NIDD, a Novel DHHC-containing Protein, Targets Neuronal Nitric-oxide Synthase (nNOS) to the Synaptic Membrane through a PDZ-dependent Interaction and Regulates nNOS Activity*

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Targeting of neuronal nitric-oxide synthase (nNOS) to appropriate sites in a cell is mediated by interactions with its PDZ domain and plays an important role in specifying the sites of reaction of nitric oxide (NO) in the central nervous system. Here we report the identification and characterization of a novel nNOS-interacting DHHC domain-containing protein with dendritic mRNA (NIDD) (GenBank™ accession number AB098078), which increases nNOS enzyme activity by targeting the nNOS to the synaptic plasma membrane in a PDZ domain-dependent manner. The deduced NIDD protein consisted of 392 amino acid residues and possessed five transmembrane segments, a zinc finger DHHC domain, and a PDZ-binding motif (~EDIV) at its C-terminal tail. In vitro pull-down assays suggested that the C-terminal tail region of NIDD specifically interacted with the PDZ domain of nNOS. The PDZ dependence was confirmed by an experiment using a deletion mutant, and the interaction was further confirmed by co-sedimentation assays using COS-7 cells transfected with NIDD and nNOS. Both NIDD and nNOS were enriched in synaptosome and synaptic plasma membrane fractions and were present in the lipid raft and postsynaptic density fractions in the rat brain. Co-localization of these proteins was also observed by double staining of the proteins in cultured cortical neurons. Thus, NIDD and nNOS were co-localized in the brain, although the co-localizing regions were restricted, as indicated by the distribution of their mRNA expression. Most important, co-transfection of NIDD and nNOS increased NO-producing nNOS activity. These results suggested that NIDD plays an important role in the regulation of the NO signaling pathway at postsynaptic sites through targeting of nNOS to the postsynaptic membrane.

Nitric oxide (NO)1 works as a volatile messenger and plays important physiological roles in the central nervous system (1, 2). N-Methyl-D-aspartate-type glutamate receptor (NMDAR) plays a critical role in synaptic plasticity, the basis for learning and memory. NMDAR-mediated increases in intracellular Ca2+ trigger various cellular responses, such as activation of the Ras-mitogen-activated protein kinase pathway, which transmits the glutamatergic signal to the nucleus and ultimately leads to long lasting neuronal responses (3). Recent studies (4–6) have suggested that NO is a key molecule linking the NMDAR-mediated increase in cytoplasmic Ca2+ and long term potentiation (LTP), a physiological model for learning and memory. NO is thought to mediate synaptic plasticity in the hippocampus and influence LTP and thus enhance memory formation. The role of NO in memory formation is also suggested by the observation that the formation of olfactory memory is dependent on NO production (7). In the brain, the production and release of NO is induced by glutamate acting on both NMDAR and a-arginine-dependent neuronal NO synthase (nNOS) (12). nNOS is unique compared with other NO synthases (NOSs), such as inducible NOS and epithelial NOS (eNOS), in that it contains a PDZ (postsynaptic density protein 95/discs-large/zona occlusens-1) domain at its N terminus. This domain may play a role in the precise localization of nNOS within the cell.

We have surveyed the postsynaptic density (PSD) fraction-associated mRNAs by random amplification and sequencing (13). Among them, the Dem2A0-5 cDNA fragment of 148 bp was not identified by our initial data base search. In this study, we cloned a full-length Dem2A0-5 cDNA and re-named it a novel nNOS-interacting DHHC domain-containing protein.

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1 The abbreviations used are: NO, nitric oxide; CAPON, C-terminal PDZ ligand of nNOS; His-NIDD, His-tagged NIDD; LTP, long term potentiation; NIDD, nNOS-interacting DHHC domain-containing protein with dendritic mRNA; NIDD-CT, C-terminal NIDD; NIDD-del3, NIDD-CT deletion mutant; NIDD-NT, N-terminal NIDD; NMDAR, N-methyl-D-aspartate type glutamate receptor; nNOS, neuronal nitric-oxide synthase; PBS, phosphate-buffered saline; PDZ, postsynaptic density protein 95/discs-large/zona occlusens-1; PSD, postsynaptic density; SPM, synaptic plasma membrane; nt, nucleotide; eNOS, epithelial NOS; GST, glutathione S-transferase; RT, reverse transcriptase.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB098078 and AB176831.

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29461
with dendritic mRNA (NIDD) (GenBank™ accession number AB998078). We also report the regulation by NIDD of nNOS activity via targeting of nNOS to the postsynaptic membrane through a PDZ domain-dependent interaction. Our results suggest that NIDD plays an important role in the regulation of synaptic plasticity by specifying the NO signaling pathway at postsynaptic sites.

EXPERIMENTAL PROCEDURES

Materials—pGEM-T easy vector was purchased from Promega (Madison, WI). Polyclonal rabbit anti-nNOS, monoclonal mouse anti-nNOS, monoclonal mouse anti-eNOS, mouse anti-PFD-95, and anti-flotillin-1 antibodies were from Transduction Laboratories (Lexington, KY). Anti-SAP97 antibody was from Stressgen (Victoria, British Columbia, Canada); mouse anti-His G antibody was from Invitrogen; anti-actin antibody was from Sigma; secondary antibodies raised against rabbit and mouse IgG and conjugated with horseradish peroxidase were from Calbiochem and Cappel (West Chester, PA), and those conjugated with rhodamine and fluorescein isothiocyanate were from Molecular Probes (Eugene, OR). The Maxi-DNA purification kit was from QIagen (Tokyo, Japan), and polyleucineimine-coated slide glasses were from Iwaki Glass (Funabashi, Japan). Neurobasal medium supplemented with B27, pcDNA3/His Max, pcDNA3.1, and LipofectAMINE 2000 were from Invitrogen. Calcium ionophore A23187 and the nNOS detection kit were from Calbiochem and Research. Rabbit anti-NIDD antiserum was raised against a fusion protein of GST with NIDD-NT (GST-NIDD-NT). Antibody was affinity-purified with GST-NIDD-NT chemically immobilized to glutathione-Sepharose 4B after removal of anti-GST antibody with GST-glutathione-Sepharose 4B. Western blotting was performed as described previously (18, 19).

Subcellular Fractionation and Sucrose Density Gradient Floation Assay—Subcellular fractions were prepared from the forebrains of Wistar rats (6 weeks old, male) as described previously (18, 19). For the subcellular fraction density gradient flotation assay, proteins (500 μg) of the SPM fraction were suspended by repeated passage through a 26-gauge needle in 1.85 ml of TNE buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA). The sample was then centrifuged at 15,000 x g for 15 min and 1:10 dilution in TEE buffer, the supernatant was incubated at 4 °C for 2 h with 30 μg of GST fusion proteins or GST alone coupled to glutathione-Sepharose 4B. After incubation, the pulled down materials were washed four times, and the bound proteins were analyzed by Western blotting. The pull-down assays for PSD-95, SAP97, and GRIP were performed using PSD proteins (20 μg) as described previously (16). Generation of Affinity-purified Anti-NIDD Antibody and Western Blotting—PSD proteins (20 μg) were immobilized to glutathione-Sepharose 4B. After removal of anti-GST antibody with GST-glutathione-Sepharose 4B, Western blotting was performed as described previously (18, 19).

Cell Culture and Transfection of cDNAs—The cerebral cortex or hippocampus of rats (embryonic day 18, Wistar) was dissociated and plated onto a polyethyleneimine-coated glass slide. Neurons were cultured essentially as described previously (20) in Neurobasal medium supplemented with B27, COS-7 cells were cultured as described previously (14). cDNAs encoding full-length NIDD, nNOS, and eNOS were amplified by PCR and inserted into appropriate restriction sites of mammalian expression vectors, pcDNA4/His Max and pcDNA3.1, respectively. The primer sets used as follows: CGCGGATCCATGGAAGAGAACACGTTTG and CGCTCGAGTTAAGGGCCATTACCCAG for NIDD-NT, amino acids 354–392; CGCGGATCCATGGAAGAGAACACGTTTG and CGCTCGAGTTAAGGGCCATTACCCAG for NIDD-CT, amino acids 354–392; CGCGGATCCATGGAAGAGAACACGTTTG and CGCTCGAGTTAAGGGCCATTACCCAG for NIDD-DE, amino acids 354–380; and CGGAATTCATGGAAGAGAACACGTTTG and CGCTCGAGTTAAGGGCCATTACCCAG for NIDD-DE, amino acids 1–159. The restriction enzyme sites introduced are underline. GST-NIDD-CT and GST-NIDD-DE3 were solubilized as described previously (17); in brief, broken and pulled-down pellets were solubilized in phosphate-buffered saline (PBS) containing 1 mM EDTA, 0.2% sodium N-lauryl sarcosinate, 15 mM diethiothreitol, and 3% Triton X-100.

For the nNOS pull-down assay, one adult rat cerebral cortex (250 mg) was homogenized in 2.5 ml of Tris-HCl buffer (25 mM, pH 7.4) containing 1 mM EDTA, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride (PMFSF) and then solubilized by addition of 20 μl of 3% Triton X-100 on a rocking platform at 4 °C for 1 h. For the nNOS pull-down assay, proteins (200 μg) of the synaptic plasma membrane (SPM) fraction were solubilized with 1% Triton X-100 at 4 °C for 1 h. After clarification by centrifugation at 15,000 x g for 15 min and 1:10 dilution in TEE buffer, the supernatant was incubated at 4 °C for 2 h with 30 μg of GST fusion proteins or GST alone coupled to glutathione-Sepharose 4B. After incubation, the pulled down materials were washed four times, and the bound proteins were analyzed by Western blotting. The pull-down assays for PSD-95, SAP97, and GRIP were performed using PSD proteins (20 μg) as described previously (16).

Production of Glutathione S-transferase (GST) Fusion Constructs and Pull-down Assay—Fusion constructs were generated by inserting appropriate restriction fragments in-frame into pGEX-T4-1. GST fusion proteins were expressed in Escherichia coli strain BL21 cells and purified according to the manufacturer’s protocol. The GST-nNOS fusion protein was analyzed by Western blotting with rabbit and mouse IgG and conjugated with horseradish peroxidase were from Calbiochem and Cappel (West Chester, PA), and those conjugated with rhodamine and fluorescein isothiocyanate were from Molecular Probes (Eugene, OR). The Maxi-DNA purification kit was from QIagen (Tokyo, Japan), and polyleucineimine-coated slide glasses were from Iwaki Glass (Funabashi, Japan). Neurobasal medium supplemented with B27, pcDNA3/His Max, pcDNA3.1, and LipofectAMINE 2000 were from Invitrogen. Calcium ionophore A23187 and the nNOS detection kit were from Calbiochem and Research. Rabbit anti-NIDD antiserum was raised against a fusion protein of GST with NIDD-NT (GST-NIDD-NT). Antibody was affinity-purified with GST-NIDD-NT chemically immobilized to glutathione-Sepharose 4B after removal of anti-GST antibody with GST-glutathione-Sepharose 4B. Western blotting was performed as described previously (18, 19).

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Generation of Affinity-purified Anti-NIDD Antibody and Western Blotting—PSD proteins (20 μg) were immobilized to glutathione-Sepharose 4B. After removal of anti-GST antibody with GST-glutathione-Sepharose 4B, Western blotting was performed as described previously (18, 19).

Cell Culture and Transfection of cDNAs—The cerebral cortex or hippocampus of rats (embryonic day 18, Wistar) was dissociated and plated onto a polyleucineimine-coated glass slide. Neurons were cultured essentially as described previously (20) in Neurobasal medium supplemented with B27. COS-7 cells were cultured as described previously (14). cDNAs encoding full-length NIDD, nNOS, and eNOS were amplified by PCR and inserted into appropriate restriction sites of mammalian expression vectors, pcDNA4/His Max and pcDNA3.1, respectively. The primer sets used as follows: CGCGGATCCATGGAAGAGAACACGTTTG and CGCTCGAGTTAAGGGCCATTACCCAG for NIDD-NT, amino acids 354–392; CGCGGATCCATGGAAGAGAACACGTTTG and CGCTCGAGTTAAGGGCCATTACCCAG for NIDD-CT, amino acids 354–392; CGCGGATCCATGGAAGAGAACACGTTTG and CGCTCGAGTTAAGGGCCATTACCCAG for NIDD-DE, amino acids 354–380; and CGGAATTCATGGAAGAGAACACGTTTG and CGCTCGAGTTAAGGGCCATTACCCAG for NIDD-DE, amino acids 1–159. The restriction enzyme sites introduced are underline. Plasmid DNAs were isolated using Maxi-DNA purification kit. Each plasmid was transfected into COS-7 cells at 90% confluence with the aid of LipofectAMINE 2000. The production of transient transfection in these studies was consistently ~60% as assessed by fluorescence derived from a control plasmid expressing green fluorescent protein.

Immunochemistry—Cultures were fixed with 4% paraformaldehyde for 20 min at room temperature, and the cells were permeabilized with 0.25% Triton X-100. The cells were incubated at 4 °C overnight with primary antibodies in PBS containing 1% goat serum. Slides were incubated with rhodamine- or fluorescein isothiocyanate-conjugated secondary antibodies for 1 h at room temperature. Cells were observed with a Leica confocal laser scanning microscope.

NOS Enzyme Assay—nNOS activity in COS-7 cells was assayed by...
Cloning and Characterization of NIDD cDNA—We have cloned a full-length cDNA of DemA20-5, whose mRNA is associated with the PSD fraction (13), and we named it NIDD. Two clones were obtained for NIDD, a long form and a short form (NIDD-L and NIDD-S, respectively). The full-length cDNAs of NIDD-L and NIDD-S were 5867 and 5756 bp long, respectively, and the encoded proteins comprised 429 and 392 amino acids, respectively. NIDD-L contained an additional 111 nucleotides at nt 1009 of NIDD-S, as shown in Fig. 1a. Domain search analysis using the SMART program revealed that NIDD protein contained five transmembrane domains and a zinc finger DHHC domain, which is highly conserved from yeast to humans and possibly involved in protein-protein or protein-DNA interactions (25). In addition, NIDD possessed an EDIV motif at its C-terminal tail, which was expected to bind to the PDZ domain of nNOS (26, 27). Homology search using the BLAST program revealed that NIDD had orthologs in the mouse and human genomes (GenBank™ accession numbers XM_285173 and NM_173570, respectively), but there was no homology to any known proteins whose function has been specified. The nucleotide sequence surrounding the first AUG codon in NIDD mRNA agreed well with the Kozak consensus sequence and was in-frame with the encoded proteins.

RESULTS

Analyses of NIDD mRNA expression.—Because NIDD was originally isolated from mRNAs associated with the PSD fraction, we considered that NIDD mRNA was likely to be localized to neuronal dendrites. To test this possibility, we performed in situ hybridization of NIDD in cultured hippocampal neuron (E18P21). Scale bar, 25 μm. a, Northern blot analysis of NIDD. Two micrograms of mRNA extracted from adult rat brain were subjected to Northern blot analysis. The positions of size markers are indicated on the left in kb. c, developmental changes of NIDD mRNA level. Total RNA was prepared from the rat forebrain at postnatal day 1 (1d) through 6 weeks (6w) and analyzed by RT-PCR. d, tissue distribution of NIDD mRNA. Total RNA was prepared from various tissues, as noted above the panel, and analyzed by RT-PCR. G3PDH, glyceraldehyde-3-phosphate dehydrogenase, was used as a control.
10% of which were applied to each indicated lane.

Bound proteins were Western-blotted with the antibodies against the proteins indicated on the panel. PSD fraction, or SPM fraction were expressed only in the brain. NIDD was highly expressed in the brain, whereas very low levels of expression were detected in muscle, spleen, lung, and testis.

**Analyses of NIDD Protein Expression**—To examine the expression of NIDD, we carried out immunoblotting analysis with rabbit anti-NIDD antibody. The 40-kDa bands were not detected in other tissues examined (Fig. 3). The brain-specific expression of NIDD protein was in good agreement with the high level of NIDD mRNA expression in the brain (Fig. 2c). The antibody also detected a weaker band of 42 kDa in muscle and a band of 90 kDa in brain, spleen, lung, and testis. The identities of these 42- and 90-kDa bands are not known at present.

**Specific Interaction between NIDD and PDZ Domain of nNOS**—We examined the interaction between nNOS and NIDD by pull-down assays, because NIDD has at its C-terminal tail an EDIV motif, which was expected to bind to the PDZ domain of nNOS (26, 27, 29–31) (Fig. 4). As expected, nNOS was pulled down by NIDD-CT but not by GST alone or by GST-NIDD-del3, a deletion mutant in which the last three amino acid residues of GST-NIDD-CT were deleted (Fig. 4b). Conversely, NIDD was pulled down by GST fusion protein with the PDZ domain of nNOS (GST-NNOS-PDZ) but not by GST alone (Fig. 4c).

Attempts to co-immunoprecipitate NIDD with nNOS, and vice versa, from detergent extracts of rat brain or co-transfected COS-7 cells, have been unsuccessful because of the insolubility of NIDD in non-denaturing detergents (data not shown). In particular, His-NIDD expressed in COS-7 cells aggregated even in the presence of 1% Triton X-100 (data not shown) as observed in Abl-phinlin 2 (Aph2), another zinc finger-DHHC-containing protein involved in endoplasmic reticulum stress-induced apoptosis (32). Therefore, we adopted a co-sedimentation assay, instead of co-immunoprecipitation, to examine further the interaction between NIDD and nNOS. COS-7 cells were co-transfected with nNOS (fixed amounts) by repeated experiments using different lots of tissue samples. This pattern was confirmed by simultaneous co-sedimentation with anti-nNOS (upper panel) and anti-His.G (lower panel) antibodies. The amounts of nNOS cDNA added were fixed (2 μg each), whereas those of His-NIDD cDNAs were graded as indicated above the gel. The pcDNA4 parent vector was also applied to the cells to make the total amounts of applied DNA constant.

**Effect of NIDD expression on the distribution of nNOS expressed in COS-7 cells**. Full-length rat nNOS and His-NIDD were expressed in COS-7 cells. The cells were lysed in PBS after 48 h of transfection, and the lysates were separated into supernatant (sup) and pellet (ppt) by centrifugation at 15,800 × g for 30 min. Equal volumes of each preparation were subjected to immunoblotting with anti-nNOS (upper panel) and anti-His.G (lower panel) antibodies. The amounts of nNOS cDNA added were fixed (2 μg each), whereas those of His-NIDD cDNAs were graded as indicated above the gel. The pcDNA4 parent vector was also applied to the cells to make the total amounts of applied DNA constant.

**Fig. 3. Tissue distribution of NIDD**. Proteins (30 μg per lane) extracted from various tissues, as indicated above the panel, were analyzed by immunoblotting with rabbit anti-NIDD antibody. The 40-kDa doublet proteins (arrow) were expressed only in the brain.

**Fig. 4. Specific interaction of NIDD with nNOS shown by pull-down assay**. Fusion proteins of GST with NIDD-CT, NIDD-del3, or nNOS-PDZ, as well as GST alone, were bound to glutathione-Sepharose 4B and incubated with cerebellar extract (a), PSD fraction (b), or SPM fraction (c). Bound proteins were Western-blotted with the antibodies against the proteins indicated on the right. Imp refers to input proteins, 10% of which were applied to each indicated lane.

**Fig. 5. Effect of NIDD expression on the distribution of nNOS expressed in COS-7 cells**. Full-length rat nNOS and His-NIDD were expressed in COS-7 cells. The cells were lysed in PBS after 48 h of transfection, and the lysates were separated into supernatant (sup) and pellet (ppt) by centrifugation at 15,800 × g for 30 min. Equal volumes of each preparation were subjected to immunoblotting with anti-nNOS (upper panel) and anti-His.G (lower panel) antibodies. The amounts of nNOS cDNA added were fixed (2 μg each), whereas those of His-NIDD cDNAs were graded as indicated above the gel. The pcDNA4 parent vector was also applied to the cells to make the total amounts of applied DNA constant.
and His-tagged NIDD (graded amounts). After 48 h of transfection, the cells were lysed in PBS, and the supernatant and the pellet fractions were subjected to immunoblotting with anti-His-G and anti-nNOS antibodies. As shown in Fig. 5, co-expression of His-NIDD caused a dose-dependent shift of nNOS from the supernatant to the pellet, suggesting an interaction between nNOS and NIDD in COS-7 cells.

**Localization of NIDD at Synaptic Sites**—The subcellular localization of NIDD was examined by immunoblotting (Fig. 6). NIDD showed a distribution pattern similar to that of nNOS, except for the soluble fraction (Fig. 6a). NIDD and nNOS proteins were enriched in synaptic fractions such as the synaptosomal and SPM fractions, indicating that the two proteins could be co-localized at synaptic sites. NIDD and nNOS were also present in the PSD fraction, although the amounts detected were reduced or minimal, respectively. Most interesting, a 90-kDa NIDD-immunoreactive protein was highly concentrated in the PSD fraction but scarce in the synaptosome and SPM fractions, indicating that the two proteins could be co-localized at synaptic sites. NIDD and nNOS were also present in the PSD fraction, although the amounts detected were reduced or minimal, respectively. Most interesting, a 90-kDa NIDD-immunoreactive protein was highly concentrated in the PSD fraction but scarce in the synaptosome and SPM fractions. The NIDD distribution in the PSD fractions were further subjected to sucrose density gradient centrifugation after treatment with 0.15% Triton X-100 or 0.5% Rubrol (see “Experimental Procedures”). Equal volumes of each fraction obtained after the centrifugation were subjected to immunoblotting. Flotillin-1 is a marker for lipid raft (41).

The NIDD distribution was further examined in primary cultures of rat cortical neurons (Fig. 7). The immunoreactivities for NIDD and nNOS were distributed in and co-localized in the dendrites as well as soma.

**Comparison of mRNA Distribution between NIDD and nNOS**—To clarify further the relationship between NIDD and nNOS, we compared their distributions in the brain by in situ hybridization analysis. Prominent expression of NIDD mRNA was detected in the hippocampal pyramidal cell layer of the CA1 region, tenia tecta, piriform cortex, and olfactory mitral cells and tufted cells (Fig. 8, a, c, e, and g). Moderate expression was observed in the anterior olfactory nuclei, caudate putamen, nucleus accumbens, cerebral cortex, amygdaloid nuclei, hippocampal pyramidal cell layer of the CA3 region, and dentate granule cell layer (Fig. 8, a, e, and g). Weak expression was detected in the septal nuclei, medial habenular nucleus, para-ventricular thalamic nucleus, anterior medial preoptic nucleus, medial preoptic area, ventromedial hypothalamic nucleus, locus coeruleus, and cerebellar Purkinje cell layer (Fig. 8, a and g). The expression of NIDD mRNAs was summarized in Table I. Dendritic localization of the NIDD mRNA was not clearly evident with this in situ hybridization protocol, possibly due to lower expression level in the dendrites compared with that in somatic areas. Autoradiographic detection of mRNA in tissue sections appeared to be unsuitable for the detection of dendritically distributed mRNAs with respect to resolution and sensitivity.

As compared with the expression pattern of nNOS mRNA, NIDD mRNA was expressed in some restricted populations of
DISCUSSION

We identified a novel nNOS-interacting synaptic membrane protein, NIDD. The major finding of this study was that nNOS distribution and activity are regulated through specific interaction with NIDD. We also found a unique molecular structure (Fig. 1), relatively specific expression in the brain (Fig. 4), early expression in the postnatal period (Fig. 2c), and dendritic distribution of the mRNA of NIDD (Fig. 2a).

The interaction of NIDD with nNOS is mediated by the PDZ-binding motif at the end of the C terminus of NIDD and the PDZ domain of nNOS, which was proven by the pull-down experiment (Fig. 4). Notably, the C-terminal sequence of NIDD is specific to the PDZ domain of nNOS (Fig. 4, a and b). The specificity of the interaction between the NIDD C-terminal sequence and nNOS PDZ domain is supported by findings reported previously (27). This type of interaction with nNOS is also seen in the interaction with C-terminal PDZ ligand of nNOS (CAPON) (29), C-terminal binding protein (31), and phosphofructokinase-M (30).
NIDD is a transmembrane protein (Fig. 1) that is co-localized with nNOS in the synaposome, SPM, and synaptic lipid raft fractions (Fig. 6). Co-localization of these proteins at synaptic sites was also confirmed in cultured cortical neurons (Fig. 7). Moreover, co-expression of NIDD in the nNOS-expressing COS-7 cells caused a shift of nNOS from the supernatant to the pellet fraction (Fig. 5). All these findings suggest that NIDD and nNOS interact in vivo and that NIDD plays a role in targeting nNOS to the synaptic membrane. The functional result of the interaction could be the regulation of NO production through nNOS at the synaptic membrane.

Membrane association of nNOS is important for its enzyme activity, because biochemical studies indicate that up to 60% of total NOS activity in the brain is found in the membrane fraction (35), and targeting of eNOS to the plasma membrane is accompanied by a large increase in NO production (36). Conversely, an nNOS-interacting protein, protein inhibitor of nNOS, inhibits nNOS enzyme activity by recruiting nNOS from the membrane to the cytosol (34). The relationship between membrane association and enzyme activity of nNOS may also apply to the NIDD-nNOS interaction. As expected, NO production by nNOS in COS-7 cells is significantly increased when the cells are co-transfected with the membrane protein NIDD (Fig. 9). Thus, NIDD regulates the nNOS enzyme activity.

In general, one of the roles of the nNOS-interacting proteins is to target the nNOS to appropriate sites and restrict the effect of NO to the region proximal to the targeted sites. NO produced by nNOS can diffuse into neighboring cells and activate soluble guanylyl cyclase or covalently modify cysteine residues of target proteins (S-nitrosylation). For example, α1-syntrophin targets nNOS to the sarcolemma (37). PSD-95 and PSD-93 target nNOS to post synaptic sites and couple the nNOS activation to Ca2+ entry through NMDAR, which is also targeted by PSD-95 and PSD-93 (38). A brain-specific adaptor protein, CAPON, competes with PSD-95 for the PDZ domain in nNOS and recruits nNOS to a small monomeric G protein, Dextras 1 (29, 39). This may facilitate the nitrosylation and subsequent activation of Dextras 1 (29, 39, 40). This generalization may also be true in the case of NIDD. The effects of NIDD on nNOS are limited to some restricted brain areas because the localization of NIDD, as far as the mRNA distribution is considered, overlapped that of nNOS only in restricted areas in the brain. We are waiting for further studies regarding this point.

In summary, we have cloned and characterized a novel nNOS-interacting synaptic membrane protein, NIDD, which increases the nNOS enzyme activity by targeting it to the synaptic membrane in a PDZ domain-dependent manner. The results suggest that NIDD plays an important role in regulating synaptic plasticity by regulating the NO signaling pathway through specification of the sites of nNOS action.

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Fig. 9. Effects of NIDD on nNOS enzyme activity. COS-7 cells transfected with indicated cDNAs were cultured in 6-well tissue culture plates. The pcDNA3.1 or pcDNA4 parent vector was also transfected to keep the applied DNA quantity constant. The enzyme activity was assayed in two ways 48 h after the transfection by counting L-[3H]citrulline. a, cells were incubated with L-[3H]arginine, and after 10 min, they were harvested and lysed for the assay. b, cells were harvested and lysed, and incubated with L-[3H]arginine at 25 °C for 15 min. c, expression of the transfected DNA was confirmed by Western blotting of the cell lysates, processed in parallel with the cells used for the activity assay. The immunoblot for actin was used as a monitor to show that cultures with equal cell density were used in these assays. Assays of nNOS and eNOS in (a or b) were carried out simultaneously. All experiments were done in triplicate and repeated at least three times with essentially the same results. Student’s t test, * p < 0.05.
A Novel nNOS-interacting Protein, NIDD

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