Nadrin, a Novel Neuron-specific GTPase-activating Protein Involved in Regulated Exocytosis*

Received for publication, May 12, 2000, and in revised form, August 30, 2000
Published, JBC Papers in Press, August 30, 2000, DOI 10.1074/jbc.M004069200

Ayako Harada‡, Birei Furuta‡§, Ken-ichi Takeuchi‡, Makoto Itakura‡, Masami Takahashi‡, and Masato Umeda‡¶

From the ¶Department of Molecular Biodynamics, The Tokyo Metropolitan Institute of Medical Science (RINSHOKEN), 3-18-22 Honkomagome, Bunkyo-ku, Tokyo 113-8613, Japan, ‡Department of Biology, Faculty of Science, Ochanomizu University, 2-1-1 Ohtsuka, Bunkyo-ku, Tokyo, 112-8610, Japan, and ¶Mitsubishi Kasei Institute of Life Sciences, 11 Minamiooya, Machida, Tokyo 194-8511, Japan

It has been proposed that the cortical actin filament networks act as a cortical barrier that must be reorganized to enable docking and fusion of the synaptic vesicles with the plasma membranes. We identified a novel neuron-associated developmentally regulated protein, designated as Nadrin. Expression of Nadrin is restricted to neurons and correlates well with the differentiation of neurons. Nadrin has a unique structure; it contains a GTPase-activating protein (GAP) domain for Rho family GTPases, a potential coiled-coil domain, and a succession of 29 glutamines. In vitro the GAP domain activates RhoA, Rac1, and Cdc42 GTPases. Expression of Nadrin in NIH3T3 cells markedly reduced the number of the actin stress fibers and the formation of the ruffled membranes, suggesting that Nadrin regulates actin filament reorganization. In PC12 cells, Nadrin colocalized with synaptotagmin in the neurite termini and also with cortical actin filaments in the subplasmalemmal regions. Expression of Nadrin or its mutant composed of the coiled-coil and GAP domain enhanced Ca2+-dependent exocytosis of PC12 cells, but a mutant lacking the GAP domain inhibited exocytosis. These results suggest that Nadrin plays a role in regulating Ca2+-dependent exocytosis, most likely by catalyzing GTPase activity of Rho family proteins and by inducing the reorganization of the cortical actin filaments.

Neurotransmitter release, a fundamental step in the process of synaptic transmission, is accomplished by the rapid membrane fusion of neurotransmitter-filled synaptic vesicles with the target plasma membrane (1, 2). The actin cytoskeleton has been proposed to play a number of roles in regulated exocytosis, particularly in endocrine and neural cells (3, 4). Morphological studies on chromaffin and neural cells demonstrated that most of the secretory vesicles are positioned at a distance of ~250 nm from the plasma membranes, suggesting the presence of a physical barrier to the movement of secretory vesicles toward the release site on plasma membranes (5–7). Localization of actin filaments using anti-actin antibodies or fluorescence-labeled phalloidin on the secretory cells has shown the presence of actin filament networks underneath the presynaptic plasma membranes and disassembly of the cortical actin filament network upon activation of the secretory cells (7–10). It has been proposed that the cortical actin filament networks act as a reservoir of vesicles ready for docking at the release sites and as a cortical barrier that must be reorganized to enable docking and fusion of synaptic vesicles with plasma membranes. Although several actin-depolymerizing proteins that mediate the actin filament disassembly, such as scinderin and gelsolin, are proposed to regulate filament disassembly and exocytosis (11–13), the molecular mechanisms underlying the precise regulation of actin filament networks during neurotransmitter release remain unknown.

The Ras superfamily of small GTP-binding proteins are molecular switches that regulate numerous cellular functions by controlling intracellular signaling events (14). The Rho family of GTP-binding proteins, consisting of Rho, Rac, and Cdc42 proteins, regulate a number of cellular functions that require the reorganization of actin-based structures (15, 16). Recent investigations lead to the idea that Rho family proteins are also involved in signaling pathways that control actin filament reorganization during exocytosis. In chromaffin cells, RhoA is specifically associated with the membrane of secretory chromaffin granules and is suggested to control the priming of exocytosis by modifying the cortical actin network (17, 18). In mast cells, activation of GTP-binding proteins by GTPγS induces reorganization of actin filaments (19). The expression of constitutively active mutants of either RhoA, Rac, Cdc42 proteins enhanced regulated exocytosis, and inhibition of endogenous Rac and Rho activities reduces the secretory response (20–22). These observations suggest that Rac and Rho are components of the signaling pathways that lead to the cytoskeleton reorganization necessary for exocytosis.

The GTP-binding proteins cycle between an active GTP-bound form and an inactive GDP-bound form. This process is regulated by three types of factors: guanine nucleotide exchange factors, which stimulate the interconversion of the GDP-bound inactive form to GTP-bound active form; guanine nucleotide dissociation inhibitors, which inhibit this reaction; and GTPase-activating proteins (GAPs), which stimulate conversion from the GTP-bound form to GDP-bound form (14, 23). GAPs for Rho family of GTP-binding proteins belong to a family whose members share significant sequence homology in a conserved GAP domain, the RhoGAP domain (14). More than 15

* 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.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB042827.

¶ To whom correspondence should be addressed. Tel.: 81-3-3823-2101 (ext.5419); Fax: 81-3-3823-2130; E-mail: umeda@rinshoken.or.jp.

1 The abbreviations used are: GTPγS, guanosine 5'-3-O-(thio)triphosphate; BSA, bovine serum albumin; GAP, GTPase-activating protein; GFP, green fluorescent protein; GH, growth hormone; GST, glutathione S-transferase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.
proteins containing RhoGAP domains have been identified in mammalian cells, and these proteins are suggested to serve as downstream effectors in signal transduction events as well as controlling the activities of Rho family proteins (14, 24, 25). Among these regulators of GTP-binding proteins, Rho guanine nucleotide dissociation inhibitors was shown to be involved in regulated exocytosis (26), but the roles of GAPs or guanine nucleotide exchange factors in the process of exocytosis remain unknown.

We report here the identification of a novel neuron-associated developmentally regulated protein, Nadrin. Nadrin contains the conserved GAP domain that is active on the Rho family proteins, and it colocalized with the cortical actin filaments in PC12 cells. Expression of either the entire protein or various domains of Nadrin in PC12 cells cotransfected with growth hormone strongly affects high K+-induced growth hormone secretion. The expression of Nadrin is restricted to neuronal cells and is highly correlated with maturation of the central nervous system. We propose that Nadrin plays an important role in controlling neurotransmitter release by regulating reorganization of the cortical actin filament network in nerve endings.

EXPERIMENTAL PROCEDURES

Cloning of Nadrin cDNA—A agt11 cDNA library constructed from the brain of a 3-wk-old female Wistar rat was screened by using a monoclonal antibody 3A10, as described previously (27). Positive clones were purified by successive rounds of plaque purification. cDNA inserts were subcloned into a pBluescript vector (Stratagene, La Jolla, CA). The library was rescreened with a partial cDNA probe of Nadrin from clone N (Ref. 27; GenBank accession number AF022966) by DNA hybridization. Positive clones were purified, and cDNA inserts were subcloned into a pBluescript vector. The samples were sequenced on an ALFExpress DNA sequence automated DNA cycle sequencing kit (Amersham Pharmacia Biotech) using an AutoCycle sequencing kit (Amersham Pharmacia Biotech). Sequence analyses were performed using Genetix Version 10.0 (Software Development Co., Ltd., Tokyo, Japan), and data base searches were performed using BLAST (28) and FASTA programs (29). The Nadrin amino acid sequence was analyzed using the COILS version 2.2 (30) and the PEST finder programs (31). A GST library—An expression vector for the GAP domain of Nadrin fused to glutathione S-transferase (GST) was made by subcloning the cDNA fragment encoding amino acid residues 218–482 of Nadrin into a pGEX-vector (Amersham Pharmacia Biotech). An expression vector for the GAP domain of 3BP-1 (amino acids 184–420) (32) was made similarly. GST fusion proteins were expressed in DH5α Escherichia coli cells, purified by affinity chromatography on glutathione-Sepharose column (Amersham Pharmacia Biotech). Monoclonal antibody 1D12 (25) was used to detect the fusion protein by Western blotting using ECL Western blotting detection reagents (Amersham Pharmacia Biotech).

Cell Culture—Subcloned PC12 cells (kindly provided by Dr. Y. Fukui, The University of Tokyo) were grown in Dulbecco’s modified minimum essential medium (Asahi Techno Glass Co., Tokyo, Japan) with 10% horse serum (Life Technologies, Inc.) and 2% fetal bovine serum (JRH Biosciences, Lenexa, KS). NIH3T3 cells were grown in Dulbecco’s modified minimum essential medium with 5% calf serum (JRH Biosciences). Neuronal and glial cell cultures were performed as described previously (27).

Construction of Expression Vectors—The cDNA constructs encoding the C-terminal GFP-tagged variants of Nadrin (amino acids 1–780), domain 1 + II (amino acids 1–465), domain I (amino acids 1–233), and domain III (amino acids 463–780), were generated by PCR using primers that engineered five XhoI and three BamHI restriction sites into XhoI and BamHI-digested pEGFP-N1 (CLONTECH Laboratories, Inc., Palo Alto, CA). To create a Nadrin mutant that lacks GAP activity, a point mutation was introduced so as to alter Arg-288, which is suggested to be required for the catalytic activity of GAP (35, 36) to Ala by overlap PCR (37). The PCR products were digested with XhoI and BamHI and ligated into pEGFP-N1 (CLONTECH Laboratories, Inc., Palo Alto, CA) to generate a Nadrin expression vector using TransFast reagent according to the manufacturer’s instructions (Promega). The cDNA inserts were transfected into NIH3T3 cells using lipofectAMINE according to the manufacturer’s instructions (Life Technologies, Inc.). The transfected cells were plated onto glass coverslips coated with poly-l-lysine (100 μg/ml, Sigma) and then cultured for 1 day or treated for 2 days with 50 ng/ml nerve growth factor (Chemicon International Inc., Temecula, CA) to induce cell differentiation. Immunocytochemistry was performed as described previously (38). Briefly, the cells were fixed with 3.7% formaldehyde for 15 min and washed three times with PBS and blocked PBS containing 2% BSA (2% BSA-PBS) for 30 min at room temperature. For detection of synaptotagmin, the fixed cells were permeabilized with PBS containing 0.1% Triton X-100 for 4 min. After being washed with PBS, the cells were incubated with monoclonal antibody 1D12 (25 μg/ml) in 2% BSA-PBS for 1 h at 4 °C. The cells were then washed with PBS and incubated with Cy3-conjugated goat-anti mouse IgG (Amersham Pharmacia Biotech) diluted 1/400 with 2% BSA-PBS for 1 h at room temperature. For actin filament staining, fixed cells were incubated with tetramethylrhodamine B isothiocyanate-labeled phalloidin (Sigma) (1/200 dilution PBS) for 45 min at room temperature. The cells were then washed five times in PBS and mounted on microscope slides for observation and photography. The cells were examined with a confocal imaging system (LSM510; Carl Zeiss, Oberkochen, Germany). NIH3T3 cells were transfected with recombinant plasmids and grown overnight. The cells were transfected with 2 μg of GAP-Nadir expression vector using TransFast reagent according to the manufacturer’s instructions (Promega). After 1 day, transfected cells were seeded onto glass coverslips and cultured for 1 day. Immunocytochemistry was performed as described above. Immunofluorescence microscopy was carried out using an Axiosvert 135 (Carl Zeiss).
Nadrin, a Neuron-specific GTPase-activating Protein

RESULTS AND DISCUSSION

Cloning of Nadrin with GAP Activity on the Rho Family Proteins—In a previous study we showed that monoclonal antibody 3A10 recognizes a series of developmentally expressed brain proteins with molecular masses of 150-, 120-, 118-, 106-, 104-, 79-, and 77-kDa, whose expression is correlated well with the maturation of the central nervous system (27). We purified the 79- and 77-kDa 3A10 antigens and identified them as synapsin Ia and Ib, respectively, by analysis of the sequences of peptide fragments (27). In this study, we cloned a gene encoding a 3A10-reactive 104-kDa protein by immunological screening of expression libraries constructed from adult rat brains. We designated the protein encoded by the gene as Nadrin (neuron-associated developmentally regulated protein) because its expression is neuron-specific and developmentally regulated (see below).

As shown in Fig. 1A, Nadrin cDNA encodes a 780-amino acid protein composed of three distinct domains that are schematically indicated in Fig. 1B. Primary sequence alignments indicated that domains I and II of Nadrin (amino acids 1–458) share 51% identity to mouse 3BP-1 (amino acids 1–406) (32) and 62% identity to the human KIAA0672 protein (amino acids 1–470) (41). Domain I (amino acids 1–248) contains the predicted coiled-coil structure, and domain II is composed of a RhoGAP domain. The overall structure of Nadrin is approximately 50%. In addition, domain III contains one region of 29 successive glutamines (amino acids 596–624) and one SH3 binding motif (amino acids 704–710) (49), and one potential PEST sequence (amino acids 653–679) (31), two nuclear localization signals (amino acids 479–481, 552–557) (49), and one potential SH3 binding motif (amino acids 704–710) (50).

Subcellular Fractionation— Cultures of PC12 cells (2–3 × 10^5 cells) were plated on ice and rinsed and resuspended in SET buffer containing 1 mM phenylmethanesulfonyl fluoride and a protease inhibitor mixture (Complete™, Life Technologies, Inc.) according to the manufacturer’s instructions. GH release experiments were performed 72 h after transfection. PC12 cells were washed with Tyrode’s-HEPES (20 mM HEPES (pH 7.4), 137 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM CaCl_2, 1 mM MgCl_2) and incubation for 4 min with low K^+ buffer (Tyrode’s-HEPES containing 137 mM NaCl, 5 mM KCl) or high K^+ buffer (Tyrode’s-HEPES containing 87 mM NaCl, 55 mM KCl). The amounts of GH released into the medium and retained in the cells were measured using a radioimmunoassay kit (Nichols institute, San Juan Capistrano, CA) or an immunoenzymatic assay kit (Toho Co., Tokyo, Japan). Secretion was expressed as a percentage of GH amounts released into medium relative to the total cellular GH amounts (40).

Subcellular Fractionation— Cultures of PC12 cells (2–3 × 10^5 cells) were plated on ice and rinsed and resuspended in SET buffer containing 1 mM phenylmethanesulfonyl fluoride and a protease inhibitor mixture (Complete™, Life Technologies, Inc.). Cells were homogenized with 20 strokes of a Teflon glass homogenizer and then centrifuged for 30 min at 100,000 × g. The supernatant was saved (cytosol), and the pellet was homogenized with a Teflon glass homogenizer in one of the extraction buffers. The suspension was then incubated for 30 min at 4 °C and centrifuged for 30 min at 100,000 × g. The supernatant and pellet were saved and dissolved in sodium dodecyl sulfate sample buffer. The extraction buffers were SET buffer containing the inhibitors mentioned above and one of 0.5% Triton X-100, 0.5% deoxycholate, or 0.5 M KCl.

In a previous study we showed that monoclonal antibody 3A10 recognizes a series of developmentally expressed brain proteins with molecular masses of 150-, 120-, 118-, 106-, 104-, 79-, and 77-kDa, whose expression is correlated well with the maturation of the central nervous system (27). We purified the 79- and 77-kDa 3A10 antigens and identified them as synapsin Ia and Ib, respectively, by analysis of the sequences of peptide fragments (27). In this study, we cloned a gene encoding a 3A10-reactive 104-kDa protein by immunological screening of expression libraries constructed from adult rat brains. We designated the protein encoded by the gene as Nadrin (neuron-associated developmentally regulated protein) because its expression is neuron-specific and developmentally regulated (see below).

As shown in Fig. 1A, Nadrin cDNA encodes a 780-amino acid protein composed of three distinct domains that are schematically indicated in Fig. 1B. Primary sequence alignments indicated that domains I and II of Nadrin (amino acids 1–458) share 51% identity to mouse 3BP-1 (amino acids 1–406) (32) and 62% identity to the human KIAA0672 protein (amino acids 1–470) (41). Domain I (amino acids 1–248) contains the predicted coiled-coil structure, and domain II is composed of a RhoGAP domain. The overall structure of Nadrin is approximately 50%. In addition, domain III contains one region of 29 successive glutamines (amino acids 596–624) and one SH3 binding motif (amino acids 704–710) (49), and one potential PEST sequence (amino acids 653–679) (31), two nuclear localization signals (amino acids 479–481, 552–557) (49), and one potential SH3 binding motif (amino acids 704–710) (50).
The sequence analyses suggest that Nadrin functions as a GAP for members of Rho-family small GTP-binding proteins. To examine the GAP activity of Nadrin on proteins of Rho family, the GAP domain (amino acids 218–482) of Nadrin was produced as a GST fusion protein in E. coli and was assayed for its ability to activate the intrinsic GTPase activity of GTP-bound RhoA, Rac1, and Cdc42. As shown in Fig. 2A, the GAP domain was able to stimulate the intrinsic GTPase activity of RhoA, Cdc42, and Rac1. In a parallel analysis, the GAP domain of 3BP-1 stimulated the GTPase activity of Rac1 and Cdc42 but not that of RhoA (data not shown), which is consistent with previously published data (32). Overexpression of Nadrin in NIH3T3 cells markedly reduced the number of actin stress fibers and formed the ruffled membranes (Fig. 2B). These results clearly demonstrate that Nadrin functions as a GAP for Rho family proteins and as a regulator of cellular actin filament organization.

Developmentally Regulated Expression of Nadrin in Rat Brain—Tissue distribution of Nadrin in adult rats was analyzed by using a rabbit polyclonal antibody raised against a synthetic peptide composed of amino acids 562–580. The anti-Nadrin antibody bound specifically to a 104-kDa protein when expressed in COS-7 cells (data not shown). In adult rat tissues, the 104-kDa protein band was specifically detected in a brain homogenate but not in other tissues, indicating that Nadrin is specifically expressed in brain (Fig. 3A). The expression of Nadrin was dependent on the developmental stage of the brain; Nadrin became detectable at the second postnatal week in the cerebral cortex and hippocampus and at the third postnatal week in the cerebellum and olfactory bulb (Fig. 3B). The expression level was maximal during the third and fourth postnatal weeks and remained high during adulthood. To examine whether Nadrin is expressed in neuronal or glial cells, primary cultures of neuronal and glial cells were established from the cerebral cortex of E18 rat brain. The neuronal and glial cultures consisted of 80% neurons and more than 95% glia, based on the immunocytochemical criteria for the expression of neuron-specific enolase and glial fibrillary acidic protein (27) (data not shown). Nadrin became detectable on the 14th day of neural cell cultivation, and the level of Nadrin expression increased during the culture period in neuronal cells (Fig. 4A). The expression of Nadrin in neuronal cells correlated well with the expression of neuron-specific enolase and synapsin I (27) (data not shown). Nadrin was not detected in glial cells (Fig. 4A). These results clearly indicate that Nadrin is a neuron-specific protein, and its expression is closely correlated with neuronal differentiation.

During development of the central nervous system, an in-
crease in the expression of various synaptic proteins, such as synaptotagmin I, synapsin I, Rab3A, and Rab guanine nucleotide dissociation inhibitors, correlated well with axon terminal differentiation and maturation of neuronal connectivity (51–54). These proteins function in the regulated exocytosis of synaptic vesicles (1). To investigate the potential role of Nadrin in exocytosis, a GFP-tagged variant of Nadrin (Nadrin-GFP) was expressed in PC12 cells, and its localization was compared with that of synaptotagmin I, which was detected by an anti-synaptotagmin monoclonal antibody 1D12 (34). Immunoblotting analysis showed that the anti-Nadrin antibody bound specifically to a 104-kDa band in PC12 cells, indicating that Nadrin is present in PC12 cells (data not shown). Fig. 4B shows a single confocal optical plane that illustrates the punctate appearance of Nadrin and synaptotagmin throughout the cytoplasm of the cell. An intense co-localization of Nadrin with synaptotagmin was observed in the neurite terminals. These data on the regional and developmental stage-specific expression of Nadrin suggest that Nadrin may have a function in regulating neurotransmitter release at the synaptic terminal.

**Involvement of Nadrin in Ca\(^{2+}\)-dependent Exocytosis**—To examine whether Nadrin functions in exocytosis, PC12 cells were co-transfected with human GH and Nadrin, and the high K\(^+\)-induced secretion of co-expressed GH was determined. The expressed GH is known to be stored in dense core vesicles and to be released in response to high K\(^+\) in the presence of extracellular Ca\(^{2+}\) (39, 40, 55). The basal secretion of GH, expressed as a percentage of the amount of GH released into medium containing 4.7 mM K\(^+\) relative to total cellular GH, is not significantly different between Nadrin-transfected cells and control cells transfected only with GH. In this assay system, an average of 37 ± 3.3% GH was secreted upon high K\(^+\) stimulation and the GH secretion was slightly, but significantly, enhanced in Nadrin-transfected cells (Fig. 5A, a). The mutant comprised of domain I significantly inhibited the release (Fig. 5A, a). It has been shown that Arg residue, which is highly conserved in GAP domains, is required for their catalytic activity (35, 36). The equivalent residue in Nadrin is Arg-288 and replacement of Arg-288 with Ala in the GAP domain of Nadrin reduced the GAP activity to one-fifth the original activity (data not shown). We replaced Arg-288 of Nadrin with Ala (Nadrin

---

**Fig. 4. Neuron-specific expression of Nadrin.** A, expression of Nadrin in cultured neuronal and glial cells was analyzed by immunoblotting with the anti-Nadrin antibody. B, co-localization of Nadrin with synaptotagmin at the neurite terminals. GFP-tagged Nadrin was expressed in differentiated PC12 cells. Distribution of the GFP-tagged Nadrin and synaptotagmin is shown by double-labeling in confocal images. Superimposed (merge) images demonstrate the overlapping distribution of these proteins at the neurite terminals. Scale bar = 20 μm.

**Fig. 5. Involvement of Nadrin in Ca\(^{2+}\)-dependent exocytosis.** A, effect of Nadrin and Nadrin mutants on GH secretion from PC12 cells. PC12 cells were co-transfected with pSI4GH, which encodes human GH, and pEGFP-N1, which contains the indicated cDNAs encoding Nadrin (amino acids 1–780), domain I (amino acids 1–233), domain I + II (amino acids 1–465) (a) and Nadrin R288A (a Nadrin mutant altered Arg-288 to Ala) (b). GH secretion was induced with a low K\(^+\) solution (5.6 mM KCl) or a high K\(^+\) solution (55 mM KCl) in the presence of extracellular Ca\(^{2+}\) (1 mM). The GH secretion is expressed as a percentage of total GH content. Data are representative of four independent measurements, and statistically significant differences (p < 0.01) are marked by asterisks. B, intracellular localization of Nadrin. PC12 cells expressing GFP-tagged Nadrin were fixed and stained with tetramethylrhodamine B isothiocyanate-labeled phalloidin. Distribution of the GFP-tagged Nadrin, F-actin, and the superimposed images is shown by double-labeling in confocal images. Arrows indicate the regions where Nadrin colocalizes with F-actin. Scale bar = 5 μm. C, subcellular fractionation of Nadrin. Homogenates of PC12 cells were centrifuged to separate the cytosol (S) and pellet (P). The resulting pellet was solubilized with either 0.5% Triton X-100 (Tx), 0.5 mM KCl, or 0.5% deoxycholate (Doc), followed by centrifugation. The presence of Nadrin in each supernatant (S) and pellet (P) fraction was examined by immunoblotting using an anti-Nadrin antibody.
Nadrin, a Neuron-specific GTPase-activating Protein

R288A) and transfected the mutant Nadrin into PC12 cells. No significant alteration in the high K+-induced exocytosis was observed with Nadrin R288A-transfected cells, suggesting that GAP activity is required for the enhancement of exocytosis by Nadrin-expression (Fig. 5A, b).

Since Nadrin is already present in PC12 cells and was not significantly overexpressed in PC12 cells in our assay conditions (data not shown), it is difficult to see the additive effect of the expressed Nadrin on exocytosis. In contrast, significant inhibitory effect was observed when the domain I (coiled-coil rich) domain was expressed in PC12 cells. Several studies have shown that the coiled-coil domain plays a critical role in formation of a ternary core complex containing synaptobrevin, syntaxin, and SNAP25, which is central to the process of synaptic vesicle docking and fusion (56). It is possible that the expression of the coiled-coil-rich domain I of Nadrin may disturb the structural organization of the exocytosis complex, which inhibits the Ca2+-dependent release of GH.

Analyses of the subcellular localization of the GFP-tagged Nadrin in PC12 cells showed a punctate appearance of Nadrin throughout the cytoplasm. Comparison of the distribution of Nadrin and F-actin labeled by tetramethylrhodamine B isothiocyanate-labeled phalloidin showed that there are clearly several subplasmalemmal regions where Nadrin and actin filaments were colocalized (indicated by arrows in Fig. 5B). To test biochemically whether the endogenous Nadrin was associated with the cytoskeletal components, cell lysates were incubated with Triton X-100 to solubilize membranes, and the Triton-insoluble cytoskeleton was pelleted by centrifugation (57, 58). Nadrin was predominantly present in the 100,000 × g-precipitated fraction, and the Triton-treatment did not release endogenous Nadrin into the supernatant, whereas 0.5 M KCl treatment readily released Nadrin (Fig. 5C). These results suggest that Nadrin was associated with cytoskeletal components in PC12 cells. We did not observe a significant change in the distribution of actin filaments after the expression of Nadrin in PC12 cells, although Nadrin regulates actin filament reorganization when expressed in NIH3T3 cells (Fig. 2B). Based on these observations, we proposed that Nadrin is involved in Ca2+-dependent exocytosis, most likely by catalyzing GTPase activity of Rho family proteins and by inducing reorganization of the cortical actin filaments.

The precise mechanisms by which Nadrin exerts its function in vivo remain to be elucidated. The C-terminal serine/threonine/proline-rich domain III of Nadrin contains a polyglutamate repeat and two nuclear localization signals. Recent studies (59, 60) show that a number of inherited neurodegenerative diseases are characterized by expanded polyglutamine repeats within the coding sequence of the disease gene. To begin the process of investigating the relationship of Nadrin with these diseases, human cDNA showing a high similarity to Nadrin was amplified by reverse transcription-PCR, and the deduced amino acid sequence was compared with that of rat Nadrin. The polyglutamine repeat was deleted from the human Nadrin cDNA, although other coding regions showed extensive homology between rat and human Nadrin (data not shown). Additional genetic studies, including chromosomal mapping and mutational analyses of Nadrin, are now under way to clarify the involvement of Nadrin in these diseases.

Concerning the functional role of the domain III of Nadrin, our recent experiments showed that the GFP-tagged domain III expressed in 3T3 fibroblasts localized specifically in the nucleus, which suggests a function for Nadrin in the nucleus (data not shown). The discovery of a novel brain-specific multifunctional protein may provide valuable information concerning the regulatory mechanisms of the actin cytoskeleton during exocytosis and may generate an interesting paradigm for possible cross-talk between synaptic terminals and the nucleus.

Acknowledgments—We thank Dr. Yoshitaka Ono and Dr. Hideki Shihata (Kobe University) for their advice on measuring GAP activity. We also thank Dr. Donald M. Marcus (Baylor College of Medicine, Houston, Texas) for helpful comments during preparation of the manuscript.

REFERENCES
1. Calakos, N., and Scheller, R. H. (1996) J. Biol. Chem. 271, 1429–1442
2. Brown, A. M., O’Sullivan, A. J., and Gomperts, B. D. (1996) Mol. Biol. Cell 7, 1053–1063
3. Trifaro, J. M., Glavinovic, M., and Gomperts, B. D. (1996) Mol. Biol. Cell 7, 1429–1442
4. Aunis, D., and Bader, M. F. (1996) J. Biol. Chem. 272, 3361–3368
5. Kaneda, M., Takeuchi, K., Inoue, K., and Umeda, M. (1997) J. Biochem. 122, 655–660
6. Leung, T. H., Man, K., and Bialek, M. (1995) J. Biol. Chem. 270, 533–537
7. Lee, R. W. H., and Trifaro, J. M. (1981) Neuron 1, 49–60
Nadrin, a Neuron-specific GTPase-activating Protein 36891

47. Settleman, J., Narasimhan, V., Foster, L. C., and Weinberg, R. A. (1992) Cell 69, 539–549
48. Barfod, E. T., Zheng, Y., Kuang, W. J., Hart, M. J., Ervins, T., Cerione, R. A., and Ashkenazi, A. (1993) J. Biol. Chem. 268, 26059–26062
49. LaCasse, E. C., and Lefebvre, Y. A. (1995) Nucleic Acids Res. 23, 1647–1656
50. Pawson, T. (1995) Nature 373, 573–580
51. Daly, C., and Ziff, E. B. (1997) J. Neurosci. 17, 2365–2375
52. Lou, X. J., and Biaby, J. L. (1995) Mol. Cell. Neurosci. 6, 252–262
53. Casticas, S., Larhammar, D., Blomqvist, A., Sanna, P. P., Milner, R. J., and Wilson, M. C. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 765–769
54. Ikonomov, O. C., Kulesa, M. C., Shisheva, A. C., and Jacob, M. H. (1998) J. Neurosci. 18, 6331–6339
55. Orita, S., Sasaki, T., Komuro, R., Sakaguchi, G., Maeda, M., Igarashi, H., and Takai, Y. (1996) J. Biol. Chem. 271, 7257–7260
56. Lin, R. C., and Scheller, R. H. (1997) Neuron 19, 1087–1094
57. Seastone, D. J., Lee, E., Bush, J., Knecht, D., and Cardelli, J. (1998) Mol. Biol. Cell 9, 2891–2904
58. Chasserot-Golaz, S., Hubert, P., Thierse, D., Dirrig, S., Vlahos, C. J., Aunis, D., and Bader, M. F. (1998) J. Neurochem. 70, 2347–2356
59. The Huntington’s Disease Collaborative Research Group (1993) Cell 72, 971–983
60. Paulson, H. L., and Fischbeck, K. H. (1996) Annu. Rev. Neurosci. 19, 79–107