THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 23, Issue of June 9, pp. 17786–17792, 2000
Printed in U.S.A.

Biochemical Interactions of the Neuronal Pentraxins

NEURONAL PENTRAXIN (NP) RECEPTOR BINDS TO TAIPOXIN AND TAIPOXIN-ASSOCIATED CALCIUM-BINDING PROTEIN 49 VIA NP1 AND NP2*

Received for publication, March 17, 2000
Published, JBC Papers in Press, March 28, 2000, DOI 10.1074/jbc.M002254200

Laura L. Kirkpatrick‡§, Martin M. Matzuk§¶‖, D'Nette C. Dodds‡‖, and Mark S. Perin‡‡‡**

*From the **Department of Neurosciences, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195 and the ‡Division of Neuroscience and Departments of ¶Pathology, §Molecular and Human Genetics, and ‖Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030*

Neuronal pentraxin 1 (NP1), neuronal pentraxin 2 (NP2), and neuronal pentraxin receptor (NPR) are members of a new family of proteins identified through interaction with a presynaptic snake venom toxin taipoxin. We have proposed that these three neuronal pentraxins represent a novel neuronal uptake pathway that may function during synapse formation and remodeling. We have investigated the mutual interactions of these proteins by characterizing their enrichment on taipoxin affinity columns; by expressing NP1, NP2, and NPR singly and together in Chinese hamster ovary cells; and by generating mice that fail to express NP1. NP1 and NP2 are secreted, exist as higher order multimers (probably pentamers), and interact with taipoxin and taipoxin-associated calcium-binding protein 49 (TCBP49). NPR is expressed on the cell membrane and does not bind taipoxin or TCBP49 by itself, but it can form heteropentamers with NP1 and NP2 that can be released from cell membranes. This is the first demonstration of heteromultimerization of pentraxins and release of a pentraxin complex by proteolysis. These processes are likely to directly effect the localization and function of neuronal pentraxins in neuronal uptake or synapse formation and remodeling.

We identified four novel proteins, NP1,[1] NP2, NPR, and TCBP49, that bind to affinity columns of the presynaptic snake venom neurotoxin taipoxin (1–4). The action of this toxin in blocking synaptic transmission and synaptic vesicle recycling led us to study the interactions between these taipoxin-binding proteins, their role in the action of taipoxin at synapses, and their potential role in general neuronal uptake. NP1, NP2, NPR, and TCBP49 were isolated by affinity chromatography of solubilized brain membranes on columns of immobilized taipoxin. NP1, NP2, and NPR define a family of neuronal pentraxins that are 50% identical to each other (3). The N-terminal half of neuronal pentraxins are 20–30% identical to previously identified pentraxins, such as serum amyloid P protein and C-reactive protein, which are elevated in the serum during acute phase response (5, 6). NP2 has been independently identified as the guinea pig sperm acrosome protein p50/apexin (7, 8) and as neuronal activity-regulated pentraxin (9). Classical pentraxins form pentamers and decamers that show calcium-dependent binding to a variety of ligands, such as bacteria, toxins, chromatin, and carbohydrates (10). C-reactive protein is thought to help mediate clearance of extracellular debris during infection and inflammation (11). We have proposed that the neuronal pentraxins function in a similar manner facilitating the uptake of synaptic material during synapse remodeling. An additional or alternative hypothesis, recently proposed by O'Brien *et al.* (12), is that NP2 mediates the synaptic clustering of AMPA glutamate receptors at a subset of excitatory synapses.

Based on cDNA sequence, NP1 and NP2 are predicted to be secreted proteins (1, 2), whereas NPR contains a putative membrane-spanning hydrophobic domain (3), raising the possibility that it is present on the neuronal cell surface. The three neuronal pentraxins are significantly larger than the classical pentraxins (>50 *versus* 30 kDa), suggesting that they may have additional novel functions. In contrast, the other taipoxin-binding protein, TCBP49, is a lumenal calcium-binding protein that is ubiquitously expressed and binds calcium through six EF-hand domains and contains the retention sequence His-Asp-Glu-Leu at its C-terminal (4). Immunofluorescence microscopy shows TCBP49 to be localized to an internal reticular compartment in neurons and glia. NP1, NP2, and NPR can all be isolated on taipoxin columns, NP1 and NP2 columns, and TCBP49 columns, suggesting that they interact *in vivo* (3). These interactions are likely to mediate aspects of taipoxin toxicity in neurons and are indicative of a normal neuronal pathway that may be involved in the uptake of extracellular material. To further understand the interactions of these proteins, we have examined native NP1, NP2, and NPR in primary hippocampal neurons and have also expressed these proteins individually and together in Chinese hamster ovary (CHO) cells. The experiments reported here examine the secretion, surface localization, multimerization, and binding characteristics of the neuronal pentraxins. In addition, we have generated mice that lack NP1 in order to confirm these interactions *in vivo*.

EXPERIMENTAL PROCEDURES

Materials—Restriction and DNA modification enzymes were from New England Biolabs and Roche Molecular Biochemicals. Protein molecular weight standards were from Bio-Rad, and DNA standards were from Life Technologies, Inc. Tissue culture media and additives were from Life Technologies, Inc. Peroxidase-conjugated and rhodamine-conjugated secondary antibodies were from Cappel. Unless noted otherwise, all other

* This work was supported by United States Public Health Service Grant NS30541. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ To whom correspondence should be addressed: Dept. of Neurosciences, The Lerner Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, OH 44195. Tel.: 216-445-9775; Fax: 216-444-7927; E-mail: perinm@ccf.org.

[1] The abbreviations used are: NP, neuronal pentraxin; NPR, NP receptor; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; UTR, untranslated region; bp, base pair(s); TCBP49, taipoxin-associated calcium-binding protein 49; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxoyole proprionic acid.

chemicals were reagent grade and used without further purification.

Antibodies and Western Blotting—The polyclonal antibodies used were as follows: NP1-2, raised against a synthetic peptide corresponding to residues 42–57 of rat NP1 (1); NP2-1, raised against a synthetic peptide corresponding to the C-terminal of rat NP2 (3); NPR-1, raised against a synthetic peptide corresponding to the C-terminal of rat NPR (3); and NP2-2, raised against GST-NP2, prepared as described in Ref. 3. In some experiments, the appropriate preimmune serum was used as a negative control.

12% SDS-PAGE gels were used for all experiments. After separation, the gels were either stained with Coomassie or used for Western blots. Gels to be blotted were transferred 1–2 h at 250 mA in standard Tris/glycine buffer and then blocked 1–2 h at room temperature in blocking buffer (6% casein, 1% polyvinyl pyrolidone-40, 10 mM EDTA in PBS, pH 7.4). Primary and secondary antibodies were generally incubated for 1–12 h in blocking buffer. Washes were done in PBST (0.3% Tween-20 in PBS). Peroxidase-conjugated antibodies were detected by ECL (Amersham Pharmacia Biotech).

Plasmids, Transfection, and Cell Culture—A 1600-bp fragment of the rat NP1 cDNA, including the entire coding sequence and 250 bp of the 3′ UTR, was subcloned into the *Bam*HI and *Sph*I sites of pcDNAI/Amp (Invitrogen). A 1650-bp piece of human NP2 cDNA, including the entire coding sequence and 260 bp of the 3′ UTR, was first subcloned into the *Xho*I and *Eco*RV sites of pBluescript (Stratagene) and then subcloned into the *Xho*I and *Xba*I sites of pcDNAI/Amp. Two different NPR constructs were generated. A 1710-bp piece of the rat NPR cDNA containing only 15 bp of the 5′ UTR, the entire coding sequence, and 200 bp of the 3′ UTR was subcloned into the *Bam*HI and *Eco*RI sites of pcDNAI/Amp. This piece was also subcloned into the *Bam*HI and *Eco*RV sites of pZeoSV2(+) (Invitrogen). Also, an 1850-bp piece containing the entire 150 bp of the 5′ UTR, the coding sequence, and 200 bp of the 3′ UTR was subcloned into the *Bam*HI and *Eco*RI sites of pZeoSV2. pSVneo plasmid was from Dr. Thomas Sudhof (University of Texas Southwestern Medical Center, Dallas, TX). All DNAs were purified by Qiagen Midi-Prep columns before use.

CHO cells were a generous gift of Dr. Mike Roth (University of Texas Southwestern Medical Center, Dallas, TX). They were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 40 μg/ml proline. For generating the initial NP1, NP2, and NPR cell lines, cells were co-transfected with the pentraxin cDNAs and, when necessary, the pSVneo DNA at a 5:1 ratio using LipofectAMINE (Life Technologies, Inc.). Typically, 6 mg of pentraxin DNA and 16 ml of LipofectAMINE were used per 100-mm dish of cells. Stable cell lines were isolated through two rounds of cloning in selection media containing 500 μg/ml Geneticin (Life Technologies, Inc.) or 500 μg/ml Zeocin (Invitrogen) and then maintained in media containing 200 μg/ml Geneticin or Zeocin. All screening for expressing clones was done by Western blotting. To generate the cell lines expressing NPR with NP1 or NP2, NPR cDNAs in pZeoSV2 were transfected into NP1 or NP2 cells, and double selection was used to identify stable clones.

Primary cultures of rat hippocampal neurons were a generous gift of Andrew Bellun (Baylor College of Medicine, Houston, TX). They were prepared from embryonic rats at day E19-E20, plated on poly-D-lysine, and maintained according to standard protocols in Neurobasal/B27 medium. Cultures were allowed to differentiate for at least 3–4 weeks before use in experiments. Culture medium was collected for analysis 4 days after the last feeding.

Immunofluorescence Microscopy—Cells were plated on collagen-coated coverslips and allowed to attach overnight. Antibodies were added directly to the tissue culture medium and allowed to bind for 2 h at 4 °C. The cells were then washed with PBS and fixed with formaldehyde, and surface-bound antibodies were visualized with rhodamine-labeled goat-anti-rabbit secondary antibody.

Affinity Chromatography—Taipoxin and recombinant TCBP49 affinity columns were generated and used as described (3). The columns were equilibrated in 20–25 column volumes of buffer containing 100 mM NaCl, 2 mM $CaCl_2$ and 20 mM HEPES, pH 7.4. In experiments using cell lysates, the column equilibration buffer also contained 0.2% TX-100. CHO or hippocampal neuron culture medium was harvested, centrifuged to remove any floating cells, and applied directly to the column. CHO and hippocampal neuron cell lysates were prepared by mixing cells in lysis buffer (1% TX-100, 100 mM NaCl, 20 mM HEPES, pH 7.4, plus protease inhibitors). The cell lysates were centrifuged to remove any insoluble material and adjusted to 2 mM $CaCl_2$ before being applied to the columns. The columns were washed with 10–15 column volumes of equilibration buffer and then eluted sequentially with 3 column volumes of the same buffer containing 200 mM, 400 mM, 600 mM, 800 mM, and 1 M NaCl. The final elution was with 5 column volumes of the same buffer containing 1 M NaCl and 10 mM EDTA. In most experiments, the eluates were concentrated in Centricon-30 concentrators (Amicon). Equal aliquots of each eluate were separated by SDS-PAGE and analyzed by Coomassie staining and Western blotting.

Generation of NP1 Knockout Mouse, Column Chromatography, and Quantitative Immunoblotting—The mouse gene encoding NP1 was identified (13) and used to generate a knockout construct that replaced the first exon encoding the signal peptide with a PGK-hprt cassette (see Fig. 6A). This construct was electroporated into 129/SvEv-derived ES cells (AB2.1), and HAT- and FIAU-resistant clones were isolated and screened for the expected restriction fragment length changes by genomic Southern blot as described (14). Correctly targeted clones were injected into blastocysts and implanted in pseudopregnant females. Chimeric mice were bred to obtain mice heterozygous for the modified NP1 gene, and heterozygous mice were bred to generate mice homozygous for the mutation.

Sucrose Gradient Centrifugation—NP1, NP2, and NPR containing samples, isolated from culture media or cell lysates by taipoxin or rTCBP49 affinity chromatography, were analyzed by sucrose gradient centrifugation (15). For these experiments, EDTA eluates containing NP1, NP2, or NPR were collected in 1-ml fractions and not concentrated. The fraction with the highest protein concentration was identified by Western blotting and used for sucrose gradient analysis. Alternatively, whole cell lysates, prepared as described above, were loaded directly onto sucrose gradients. 5–20% sucrose gradients were poured into buffer (20 mM HEPES, 100 mM NaCl, and 0.2% TX-100), 1 ml of sample was loaded, and the gradients were centrifuged at 40,000 × *g* in SW41 rotor for 14 h at 4 °C. 500-μl fractions were collected from the top by pushing the gradient from the bottom with 40% sucrose. Equal aliquots of each fraction were analyzed by Western blotting. Bio-Rad gel filtration standards were run in a separate gradient as size controls.

RESULTS

NP1, NP2, and NPR are all expressed in the hippocampus, as revealed by *in situ* hybridization (1, 3, 9), and primary cultures of rat hippocampal neurons are sensitive to taipoxin, as evidenced by the swelling of presynaptic terminals and the cell surface exposure of synaptic vesicle proteins (1). To confirm the presence of NP1, NP2, and NPR proteins in hippocampal neurons, detergent lysates of cultured hippocampal neurons were chromatographed on a taipoxin column to isolate neuronal pentraxins. As in original purifications from solubilized rat brain membranes (1, 3), the column was loaded and washed in the presence of 2 mM calcium and eluted with 10 mM EDTA. All three of the pentraxins can be purified from detergent-solubilized hippocampal neurons by taipoxin chromatography (Fig. 1*A*). NP1 and NP2, purified off taipoxin columns, run as single bands on SDS-PAGE. NPR, in contrast, runs as two separate bands of apparent molecular masses of 55 and 65 kDa. The 65-kDa form of NPR is initiated with a hydrophobic sequence of sufficient length to be a transmembrane domain (3). To test whether the neuronal pentraxins are secreted or released from neurons, conditioned medium was collected from hippocampal cultures 4 days after feeding and chromatographed on the taipoxin column. NP1 and NP2 are purified from media by taipoxin chromatography and run as single bands on SDS-PAGE gels. Secretion of NP1 and NP2 would be expected as they have apparent N-terminal signal peptide sequences of 9–16 amino acids (1, 2). NPR also can be purified from neuronal media, but only the 55-kDa band was isolated (Fig. 1*B*), suggesting that NPR is proteolytically released from the cell surface or that a distinct 55-kDa form of NPR is secreted.

To examine the properties of NP1, NP2 and NPR individually, we generated stable CHO cell lines that express each of these proteins. NP1 and NPR both have relatively long 3′ UTRs, over 3 kilobases in length (1, 3). Initial attempts to express NP1 using the full-length cDNA were unsuccessful.[2] We therefore used smaller expression constructs, consisting of only the coding sequences and minimal amounts of the 5′ and

[2] M. S. Perin, unpublished data.

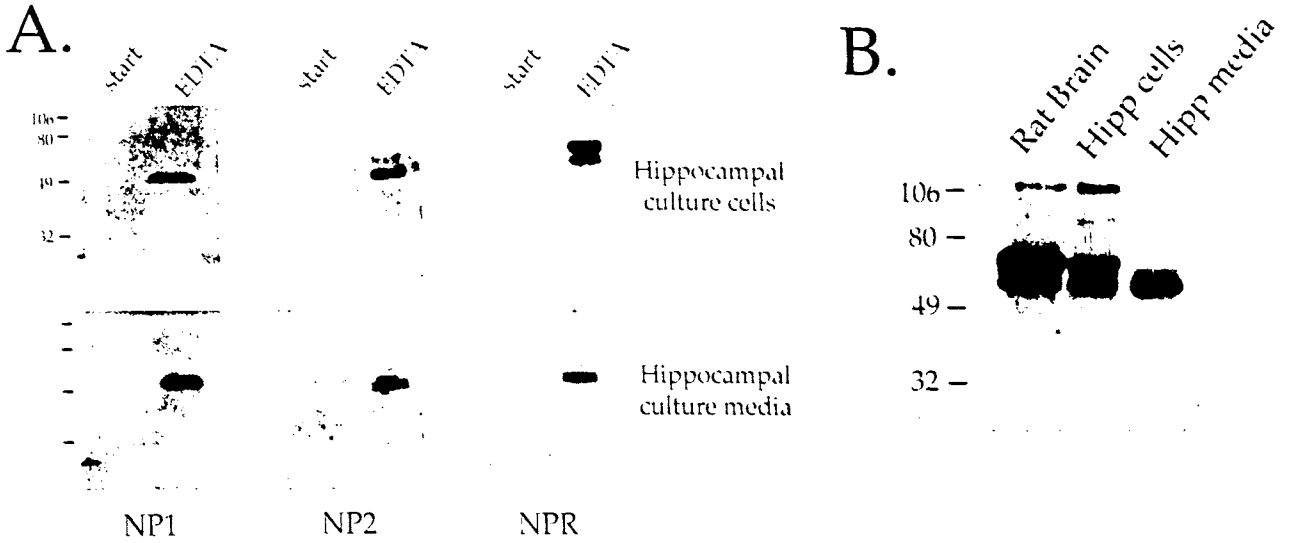

FIG. 1. **Hippocampal neurons produce NP1, NP2, and NPR but release only low molecular weight form of NPR into media.** Detergent-solubilized cultured hippocampal neurons or conditioned media from these neurons was chromatographed over taipoxin columns. 25 μg of starting material and ⅛ of the EDTA column eluate were run on SDS-PAGE gels and detected by Western blotting with NP1, NP2, or NPR antibodies (*A*). EDTA eluates from solubilized rat brain membranes, solubilized hippocampal neurons, and conditioned media were analyzed by Western blotting with NPR antibody to show the absence of the high molecular weight form of NPR from media (*B*).

3′ UTRs. Two different NPR constructs were generated, differing only in the length of the 5′ UTR included. The full-length rat NPR cDNA contains only 150 bp of the 5′ UTR. It does not contain an in-frame stop codon before the initiator CTG, and it is extremely G/C-rich (3). Because it has been suggested that such a sequence may form secondary structure necessary for initiation at cryptic upstream CTG sites (16), we made one NPR construct with the entire 5′ UTR and another containing only 15 bp of the 5′ UTR. These were designated "long" and "short" NPR, respectively. The short NPR construct was made in pcDNAI/Amp and in pZeoSV2. The long NPR construct was made only in pZeoSV2. NP1 and NP2 constructs were made in pcDNA1/Amp. The use of two selectable markers allowed us to isolate stable cell lines co-expressing NP1 and NPR and lines co-expressing NP2 and NPR. The expression of NP1, NP2, and NPR by these cell lines is shown in Fig. 2. NP1 and NP2 are expressed as proteins that run as single bands on SDS-PAGE gels. NPR expressed by these cells run as multiple bands on SDS-PAGE gels. Both the short and long NPR constructs produce the same bands, although the long NPR construct produces more of the highest molecular weight band.

We tested whether NP1, NP2 and NPR expressed individually in CHO cell lines could bind to taipoxin as assayed by affinity chromatography of CHO cell lysates and conditioned culture media on taipoxin columns. Cell lysates were adjusted to 2 mM free calcium before loading, and the column was washed in the presence of calcium. The calcium concentration of the tissue culture media was not adjusted. Results are shown in Fig. 3*A*. NP1 from both cell lysates and culture media binds to taipoxin columns, remains bound in the presence of 1 M NaCl, and is only eluted with EDTA, suggesting that the binding is dependent on divalent cations. NP1 does not bind to taipoxin when the chromatography is done in the presence of magnesium instead of calcium (data not shown), suggesting that the binding of NP1 to taipoxin is indeed calcium-dependent. Like NP1, NP2 in both cell lysates and conditioned culture media binds to taipoxin columns and only elutes with EDTA. These results demonstrate that NP1 and NP2 can bind taipoxin independently of each other and independently of NPR. In contrast, NPR, from NPR CHO cell lysates, fails to bind to taipoxin columns. Interestingly, we could not detect or immunoprecipitate NPR in conditioned medium from these cells (data not shown). NPR fails to bind to taipoxin columns when made from either the short or long NPR construct, suggesting that the purification of NPR from whole rat brain on taipoxin columns occurs as a result of its binding to another protein on the column, perhaps NP1 or NP2. We tested this by loading the taipoxin column with NP1 and then running NPR cell lysate over the column. Only the prebound NP1 was eluted from the column after washing (data not shown). We also tried mixing equal amounts of solubilized NP1 cells with solubilized NPR cells and then running the mixed cell lysate over the taipoxin column (Fig. 3*B*). Again, only NP1 bound to the column. Finally, we co-expressed NP1 and NPR in a single cell line and

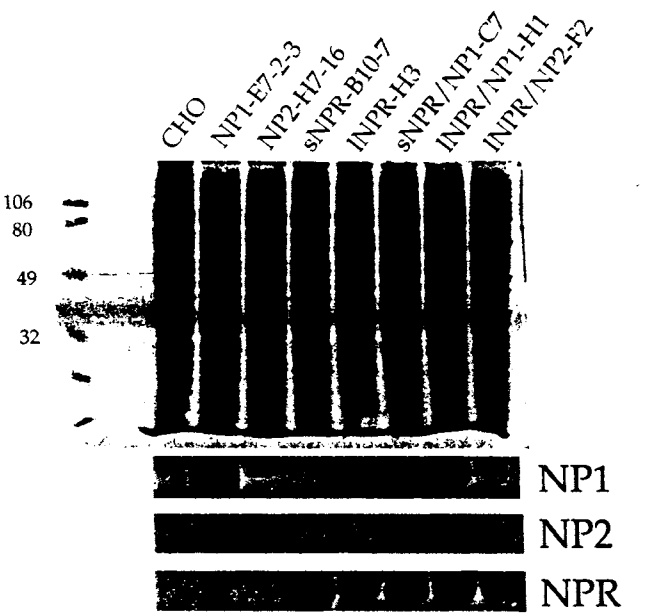

FIG. 2. **CHO cell lines that express NP1, NP2, NPR, NPR/NP1, or NPR/NP2.** 50 μg of CHO cell lines expressing neuronal pentraxins were subjected to SDS-PAGE and either stained with Coomassie or transferred to nitrocellulose and detected with antibodies to NP1, NP2, or NPR.

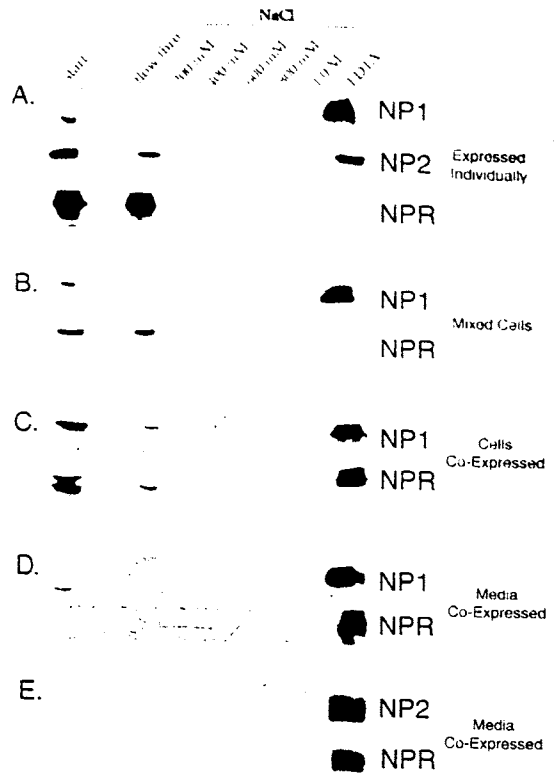

FIG. 3. **NPR only binds to taipoxin columns when co-expressed with NP1 or NP2.** Triton X-100-solubilized CHO cells expressing NP1, NP2, and NPR (*A* and *B*), CHO cells co-expressing NP1 and NPR (*C*), or media of CHO cells co-expressing NP1/NPR or NP2/NPR (*D* and *E*) were chromatographed over taipoxin columns in the presence of 1 mM calcium. Columns were eluted with a step salt gradient with 1 mM calcium and a final elution with 10 mM EDTA. 25 μg of starting material or 1/25 of the EDTA column eluate was run on SDS-PAGE gels and detected by Western blotting with NP1, NP2, or NPR antibodies. Cell lines E7, H7, B10, C7, and F2 were used.

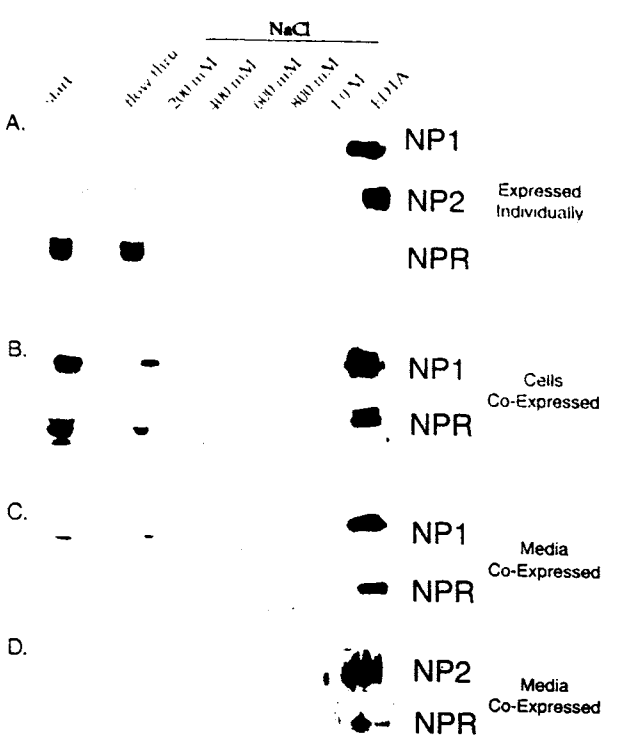

FIG. 4. **NPR only binds to TCBP49 columns when co-expressed with NP1 and NP2.** Triton X-100-solubilized CHO cells expressing NP1, NP2, and NPR (*A*), CHO cells co-expressing NP1 and NPR (*B*), or media of CHO cells co-expressing NP1/NPR or NP2/NPR (*C* and *D*) were chromatographed over a recombinant TCBP49 column in the presence of 1 mM calcium. Columns were eluted with a step salt gradient with 1 mM calcium and a final elution with 10 mM EDTA. 25 μg of starting material or 1/25 of the EDTA column eluate was run on SDS-PAGE gels and detected by Western blotting with NP1, NP2, or NPR antibodies. Cell lines E7, H7, B10, C7, and F2 were used.

tested the cell lysate and media from these cells on the taipoxin column (Fig. 3, *C* and *D*). Interestingly, NPR from both the cell lysate and the media was found bound to the taipoxin column with the NP1. Again, binding is calcium-dependent, as NP1 and NPR are only eluted with EDTA. This result indicates that the interaction between NP1 and NPR is quite strong, as it withstands 1 M NaCl. NPR from cell lysates and media can also bind the taipoxin column when co-expressed with NP2 (Fig. 3*E*). Again, both NP2 and NPR remain bound in 1 M NaCl and are released by EDTA. This suggests that NP1 and NP2 are similar in forming complexes with NPR. Given that the formation of NP1·NPR and NP2·NPR complexes requires co-expression, it is likely that these complexes are formed shortly after translation during their passage through the secretory pathway.

We have previously shown NP1, NP2, and NPR can also be enriched by chromatography on columns of recombinant TCBP49, a fourth taipoxin-binding protein with six EF-hand calcium binding domains that is present in lumenal reticular compartments (4). We tested whether neuronal pentraxins expressed in our CHO cell lines could bind TCBP49. TCBP49 (rTCBP49) was expressed in bacteria (4), purified, and coupled to activated CH-Sepharose. NP1, NP2, and NPR cell lysates or conditioned medium were chromatographed over rTCBP49 columns using the same buffers and procedures used for chromatography on taipoxin columns (Fig. 4). NP1 from cell lysates or conditioned media bound to rTCBP49 columns and required EDTA for elution. Similarly, NP2 from cell lysates or conditioned media bound tightly to rTCBP49 columns. In contrast, NPR from cell lysates did not bind to rTCBP49 columns. When cell lysates or conditioned media from cells expressing both NP1 and NPR or cells expressing both NP2 and NPR were used, NPR bound rTCBP49 columns and was eluted in the final EDTA elution.

The column chromatography data imply that NP1 and NPR must form a complex within the cell in order for NPR to be purified on either taipoxin or rTCBP49 columns. Because the classical pentraxins form pentamers and decamers *in vivo* (10), we tested whether NP1, NP2, and/or NPR exist as pentamers, or higher order multimers, in our CHO cell lines. Whole cell lysates or EDTA eluates from taipoxin or rTCBP49 columns were analyzed using sucrose gradient sedimentation (Fig. 5). First, NP1 from conditioned media was purified by taipoxin chromatography, and the EDTA eluate was loaded directly on sucrose gradients and centrifuged. Comparison of the position of NP1 in these gradients to molecular size standards demonstrates that NP1 exists as higher order multimers; the peak of these gradients corresponds to an approximate size of 250 kDa or to the size of a pentamer (Fig. 5*A*). No NP1 was detectable at positions corresponding to a monomer. NP2 also sedimented into fractions corresponding to a pentamer. The samples used for these NP1 and NP2 gradients were EDTA eluates, indicating that the multimeric complexes are stable in EDTA and do not depend on calcium for their formation. This also indicates that EDTA does not cause the elution of NP1 or NP2 from the taipoxin column merely by breaking up a pentameric NP1 or NP2 complex. When detergent lysates of NP1-expressing cells were run on the same gradients, NP1 again ran as a pentameric-sized complex (Fig. 5*B*). In contrast, when NPR-expressing cells were run on these sucrose gradients, almost all of the NPR

was present in fractions consistent with monomers or dimers (Fig. 5*B*). The existence of NPR as a monomer or dimer is unique for any of the pentraxins analyzed to date. Given our previous results suggesting that NP1 and NP2 form complexes with NPR, we analyzed whether NP1, NP2, and NPR form large multimeric complexes in cells co-expressing either NP1 and NPR or NP2 and NPR. Cells were lysed with detergent and chromatographed on taipoxin columns. Taipoxin column EDTA eluates were run on sucrose gradients. As shown in Fig. 5, *C* and *D*, when co-expressed with NP1 or NP2 (data not shown), NPR ran on sucrose gradients as a large multimeric complex at least the size of a pentamer. This indicates that co-expression of NP1 or NP2 with NPR enables the NPR to be assembled into large complexes and that only such multimeric complexes are capable of binding to taipoxin or rTCBP49. The existence of multimeric complexes of NP1, NP2, and NPR can also be shown using nondenaturing polyacrylamide gel electrophoresis and size-exclusion chromatography (data not shown). When taipoxin column eluates or lysates of NP1/NPR or NP2/NPR co-expressing cells are run on nondenaturing gels, NP1, NP2, and NPR all run near the top of 6% gels at positions indicative of large multimeric complexes. When the same eluates or lysates from co-expressing cells are run on Superose 6 size exclusion columns, NP1, NP2, and NPR all elute at positions indicative of a large complex that is larger than a 158-kDa gel filtration standard.

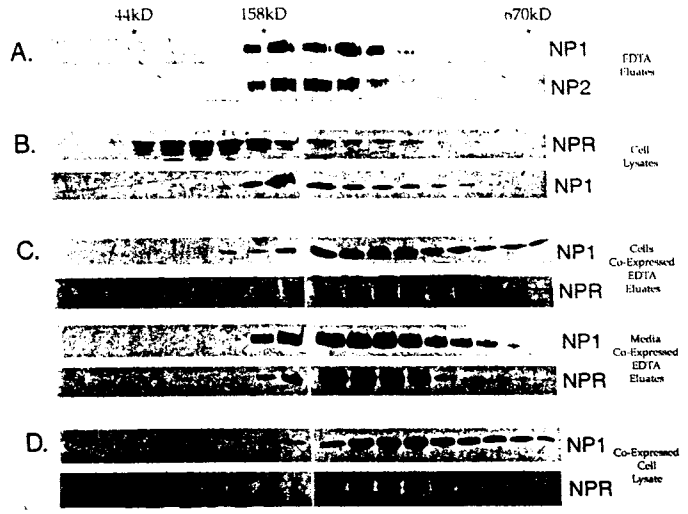

FIG. 5. **NPR only forms oligomeric complexes when co-expressed with NP1 and NP2.** Triton X-100-solubilized CHO cells expressing NP1, NP2, NPR, or NP1/NPR or media from these lines were used for sucrose gradient analysis either before or after purification from taipoxin columns. *A*, taipoxin column eluates of NP1- or NP2-expressing cell lines. *B*, cell lysates of NP1- or NPR-expressing cells. *C*, taipoxin column eluates of NP1/NPR-solubilized cells or media. *D*, detergent cell lysates of cells co-expressing NP1/NPR. Position of neuronal pentraxins in the gradient was determined by Western blotting and compared with molecular size standards run on parallel gradients. Cell lines E7, H7, B10, H3, C7, and F2 were used.

To investigate these interactions *in vivo*, we generated mice that lack NP1. Fig. 6*A* shows the targeting construct used to knock-out NP1 by homologous recombination in ES cells. The first exon, including the translation start site and the signal peptide, was targeted for removal. Southern blot analysis of heterozygous and homozygous mice demonstrated the expected mutant allele structure after recombination (Fig. 6*B*). Heterozygous crosses displayed the expected Mendelian numbers of wild type, heterozygous, and null mice (28 wild type homozygous, 67 heterozygous, and 30 homozygous recombinant). Mice homozygous for the mutated gene are viable and fertile and show no obvious alteration in cerebral, cerebellar, or hippocampal ultrastructure (data not shown) or obvious changes of gait or behavior. These mice do not produce any NP1 as determined by Western blot analysis. Because NP1 is a very low abundance protein, chromatography of solubilized brain membranes from wild type and NP1 knockout mice on taipoxin columns was used to conclusively demonstrate the absence of NP1 protein. 20 wild type and 20 knockout mouse brains were solubilized in 100 mM NaCl, 20 mM HEPES, 1% Triton X-100 and chromatographed on taipoxin columns in the presence of 1 mM calcium chloride. After an extensive wash, the columns were eluted with steps of 200 mM, 400 mM, 600 mM, 800 mM, or 1 M NaCl in the presence of 1 mM calcium chloride and a final elution with 1 M NaCl 10 mM EDTA. Western blot analysis of column fractions demonstrated that NP1 is not present in any fractions from the NP1 knockout mouse brains. Four different NP1 antibodies raised against C-terminal and N-terminal epitopes fail to detect NP1 in these fractions (Fig. 6). This confirmed that the mutation, which deleted the signal peptide-encoding exon, was null. Control chromatography with wild type mice show the expected enrichment of NP1 in the 1 M NaCl 10 mM EDTA fraction. Interestingly, chromatography of NP1 null and wild type mouse brains show similar enrichment of NP2 in EDTA eluates but a dramatic reduction in the amount of NPR that is purified from NP1 null mice. To quantify this, these chromatographies were repeated three times with solubilized membranes

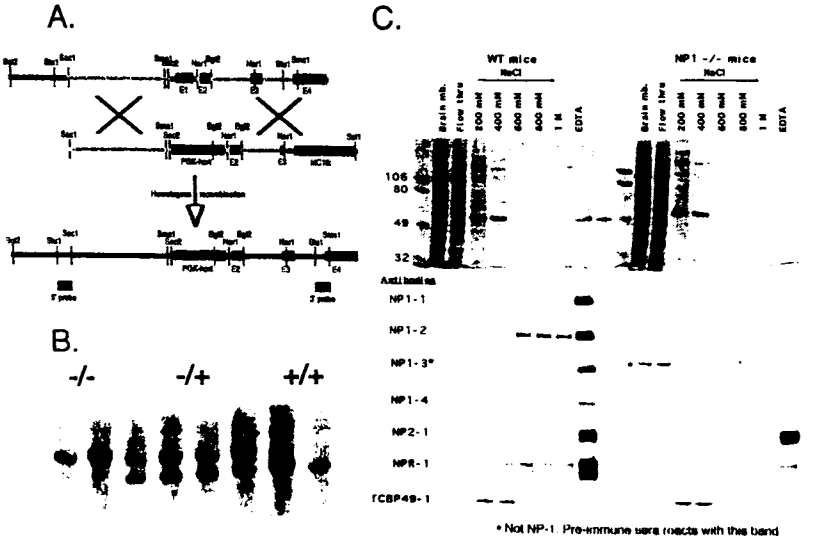

FIG. 6. **Mice lacking NP1 show a reduction in the amount of NPR that can be purified by taipoxin chromatography.** Mice lacking NP1 were generated through homologous recombination in ES cells using a targeting strategy shown in *A*. Alteration of genomic Bgl2 restriction fragment lengths in wild type, heterozygous, and homozygous null mice is shown in *B*. Chromatography of solubilized wild type and NP1 null mouse brains on taipoxin columns is shown in *C*. 50 μg of solubilized brain membranes and flow through and 1/25 of each elution fraction were subjected to SDS-PAGE and stained with Coomassie or transferred to nitrocellulose and probed with antibodies shown. Preimmune serum from NP1-3 recognizes a band of 51 kDa that is not NP1 (*).

from wild type and NP1 null brains. Western blots were probed with NP2 or NPR antibodies followed by incubation with ^{125}I-labeled protein A. NP2 and NPR immunoreactive bands were identified by autoradiography, cut out, and counted by liquid scintillation. The mean amount of NP2 from chromatographies of null brains was 61,000 cpm, with a standard deviation of 13,000. The mean amount of NP2 from wild type was 64,000 ± 14,000 cpm. The amount of NP2 in the EDTA eluates from NP1 knockout and wild type mouse brains was not significantly different. In contrast to NP2, there was a 3-fold reduction of the amount of NPR present in the EDTA eluate of NP1 knockout mouse brains compared with wild type (32,000 ± 7700 cpm *versus* 100,000 ± 12,000 cpm, $p < 0.05$). This reduction of NPR strongly suggests that NPR forms heteromultimeric complexes *in vivo* with NP1 and NP2.

The 65-kDa form of native rat brain NPR contains an apparent transmembrane domain (3), suggesting that this form of NPR can localize NP1 or NP2 to the cell surface. We investigated the cell surface exposure of NP1, NP2, and NPR by immunofluorescence using our stable CHO cell lines. CHO cells expressing NP1, NP2, or NPR, cells co-expressing NP1 and NPR, and cells co-expressing NP2 and NPR were washed with PBS and incubated with antibodies to neuronal pentraxins. Cells were fixed and incubated with rhodamine-labeled secondary antibodies. These procedures should only detect cell surface bound NP1, NP2, or NPR. Cells expressing NP1 or NP2 alone show little cell surface staining with NP1 or NP2 antibodies, suggesting that NP1 and NP2 are secreted directly into media. In contrast, cells expressing NPR have substantial cell surface staining with NPR antibodies (Fig. 7). Cells co-expressing NP1/NPR or NP2/NPR have cell surface staining for both NP1 and NPR and for both NP2 and NPR, respectively (Fig. 7). These results suggest that co-expression with NPR results in the retention of NP1 and NP2 at the cell surface.

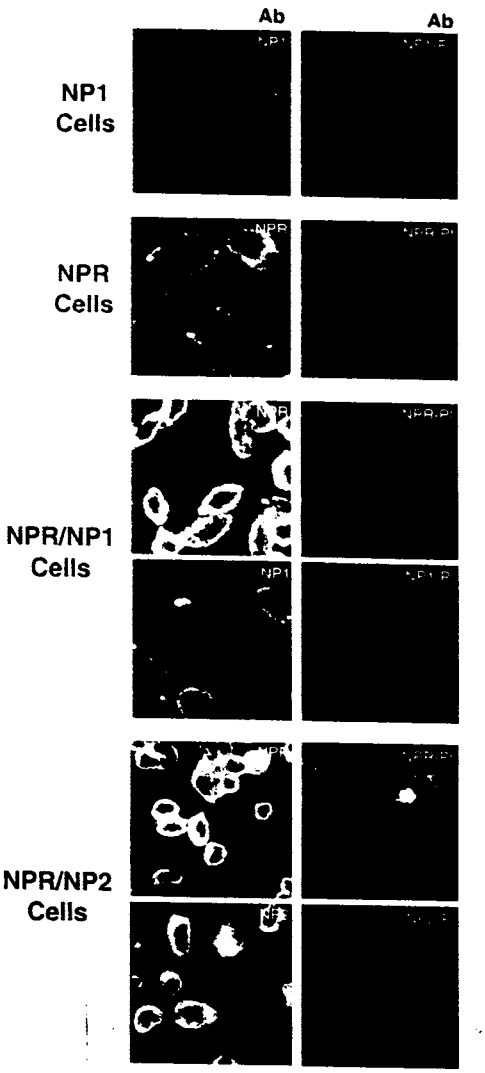

FIG. 7. **Co-expression of NPR with NP1 or NP2 allows extensive cell surface expression of NP1 and NP2.** CHO cells expressing NP1 or NPR or co-expressing NP1/NPR or NP2/NPR were stained for extracellular surface staining with NP1, NP2, and NPR antibodies. Cell lines E7, B10, C7, and F2 were used.

DISCUSSION

We have previously identified NP1, NP2, NPR, and the six EF-hand, calcium-binding protein TCBP49 as taipoxin-binding proteins. We proposed that these proteins bind each other and are involved in the uptake of material at synapses. The potential existence of three different neuronal pentraxins at the synapse raises questions about their individual activities, their multimerization state, their presence at the cell membrane, and their interactions with taipoxin and TCBP49, questions that can be addressed by expressing these proteins in mammalian cells.

When expressed individually in CHO cells, NP1 and NP2 display many characteristics of classical pentraxins. They are constitutively secreted or released from cells, show calcium-dependent ligand binding, and form pentamers. In contrast, when NPR is expressed individually, it is apparently not secreted or is degraded rapidly when secreted, does not bind taipoxin, and does not multimerize completely. As such, NPR is the first pentraxin that does not form pentamers or decamers by itself. NPR is also unusual in that it is the only pentraxin to date initiated by a transmembrane domain. Our experiments with cell lines expressing neuronal pentraxins clearly demonstrate that NP1 and NP2 are capable of binding taipoxin and TCBP49 individually. In contrast, NPR by itself is incapable of interacting with taipoxin or TCBP49. This suggests that NP1 and NP2 share similar functions that do not extend to NPR. This is interesting because NP1, NP2, and NPR form an otherwise distinct pentraxin protein subfamily. They have similar conservation to each other, showing approximately 50% amino acid sequence identity. This conservation is much higher than the 20–30% conservation of neuronal pentraxins to classical pentraxins, such as C-reactive protein (5) and serum amyloid protein SAMP (6), and other long pentraxins such as TG14/PTX3 (17, 18).

When we previously identified NPR by purification on GST-NP1 and GST-NP2 columns, we proposed that NP1 and NP2 bind ligands and then bind NPR to facilitate uptake (3). The data we have presented here make this less likely. First, NPR from solubilized transfected CHO cells does not bind to taipoxin columns preloaded with NP1. Second, NPR cannot be isolated by taipoxin column chromatography when solubilized NP1-expressing cells and NPR-expressing cells are mixed. However, the co-expression of NP1 and NPR, or NP2 and NPR in the same cells, does allow for NPR purification on taipoxin columns. Together, these results suggest that NP1, NP2, and NPR must interact within a single cell, perhaps during synthesis, in order for NPR to be able to bind NP1 or NP2 in a way that allows for its purification on taipoxin. This does not completely rule out the possibility that neuronal pentraxins from separate cells could bind each other, but it does suggest that any such binding is blocked or inhibited by taipoxin binding. NP1·NPR and NP2·NPR complexes are extremely stable. When these complexes are bound to taipoxin columns, they cannot be eluted with high salt concentrations and are only eluted by removing calcium. We have yet to find a condition that elutes NPR in the

absence of NP1 or NP2. We also know that the NP1·NPR complex that elutes from taipoxin columns is still intact, as it runs as a pentamer or higher order multimer in sucrose gradients. The simplest interpretation of these results is that NP1 and NPR co-assemble into a pentamer that is stable in 1 M NaCl and in EDTA, although we cannot rule out the possibility that NP1 and NPR form higher order oligomers, such as decamers. The generation of mice that lack NP1 confirms that neuronal pentraxins generate hetero-oligomers *in vivo*. Taipoxin chromatography of solubilized brain tissue from these mice shows an absence of NP1 and the same amount of NP2 but a great reduction of NPR.

NP1 and NP2 must have at least two binding domains that do not overlap. With one, NP1 can bind itself or NPR in the process of multimerization. This binding site does not require calcium, and the resulting oligomer is very stable. Isolated pentameric neuronal pentraxins are resistant to proteolysis. We have kept them on ice for days at a time and find they still behave normally in column chromatography and sucrose gradient analysis, with no protein degradation detected by SDS-PAGE. A second binding site mediates interaction with taipoxin and presumably other ligands. This site is calcium-dependent. As the classical pentraxins also have sites for oligomerization and ligand binding, both of these sites may exist on the C-terminal half of NP1 and NP2. This suggests the possibility of additional functions for the N-terminal half of neuronal pentraxins. One potential function could be the binding of TCBP49 by NP1·NPR and NP2·NPR heteromultimers, through their NP1 or NP2 subunits. NPR also has a pentraxin domain at its C-terminal end. Although it is clear that NPR can multimerize with NP1 and NP2, calcium-dependent ligand binding by the NPR subunit has not yet been demonstrated. Future experiments utilizing these cell lines and lines expressing smaller portions of all three neuronal pentraxins will allow direct tests of these questions and of the ligand specificity of these proteins.

It is interesting to note that NPR expression allows cell surface localization of NP1 and NP2, but at the same time, NP1·NPR or NP2·NPR complexes can be isolated from conditioned media of hippocampal neurons and transfected CHO cells. Cell surface localization suggests that NPR with a transmembrane domain can anchor the complex to the cell surface. Isolation of these complexes in conditioned media suggests that tethered NP1·NPR or NP2·NPR can be subsequently released by regulated or unregulated proteolysis.

We have suggested that neuronal pentraxins would be ideal candidates to mediate uptake of debris at synapses based on their sequence homology to classical pentraxins such as C-reactive protein, on their potential to localize taipoxin to synapses, and on the ability of taipoxin to block synaptic vesicle recycling. Although we find NP1 enriched at cerebellar glomeruli (data not shown), and although O'Brien *et al.* (12) have shown that NP2 is selectively enriched at some excitatory synapses in the hippocampus, direct demonstration of the function of neuronal pentraxins awaits further experiments. As a step for such a demonstration, we have generated mice that lack NP1. These mice are viable, fertile, and appear grossly normal. Given our demonstration that NP1 and NP2 are very similar in amino acid sequence, can both bind taipoxin, both interact with NPR, and are expressed in similar if not the same neurons in the brain, it is likely that these proteins mediate similar functions and would allow redundancy in knockout mice. We are generating mice lacking both NP1 and NP2, as well as mice lacking NPR, to directly test whether neuronal pentraxins are required for uptake of synaptic material associated with synapse formation and remodeling and whether loss of neuronal pentraxins affect synaptic formation or plasticity. Mice lacking both NP1 and NP2 will allow direct tests of the whether neuronal pentraxins are required for synaptic targeting of taipoxin and should allow experiments testing whether taipoxin needs to be endocytosed to block neurotransmission.

A separate or additional synaptic function for neuronal pentraxins has been suggested by data showing that NP2 is associated with AMPA receptor clustering (12). Cotransfection of HEK cells with NP2 and GLUR1–3 receptors results in coaggregates of AMPA receptors and NP2. Interestingly, soluble NP2 was ineffective in clustering AMPA receptors. Heteromultimerization of NP2 and NPR is likely to more firmly attach NP2 to the cell membrane. Missing in such studies is a demonstration that loss of NP2 (or all neuronal pentraxins) results in a loss or reduction in AMPA receptors clusters. Mice lacking NP1 and NP2 should prove ideal to test this, although a loss of AMPA receptors due to a loss of clustering activity may be difficult to differentiate from a loss of synapses.

Acknowledgments—We thank Cathy Guo and Tyna Yang for excellent technical assistance. We thank Lisa Bjartmar for critically reading the manuscript.

REFERENCES

1. Schlimgen, A. K., Helms, J. A., Vogel, H., and Perin, M. S. (1995) *Neuron* **14,** 519–526
2. Hsu, Y.-C., and Perin, M. S. (1995) *Genomics* **28,** 220–227
3. Dodds, D. C., Omeis, I. A., Cushman, S. J., Helms, J. A., and Perin, M. S. (1997) *J. Biol. Chem.* **272,** 21488–21494
4. Dodds, D., Schlimgen, A. K., Lu, S.-Y., and Perin, M. S. (1995) *J. Neurochem.* **64,** 2339–2344
5. Whitehead, A. S., Zahedi, K., Rits, M., Mortensen, R. F., Lelias, J. M. (1990) *Biochem. J.* **266,** 283–290
6. Dowton, S. B., and McGrew, S. D. (1990) *Biochem. J.* **270,** 553–556
7. Noland, T. D., Friday, B. B., Maulit, M. T., and Gerton, G. L. (1994) *J. Biol. Chem.* **269,** 32607–32614
8. Reid, M. S., and Blobel, C. P. (1994) *J. Biol. Chem.* **269,** 32615–32620
9. Tsui, C. C., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., Barnes, C., and Worley, P. F. (1996) *J. Neurosci.* **16,** 2463–2478
10. Gewurz, H., Zhang, X.-H., and Lint, T. F. (1995) *Curr. Opin. Immunol.* **7,** 54–64
11. Steel, D. M., and Whitehead, A. S. (1994) *Immunol. Today* **15,** 81–88
12. O'Brien, R. J., Xu, D., Petralia, R. S., Steward, O., Huganir, R. L., and Worley, P. (1999) *Neuron* **23,** 309–323
13. Omeis, I., Hsu, Y.-C., and Perin, M. S. (1996) *Genomics* **36,** 543–545
14. Matzuk, M. M., Finegold, M. J., Su, J.-G. J., Hsueh, A. J. W., and Bradley, A. (1992) *Nature* **360,** 313–319
15. Perin, M. S., Brose, N., Jahn, R., and Südhof, T. C. (1991) *J. Biol. Chem.* **266,** 623–629
16. Kozak, M. (1999) *Gene* **234,** 187–208
17. Lee, G. W., Lee, T. H., and Vilcek, J. (1993) *J. Immunol.* **150,** 1804–1812
18. Breviario, F., d'Aniello, E. M., Golay, J., Peri, G., Bottazzi, B., Bairoch, A., Saccone, S., Marzella, R., Predazzi, V., Rocchi, M., Della Valle, G., Dejana, E., Mantovani, A., and Introna, M. (1992) *J. Biol. Chem.* **267,** 22190–22197