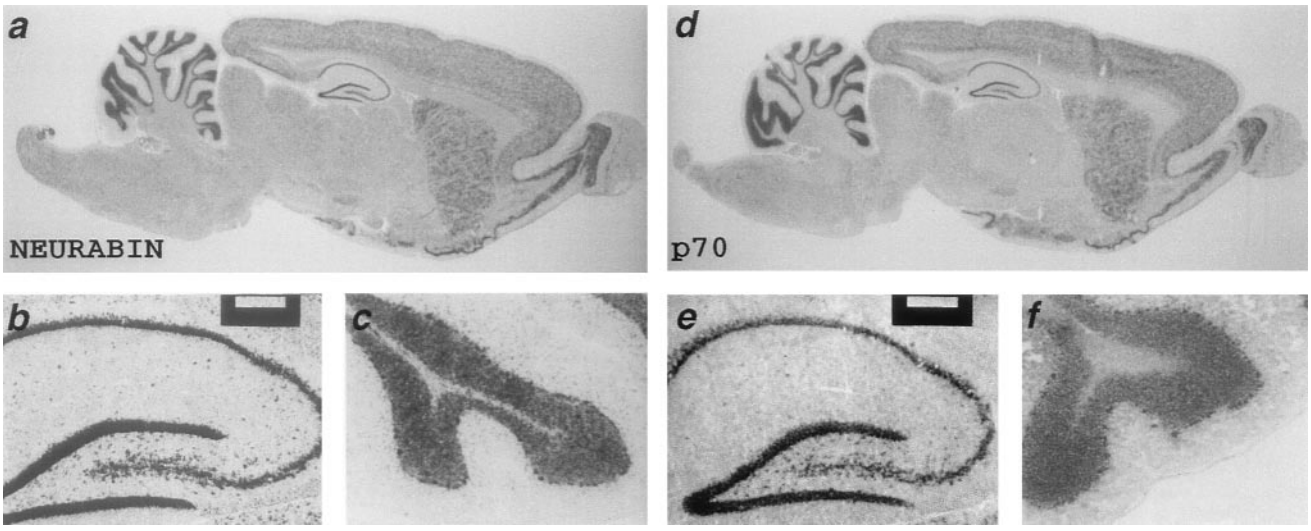

FIG. 4. *In situ* hybridization of neurabin and p70^{S6k} mRNA. Sagittal rat brain sections showing (*a*) neurabin mRNA and (*d*) p70^{S6k} mRNA. Higher magnification of (*b*) neurabin and (*e*) p70^{S6k} in the hippocampus and (*c*) neurabin and (*f*) p70^{S6k} message in the cerebellum.

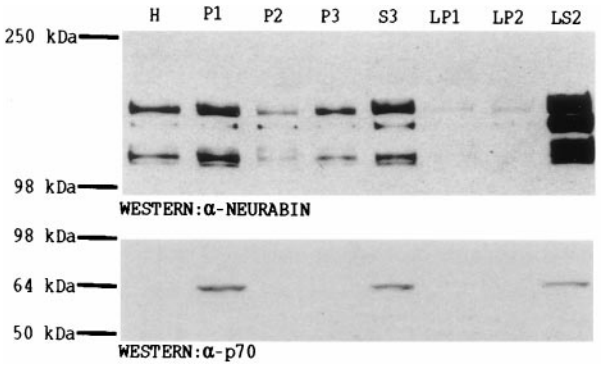

FIG. 5. Neurabin and p70^{S6k} are found in the soluble fraction of synaptosomes from rat brain. Fractions are labeled as described in *Materials and Methods*: H, homogenate; P, pellet; and S, supernatant.

does not abolish normal serum activation and rapamycin sensitivity (data not shown).

We directly evaluated the influence of neurabin on the phosphotransferase activity of p70^{S6k} by cotransfecting HEK293 cells with myc-neurabin and HA-p70^{S6k} cDNAs, then assaying the kinase activity of HA immunoprecipitates (Table 2). Neurabin cotransfection produces a 50% augmentation of p70^{S6k} activity. Transfection of the cells with neurabin in which the PDZ domain has been mutated (Gly-Leu-Gly-Ile to Gly-Leu-Ala-Ala) to prevent binding to p70^{S6k} abolishes the stimulation. This augmentation is not found in cells coexpressing wild-type myc-neurabin and the p70^{S6k} mutant lacking the C-terminal five amino acids (Table 2).

Neurabin is Brain Selective with Regional and Subcellular Localizations Closely Resembling p70^{S6k}. In the brain, Northern analysis revealed a prominent band for neurabin mRNA of ≈9.5 kilobases (Fig. 3*a*). Testis displayed a faint band at ≈4.2 kilobases. Western blot analysis revealed brain selectivity for neurabin that was visualized as a doublet consisting of a stable band at 180 kDa and a more variably represented band at 140 kDa (Fig. 3*b*). No immunoreactivity was observed in a variety of other peripheral tissues except for testis, which displayed a 120-kDa band.

In situ hybridization revealed very similar localizations for the mRNA of neurabin and p70^{S6k} (Fig. 4). Highest expression of both occurred in granule cells of the cerebellum and in the hippocampus, with levels greater in the dentate gyrus than in pyramidal cells. Neurabin mRNA also was enriched in the striatum and olfactory bulbs, both main and accessory. One discrepancy between the two proteins involved the substantial expression of neurabin in the thalamus, where p70^{S6k} message levels were modest.

Subcellular fractionation revealed neurabin protein greatly enriched in the soluble portion of both cytosolic and synaptosomal fractions (Fig. 5). p70^{S6k}, a soluble cytosolic protein, also was enriched in the soluble portion of synaptosomes.

DISCUSSION

Our findings establish neurabin as a nervous system-selective protein that interacts with p70^{S6k}. Although neurabin is most concentrated in the brain, it occurs in testis, and low levels of mRNA can be detected in most tissues (data not shown). This may reflect localization of neurabin to neuronal components of these tissues. *In situ* hybridization showed substantial colocalization of neurabin and p70^{S6k} mRNA in the brain.

Mutagenesis analysis suggests that interaction of neurabin with p70^{S6k} is through its PDZ domain located in the middle portion of the protein. Because p70^{S6k} does not share homology with PDZ domain containing proteins, and because deletion of the C-terminal five amino acids of p70^{S6k} abrogates binding, this interaction is an example of heterotypic binding of a PDZ domain to the C terminus of a substrate protein. In addition, stimulation of p70^{S6k} kinase activity by neurabin requires a functional PDZ domain.

Nakanishi *et al.* (10) independently identified neurabin as an F-actin binding protein. Neurabin binds to F-actin through a unique N-terminal domain that targets the protein to the cytoskeletal compartment. This actin-binding domain is some distance from the PDZ domain, so actin and p70^{S6k} may bind independently.

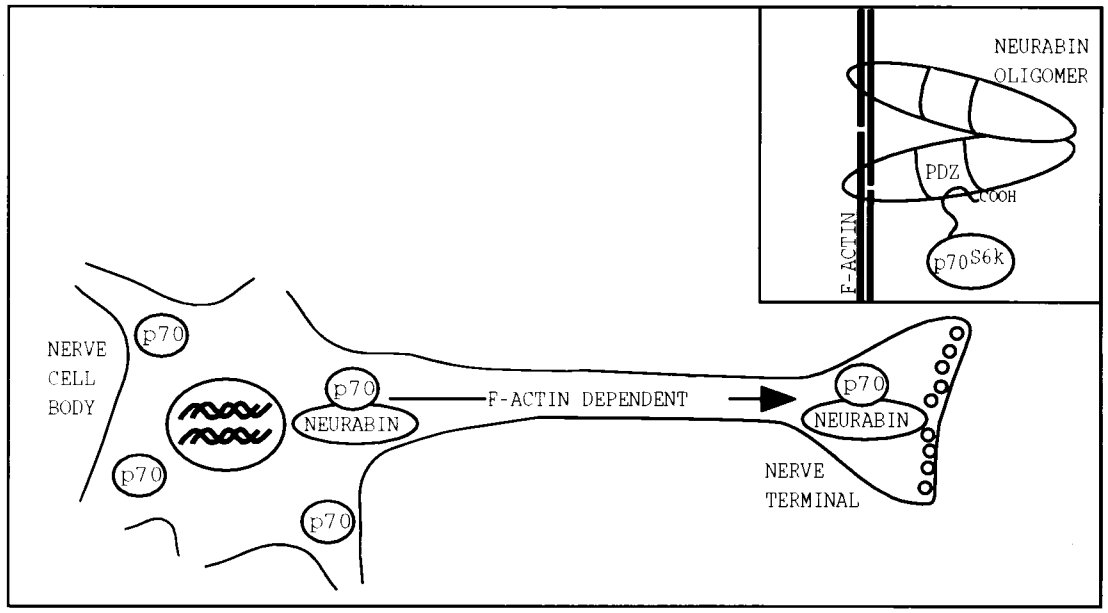

FIG. 6. Model of p70^{S6k} regulation and targeting by neurabin. In nerve cell bodies, neurabin binds cytosolic p70^{S6k}. Subsequently, neurabin binds F-actin and is targeted to nerve terminals. p70^{S6k} kinase activity is enhanced by its interaction with neurabin, but its function at the synapse is unknown. Kinase activity may be regulated in response to mitogenic stimuli or possibly neuronal activity. (*Inset*) Neurabin oligomers bind the C terminus of p70^{S6k} via a single PDZ domain and bind F-actin with a unique N-terminal actin binding region. Oligomerization is potentially mediated by coiled–coiled structure at the C terminus of neurabin.

How might the interaction of neurabin and $p70^{S6k}$ influence neuronal physiology? The extension of processes is an essential neuronal function involving actin and actin-associated proteins that regulate cytoskeletal function. During development, neuronal growth cones respond to environmental clues to find their proper synaptic targets. The central role of actin and actin binding proteins in growth-cone formation, and guidance has been documented well (21). A role for neurabin in growth-cone function is suggested by its ability to bind actin and its localization to the synapse. This role is confirmed by the disruption of neurite outgrowth in hippocampal cultures by neurabin antisense oligonucleotides (10).

Rapamycin, which potently inhibits $p70^{S6k}$ kinase activity via RAFT1, is neurotrophic; it stimulates neurite outgrowth in a variety of systems at low nanomolar concentrations (22, 23). A number of immunophilin ligands, including the immunosuppressant FK506, which also binds to FKBP12, are neurotrophic (22). Although the immunosuppressant actions of FK506 derive from the binding of the FK506–FKBP12 complex to the calcium-activated phosphatase calcineurin, the neurotrophic activity appears to be mediated by an undetermined calcineurin-independent process (22, 24). Because RAFT1 and $p70^{S6k}$ are not involved in actions of FK506, the various drugs might exert their neurotrophic actions via differing molecular mechanisms, in which case rapamycin could be neurotrophic via RAFT1, $p70^{S6k}$, and neurabin. Consistent with this possibility are features of neurotrophic actions of rapamycin that differ from those of FK506 (23).

The localization and function of $p70^{S6k}$ and other regulators of protein translation have not been studied well in neurons. Although dendritic mRNA transport and translation have been established (25), the case for translational apparatuses in nerve endings is less certain. The observation of polyribosomes, mRNA, and local protein synthesis in the axons of model systems such as the squid and goldfish suggests the existence of a similar capability in mammals (26). This protein synthetic capacity would require the targeting of regulatory components of the translational machinery to nerve endings. A function for $p70^{S6k}$ outside of its role in translational control is suggested by its phosphorylation of the cAMP response element modulator (27). Therefore, the study of $p70^{S6k}$ in neurons may reveal extratranslational and potentially neuronal specific roles for this enigmatic kinase at the intersection of numerous disparate signal transduction pathways.

We present a model that explores the role of neurabin and $p70^{S6k}$ in neurite outgrowth (Fig. 6). Previous work demonstrated that neurabin binds F-actin via an N-terminal domain (10). It appears that this domain is responsible for localization of neurabin to both inchoate and established nerve endings. Subcellular fractionation shows that $p70^{S6k}$, which does not have a known neuronal isoform, is targeted to nerve endings. Consistent with these findings is the hypothesis that the PDZ domain of neurabin is responsible for $p70^{S6k}$'s presence at nerve terminals. There are a number of roles $p70^{S6k}$ could play at the synapse. Although protein translation at the nerve ending has not been established firmly, it is possible that $p70^{S6k}$ is required to modulate translation in response to synaptic demands. Alternatively, $p70^{S6k}$ could be functioning in a neuronal-specific capacity that is independent of its well characterized function as a mitogen activated ribosomal kinase.

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