Recent work suggests a role for PDZ domains in the targeting of binding partners to specific sites in the cell. To identify whether the PDZ domain of neuronal nitric-oxide synthase (nNOS) can play such a role, we performed affinity chromatography of brain extract with the nNOS PDZ domain. We identified the carboxyl-terminal-binding protein (CtBP), a phosphoprotein first identified as a binding partner to adenosine E1A, as a nNOS binding partner. CtBP interacts with the PDZ domain of nNOS, and this interaction can be competed with peptide that binds to the PDZ peptide-binding site. In addition, binding of CtBP to nNOS is dependent on its carboxyl-terminal sequence -D-x-x-L, residues conserved between species that fit the canonical sequence for nNOS PDZ binding. Immunoprecipitation studies show that CtBP and nNOS associate in the brain. When CtBP is expressed in Madin-Darby canine kidney cells, its distribution is primarily nuclear; however, when CtBP is co-expressed with nNOS, its localization becomes more cytosolic. This change in CtBP localization does not occur when its carboxyl-terminal nNOS PDZ binding motif is mutated or when CtBP is co-expressed with postsynaptic density 95, another PDZ domain-containing protein. Taken together, our data suggest a new function for nNOS as a regulator of CtBP nuclear localization.

Nitric oxide (NO) is a gaseous molecule that plays an important role in the central nervous system (1, 2). It is produced by the enzyme neuronal nitric-oxide synthase (nNOS), which can be stimulated when glutamate receptors of the N-methyl-D-aspartate receptor family are activated, and calcium enters the neuron (2). Calcium binds to calmodulin, and the complex then activates nNOS. Furthermore, the NO produced can increase the production of 3',5'-cyclic guanosine monophosphate levels, which can activate other enzymes including protein kinases (3, 4). Recent evidence supports a role for NO in synaptic plasticity (5), long-term potentiation (6), and aspects of learning and memory (7).

nNOS contains a PDZ (PSD-95, discs-large and zona occludens-1) domain, a consensus sequence of approximately 90 amino acids that has been shown to mediate protein-protein interactions (8). In neurons, nNOS is targeted to synaptic sites via its interaction with the PDZ domains of PSD-95 and PSD-93 (9). In fact, PSD-95 also interacts with N-methyl-D-aspartate receptors via one of its PDZ domains. Through its concurrent interaction with nNOS, PSD-95 serves as a physical tether to allow nNOS signaling by N-methyl-D-aspartate receptor activity (10, 11). By abolishing expression of PSD-95 protein either by knockout technology in mice or by antisense technology in tissue culture, it has been shown that the presence of PSD-95 is essential for NO production by glutamate stimulation (12, 13). Thus, the PDZ domain of nNOS plays an important role in helping to localize nNOS to appropriate sites in the neuron.

Subcellular fractionation shows that roughly half of nNOS is soluble, and half is particulate (14). As such, binding partners of the PDZ domain of nNOS can serve to regulate nNOS localization in the cell. In fact, it has been shown that the soluble CAPON (carboxy-terminal PDZ ligand of nNOS) competes with PSD-95 and PSD-93 for binding of nNOS and thus may participate in localization of nNOS to the cytoplasm (15). Furthermore, phosphofructokinase binds to the PDZ domain of nNOS and co-localizes with nNOS in the cytosol of inhibitory interneurons in the hippocampus (16). The binding of phosphofructokinase to nNOS does not alter the enzymatic activity of either phosphofructokinase or nNOS (16); thus, the possibility exists that the binding of these two enzymes is important for subcellular localization.

Based on the fact that the PDZ domain of nNOS is important for protein localization, we tested whether other protein complexes may bind to this domain. To address this question, we performed affinity chromatography using a GST fusion protein column containing the PDZ domain of nNOS. Purification of brain lysates on the column yielded a protein of 48 kDa, which was identified as the carboxyl-terminal-binding protein (CtBP), a protein originally identified as a phosphoprotein that interacts with the adenosine E1A protein (17) and acts as a transcriptional co-repressor (18, 19). Endogenous CtBP associates with nNOS in the brain and binds to the peptide-binding site of the nNOS PDZ domain via a consensus sequence at its carboxyl terminus. At the cellular level, CtBP protein is expressed in the nucleus, whereas when CtBP is co-expressed with nNOS, it relocalizes to the cytosol. Furthermore, this translocation is dependent on nNOS binding. Taken together, these results
suggest a novel function for a PDZ domain-containing protein, namely nNOS, in regulation of the transcriptional co-repressor to the cytosol.

**EXPERIMENTAL PROCEDURES**

**Materials**—Peptides were from Research Genetics (Birmingham, AL). Glutathione-Sepharose and pGEX4T-1 vector were from Amer sham Pharmacia Biotech. Affi-Gel 10 beads were from Bio-Rad (Hercules, CA). Horseradish peroxidase-coupled donkey anti-guinea pig antibody was from Sigma. Supersignal Chemiluminescent Substrate was from Pierce. Mouse antibody raised against nNOS was from Transduc tion Laboratories (Lexington, KY), mouse antibody raised against T7 was from Novagen (Madison, WI), mouse antibody 9E10 raised against myc and rabbit antibody raised against rab 3a were from Dr. Shu-Chan Hsu (Rutgers University), and rabbit antibody raised against nNOS and mouse antibody raised against lamin B2 were from Zymed Laboratories, Inc. cDNA encoding CTBP with Asp437 and Leu439 mutated to alanines (CTBP-AXA) was constructed by using polymerase chain reaction and subcloning the fragment into pGEX4T-1 with a myc tag at the EcoRI and XhoI sites. Rabbit antibody raised against human CTBP and cDNA encoding T7-tagged CTBP were generously provided by Drs. G. Chinnadurai and T. Subramanian (St. Louis University Medical Center) or generated by immunizing rabbits using full-length CTBP as an antigen. All other antibodies were from Sigma.

**Fractionation of Rat Brain**—One adult rat brain was added to Buffer A (0.25 M sucrose, 10 mM NaCl, 2 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, and 10 mM Tris, pH 7.5) and homogenized for 18 strokes in a Dounce homogenizer. The extract was centrifuged at 800 × g for 15 min at 4 °C, and the supernatant was spun at 100,000 × g for 1 h at 4 °C to yield the cytosolic fraction. The pellet from the 800 × g spin was resuspended in Buffer A and layered over a 2.5 M sucrose, 10 mM NaCl, 1.5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, and 10 mM Tris, pH 7.5, and nuclei were pelleted by centrifugation at 12,400 × g for 1 h at 4 °C. The nuclei were washed by resuspending them in Buffer A and centrifuged at 800 × g for 10 min at 4 °C.

**Expression and Purification of GST, GST-CTBP, GST-CTBP-AXA, and GST-CTBP-Asp**—Proteins—The GST and GST-tagged proteins encoding the first 195 amino acids of nNOS was constructed by polymerase chain reaction and expressed and purified from Escherichia coli as described previously (16). cDNAs encoding GST fusion proteins of CTBP and CTBP with Asp437 and Leu439 mutated to alanines (CTBP-AXA) were constructed using polymerase chain reaction and subcloned into pGEX4T-1 at the XhoI and XhoI sites. For protein affinity chromatography studies, GST fusion proteins were not eluted from the beads.

**Protein Affinity Chromatography**—Affinity chromatography using nNOS-1-195 was performed as described previously (16). Briefly, GST fusion proteins were cross-linked to Affi-Gel 10, and the columns were pre-cleared of any unlinked GST fusion protein that might leach off of the Affi-Gel beads during incubation with brain extract. Bovine serum albumin (BSA; 1 mg/ml) and unlinked GST-Affi-Gel 10 beads were constructed in a similar manner and used to clear the extract before its application to GST- and nNOS-Affi-Gel 10 to reduce the background binding of nonspecific proteins.

Twenty-five adult rat brains were homogenized, and proteins were extracted in 1% Triton X-100 for 1 h at 4 °C. The homogenate was spun at 12,000 × g for 10 min, and the supernatant was pre-cleared with BSA-Affi-Gel 10 beads. The precleared extract was incubated with nNOS-Affi-Gel 10 at 4 °C for 3 h. As controls, extract was incubated with GST-Affi-Gel 10, and buffer containing 1% Triton X-100 was incubated with nNOS-Affi-Gel 10. All incubations were done in batch. Extract/beads were washed, and proteins were eluted consecutively with 2.5 and 4 mM urea. Each fraction was precipitated with 20% trichlo roacetic acid, resuspended in 100 mM Tris (pH 8.8), and 1% SDS, and proteins were resolved by 8% SDS-polyacrylamide gel electrophoresis and visualized using silver.

**Western Transfer and Protein Sequencing**—The 2.5 and 4 mM urea fractions were combined, and proteins were separated on an 8% polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Immobilon P; Millipore). The membrane was stained with Coomassie Blue to visualize bands, the desired protein bands were excised, and proteins were performed by ProSeq (Salem, MA). Proteins were cleaved with CNBr and then coupled with O-phthalaldehyde as described previously (20). O-Phthalaldehyde treatment blocks all peptides that do not contain an amino-terminal proline. Proteins were then sequenced by Edman degradation. The single peptide sequence was used to search the National Center for Biotechnology Information using the BLAST programs.

**GST Fusion Protein Binding**—One adult rat brain or two 10-cm dishes of Cos-7 cells transfected with cDNA encoding T7-CTBP were homogenized in 10 ml of TEE (25 mM Tris·HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride). Triton X-100 was added to a final concentration of 1%, and the homogenates were incubated on ice for 1 h. The extract was spun at 12,000 × g for 15 min. One ml of brain or Cos-7 cell extract was incubated with glutathione-Sepharose bound to 25 μg of the appropriate GST fusion protein at 4 °C for 1 h. Extract/beads were loaded into the appropriate size column and washed three times with 20 ml of TEE containing 0.2% Triton X-100. Bound proteins were eluted with 200 μl of 0.2% Triton X-100 by incubation for 1 h at 4 °C. The eluates were loaded into SDS-polyacrylamide gels, and transferred to polyvinylidene difluoride membrane as described above. Blots were probed using a rabbit antibody raised against nNOS or a monoclonal antibody raised against T7 and visualized using a secondary antibody coupled to horseradish peroxidase and Supersignal Chemiluminescent Substrate (Pierce).

**Cos-7 and MDCK Cell Transfection**—For biochemical assays, Cos-7 cells were plated at 70–80% confluence. For immunofluorescence, MDCK cells were plated at 480,000 cells/35-mm dish. Cells were transfected with cDNAs encoding T7-tagged CTBP, myc-tagged CTBP-AXA, and nNOS-green fluorescent protein using LipofectAMINE 2000 (Life Technologies, Inc.).

**Immunoprecipitation**—Frozen rat brains were homogenized in 5 ml of lysis buffer (10 mM Tris·HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride). Soluble proteins were extracted with 1% Triton X-100 by incubation for 1 h at 4 °C. Insoluble proteins were pelleted by centrifugation at 20,000 × g for 20 min, and the supernatant was pre-cleared with protein A-Sepharose for 1 h. Lysates were diluted 10-fold in TEE, and GST was immunoprecipitated with 10 μg/ml polyclonal anti-GST antibody or preimmune antiserum linked to protein A-Sepharose (Amersham Pharmacia Biotech) for 2 h at 4 °C. Beads were washed, and bound proteins were eluted in SDS loading buffer and analyzed by immunoblotting using a mouse antibody raised against nNOS.

For co-immunoprecipitations from crude brain nuclei, one frozen rat brain was homogenized in 10 ml of TEE and 1 mM dithiothreitol using a Potter-Gel 10 beads were constructed in a similar manner and used to clear the extract before its application to GST- and nNOS-Affi-Gel 10 to reduce the background binding of nonspecific proteins.

**Quantitation of nNOS in the Nucleus**—Using the software analysis program ImagePro (Media Cybernetics), the percentage of nNOS protein in the nucleus was calculated using the following formula: (percent of nNOS protein in the nucleus = (sum of pixels in nucleus)/(sum of pixels in cell) × 100).

**RESULTS**

**Isolation of a 48-kDa Protein That Binds to the PDZ Domain of nNOS**—To identify proteins that bind to the PDZ domain of nNOS in brain, we constructed an Affi-Gel 10 column covalently linked with the PDZ domain of nNOS (nNOS-Affi-Gel) and incubated this column with brain extract (Fig. 1, nNOS-Extract). The column was washed with buffer to release unbound proteins, and proteins tightly bound to the column were eluted off with urea in buffer. Two control columns were run as follows: GST coupled to Affi-Gel 10 (GST-Affi-Gel) was incubated with brain extract (Fig. 1, GST-Extract) to identify nonspecific binding proteins, and the PDZ domain of nNOS coupled to Affi-Gel 10 was incubated with buffer (Fig. 1, nNOS-Buffer) to identify nNOS fusion protein that leached off of the column. As controls in Fig. 1, proteins of 50 and 70 kDa leached from the nNOS-Affi-Gel buffer control column (lane 1). Additionally, a nonspecific protein (55 kDa) eluted from both the nNOS- and GST-Affi-Gel columns (lanes 2 and 3). This protein either binds to GST or nonspecifically binds to Affi-Gel beads. A 48-kDa and an 80-kDa doublet protein elute specifically from the nNOS column (lane 2). These proteins do not bind to a control GST column and are not a product of proteolysis of the linked fusion protein. We have previously identified the 80-kDa doublet to be
phosphofructokinase (16). To identify the 48-kDa protein, the protein was isolated, and two peptides were sequenced by Edman degradation as described under “Experimental Procedures.” The sequence obtained (Fig. 2) showed high homology to the previously sequenced murine CtBP.

**nNOS Binds CtBP at Its Peptide-binding Site**—It has been shown previously that the PDZ domain of nNOS preferentially binds to peptides ending with the sequence G(D/E)AV (22, 23) or EXAV (15, 16). The last four amino acids of CtBP are SDQL, where the D fits the consensus for the second amino acid from the carboxyl terminus, and the L is a conservative substitution for the carboxyl-terminal V. To determine whether CtBP binds to the peptide-binding site in the PDZ domain of nNOS, we performed affinity chromatography of Cos-7 cells transfected with T7-tagged CtBP using a GST-nNOS-PDZ column. Fig. 3 shows that CtBP does in fact bind to the PDZ domain of nNOS and that this binding can be competed with the peptide VSP-DFGDAV, suggesting that CtBP binding is at the peptide-binding site in nNOS-PDZ. The peptide KLLSIE5D, which binds to the second PDZ domain of PSD-95, competes the binding of CtBP to the PDZ domain of nNOS with much lower potency (Fig. 3, top panel), suggesting that this interaction is specific.

To identify whether the carboxyl-terminal sequence of CtBP plays a role in nNOS binding, we performed affinity chromatography of rat brain using GST-CtBP or GST-CtBP with the amino acids conserved for PDZ binding (D and L) mutated to alanines (GST-CtBP-AXA). As seen in Fig. 3 (bottom panel), nNOS binds only to wild type CtBP. Mutation of the carboxyl terminus of CtBP attenuates nNOS binding. Thus, the PDZ consensus sequence DXL is important for CtBP binding to nNOS.

**nNOS Co-immunoprecipitates with CtBP from Brain Extract**—To determine whether CtBP and nNOS interact in vivo, we immunoprecipitated CtBP from brain extract. As shown in Fig. 4A, a silver-stained SDS-polyacrylamide gel of the immunoprecipitates shows proteins of 95, 100, 140, and 200 kDa when CtBP is immunoprecipitated. Most co-immunoprecipitated proteins are not usually isolated at levels sufficient for detection by silver staining; because these proteins can be detected by silver staining, they are major components of the CtBP immunocomplex. These proteins are not seen when the extract was incubated with preimmune serum. To determine whether the 140-kDa protein is nNOS, we performed Western blotting of the immunoprecipitates. As seen in Fig. 4B, nNOS immunoprecipitated from brain incubated with anti-CtBP but not from brain incubated with preimmune serum. These data suggest that CtBP and nNOS interact in vivo.

**nNOS Changes CtBP Localization from the Nucleus to the Cytosol**—Because proteins that contain PDZ domains play a role in localizing their binding partners to subcellular sites, we investigated whether the binding of CtBP to nNOS would regulate its localization. We transfected MDCK cells with CtBP, nNOS, or both proteins. Cells that express CtBP most often show nuclear localization (Table I; Fig. 5A). Many cells show a doughnut-shaped nuclear expression pattern that suggests association of CtBP with the nuclear membrane or exclusion from the nucleolus. In contrast, nNOS expression is both cytosolic and nuclear (100% of cells; Fig. 5B) with 21.03 + 2.07% of nNOS protein in the nucleus, based on pixel intensity (n = 16). However, when CtBP is co-expressed with nNOS, CtBP distribution changes dramatically. Binding to nNOS changes the localization of CtBP from the nucleus to the cytosol (Table I; Fig. 5C), but it does not change nNOS distribution (100% of cells cytosolic and nuclear; 20.02 + 1.14% nNOS in nucleus; n = 16). Mutating the carboxyl terminus of CtBP does not disrupt its nuclear localization (Table I; Fig. 5D); however, the mutation does disrupt its binding to nNOS and its localization by nNOS (Table I, Fig. 5E). Thus, nNOS regulates the expression pattern of CtBP protein in the cell.

To address whether CtBP specifically interacts with the PDZ domain of nNOS (a type III PDZ domain), we transfected MDCK cells with another PDZ-containing protein, PSD-95 (which contains type I PDZ domains), and CtBP and analyzed the localization of CtBP. As expected, we found that CtBP was localized to the nucleus when PSD-95 was co-expressed (data not shown). Thus, the change in distribution of CtBP protein from the nucleus to the cytosol is specific to the type III PDZ domain of nNOS.

**nNOS Localizes to Both Cytosol and Nucleus in Brain**—Because it has been reported previously that some PDZ-containing proteins localize to the nucleus, we performed subcel-
the PDZ domain of nNOS because its binding can be competed with the peptide VSPDFGDAV. The carboxyl terminus of nNOS contains a consensus sequence for nNOS changes CtBP localization.

**Table I**

|                | Nuclear | Cytosolic | Nuclear + Cytosolic |
|----------------|---------|-----------|-------------------|
| CtBP (n = 78)  | 66%     | 0%        | 34%               |
| CtBP + nNOS* (n = 69) | 24.5% | 10%       | 65.5%              |
| CtBP-AXA (n = 30) | 77%    | 0%        | 23%                |
| CtBP-AXA + nNOS (n = 31) | 71%    | 0%        | 29%               |

* *p < 0.001 by *x*2 test.

**DISCUSSION**

The primary finding of this study is that nNOS associates with the transcription factor CtBP. CtBP specifically binds to the peptide-binding site in the PDZ domain of nNOS because its binding can be competed with the peptide VSPDFGDAV. The carboxyl terminus of nNOS contains a consensus sequence for CtBP association with the Peptide-Binding Site.

**FIG. 3.** CtBP binds competitively to the peptide-binding site in the nNOS PDZ domain via its carboxyl terminus. Top panel, Cos-7 cells were transfected with a cDNA encoding CtBP tagged with T7 at its amino terminus. Detergent-soluble extracts of these cells were incubated with glutathione-Sepharose bound to 25 g of GST, GST-nNOS-PDZ in the presence of increasing concentrations (nM) of the peptide VSPDFGDAV, which binds to the PDZ binding site, or KLISSIESD, which binds to the second PDZ domain of PSD-95 but does not bind to the PDZ domain of nNOS. The Sepharose was washed and eluted, and proteins were resolved by 8% SDS-polyacrylamide electrophoresis and transferred to Immobilon-P. Western blotting of eluates demonstrates that CtBP specifically binds to the PDZ domain of nNOS and that binding is blocked by the peptide VSPDFGDAV with an IC50 of ~25 nM. The peptide KLISSIESD is much less potent in blocking CtBP binding. Bottom panel, brain extract was incubated with glutathione-Sepharose bound to 25 g of GST, GST-CtBP, or GST-CtBP-AXA (CtBP with D and L mutated to A). The Sepharose was washed and eluted, and proteins were resolved by 8% SDS-polyacrylamide electrophoresis and transferred to Immobilon-P. Western blotting of eluates demonstrates that nNOS binds to the wild type CtBP but not to the mutant.

**FIG. 4.** nNOS co-immunoprecipitates with CtBP. Detergent-soluble brain extract was incubated with rabbit preimmune serum or rabbit anti-CtBP. Antibody complexes were collected with protein A-Sepharose, and the immunoprecipitated complexes were separated by 8% SDS-polyacrylamide electrophoresis. A, a silver-stained gel shows an IgG band in both preimmune and anti-CtBP lanes. CtBP runs just below the IgG band. A 140-kDa band is seen when extracts are incubated with anti-CtBP, but not when extracts are incubated with preimmune serum. B, the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and transferred to Immobilon-P. Western blotting for nNOS reveals a nNOS band at ~140 kDa that is much more intense in the anti-CtBP complexes than in the preimmune complexes. Immunoblot for CtBP is not shown because CtBP co-migrates with IgG. Load represents 10% of extract proteins.

**TABLE I**

|                | Nuclear | Cytosolic | Nuclear + Cytosolic |
|----------------|---------|-----------|-------------------|
| CtBP (n = 78)  | 66%     | 0%        | 34%               |
| CtBP + nNOS* (n = 69) | 24.5% | 10%       | 65.5%              |
| CtBP-AXA (n = 30) | 77%    | 0%        | 23%                |
| CtBP-AXA + nNOS (n = 31) | 71%    | 0%        | 29%               |

* *p < 0.001 by *x*2 test.
Fig. 5. nNOS localizes CtBP from the nucleus to the cytosol. A–C, MDCK cells were transfected with a cDNA encoding CtBP tagged with T7 at its amino terminus (red; A), nNOS fused to green fluorescent protein (green; B) or both constructs (C). The cells were fixed and stained with an antibody raised against the T7 epitope to detect CtBP expression and with Hoechst 33258 or 4′,6-diamidino-2-phenylindole (blue; A–C, DAPI) to stain nuclei. When CtBP is overexpressed alone, its localization is primarily nuclear (A, arrows). Some cells show some cytosolic expression with
nNOS Changes CtBP Localization

Until now, reports have suggested that the PDZ domain of nNOS serves to regulate the localization of nNOS protein. Interactions with other proteins containing PDZ domains function to target nNOS to noncytosolic sites. For example, in brain, nNOS binding to PSD-95 localizes nNOS to the postsynaptic density (9). Similarly, in skeletal muscle, association of nNOS with 1-syntrophin localizes nNOS to the sarcolemma (9, 29). In contrast, proteins that contain carboxyl-terminal motifs that bind to the PDZ domain of nNOS often target nNOS to the cytosol or inhibit nNOS activity. For example, when CAPON binds to nNOS, nNOS is not targeted to postsynaptic sites in the brain; instead, it is localized to the cytosol (15). PIN (protein inhibitor of nNOS) associates with nNOS in the cytosol and inhibits nNOS activity (30, 31). Interestingly, we found no effect on nNOS activity when we added purified GST-CtBP to brain extract. Thus, we believe that the binding of CtBP to nNOS plays a role in protein localization rather than regulation of enzymatic activity, suggesting a novel role for nNOS as a targeting molecule.

Recently, there have been a number of reports describing the role of PDZ domain-containing proteins in localization to the nucleus. For example, the PDZ-containing protein zona occludens-1 shuttles between tight junctions and the nucleus in epithelial cells and is thought to regulate gene expression (32). When it is localized to the nucleus, the SH3 domain of zona occludens-1 binds to a nucleic acid-binding protein known as ZONAB, and together zona occludens-1 and ZONAB bind to the Erb-2 promoter and regulate Erb-2 transcription (33). Similarly, CASK/LIN-2, a multi-PDZ-containing protein, can translocate from the cytosol to the nucleus and bind to Tbr-1, a T-box transcription factor involved in brain development (34). The binding of the guanylate kinase domain of CASK to Tbr-1 induces gene transcription. Thus, evidence is beginning to emerge to support a role for PDZ-containing proteins in transcription factor activity. Our data support the idea that nNOS protein is present in the nucleus; however, unlike the other PDZ proteins mentioned above, nNOS may traffic nuclear proteins to the cytosol, thereby making them inaccessible to regulate transcription. Furthermore, targeting of proteins by nNOS occurs via its PDZ domain, unlike the proteins described above, which contain PDZ domains but regulate nuclear events via other domains.

There are two possible mechanisms by which nNOS may target proteins from the nucleus. Because a majority of nNOS protein is found in the cytosol, nNOS may act to “trap” nuclear proteins in the cytosol once they diffuse or are transported from the nucleus. Alternatively, nNOS may bind nuclear proteins and actively transport them from the nucleus to the cytosol. In line with this mechanism, we find some nNOS protein, although only a small amount, in the nucleus (Fig. 6A). In addition, this localized nNOS associates with CtBP in brain nuclei (Fig. 6B). nNOS contains putative nuclear export sequences; however, treatment of cells with leptomycin B, a drug that inhibits CRM-1-mediated nuclear transport, has no effect on nNOS protein localization. This suggests that nNOS does not shuttle from the nucleus to cytosol by binding CRM-1. Future studies will serve to identify which mechanism plays a role in the targeting of nuclear proteins by nNOS.

Thus, we believe that we have found a novel function for nNOS as a regulator of nuclear protein localization. Although other forms of nitric-oxide synthase, such as endothelial and inducible nitric-oxide synthase, also produce NO, nNOS is the only isoform that contains a PDZ domain. Furthermore, nNOS is expressed at the highest levels in brain and skeletal muscle, with very little expression in other tissues (35). As such, the PDZ of nNOS may play a novel role in nNOS function in these two tissues.

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2 B. L. Firestein, unpublished observations.

3 G. M. Riefler and B. L. Firestein, unpublished observations.
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