Neuron, Vol. 20, 115–124, January, 1998, Copyright ©1998 by Cell Press

CAPON: A Protein Associated with Neuronal Nitric Oxide Synthase that Regulates Its Interactions with PSD95

Samie R. Jaffrey, Adele M. Snowman, Mikael J. L. Eliasson, Noam A. Cohen, and Solomon H. Snyder*
The Johns Hopkins University
School of Medicine
Departments of Neuroscience, Pharmacology and Molecular Sciences, and Psychiatry
Baltimore, Maryland 21205

Summary

Nitric oxide (NO) produced by neuronal nitric oxide synthase (nNOS) is important for N-methyl-D-aspartate (NMDA) receptor-dependent neurotransmitter release, neurotoxicity, and cyclic GMP elevations. The coupling of NMDA receptor-mediated calcium influx and nNOS activation is postulated to be due to a physical coupling of the receptor and the enzyme by an intermediary adaptor protein, PSD95, through a unique PDZ–PDZ domain interaction between PSD95 and nNOS. Here, we report the identification of a novel nNOS-associated protein, CAPON, which is highly enriched in brain and has numerous colocalizations with nNOS. CAPON interacts with the nNOS PDZ domain through its C terminus. CAPON competes with PSD95 for interaction with nNOS, and overexpression of CAPON results in a loss of PSD95/nNOS complexes in transfected cells. CAPON may influence nNOS by regulating its ability to associate with PSD95/NMDA receptor complexes.

Introduction

Studies of neuronally derived nitric oxide (NO) have revealed many roles for this gaseous messenger molecule (Moncada, 1994; Yun et al., 1996). In the peripheral nervous system, NO mediates nonadrenergic, noncholinergic neurotransmission, serving as an effector of autonomic neurons on smooth muscle. NO has been implicated in several forms of neuronal plasticity, such as LTP (for a review, see Huang, 1997). Studies in mice with a targeted genomic deletion of the NO biosynthetic enzyme neuronal NO synthase (nNOS) have shown that NO mediates a substantial portion of the neurotoxicity associated with stroke (Huang et al., 1994). In the brain, NO and citrulline are produced from arginine predominantly by nNOS (Huang et al., 1993), although endothelial NOS (eNOS) may also occur in neurons (Dinerman et al., 1994; O'Dell et al., 1994). Most neurotransmitters are stored in synaptic vesicles, and neurotransmitter effects are elicited following the exocytosis of transmitter into the synaptic space. For an evanescent transmitter such as NO, there is no storage pool, and newly synthesized NO is used as it is made. NO synthesis is triggered by the influx of calcium, which, when complexed with calmodulin, activates the biosynthetic activity of NOS (Bredt and Snyder, 1990).

Because NO lacks vesicular storage and depends on new synthesis for its release, nNOS must be associated with the plasma membrane. Subcellular fractionation indicates that roughly half of brain nNOS is soluble and half particulate (Hecker et al., 1994; Bredt, 1996). Recently, Bredt and associates showed that nNOS is targeted to membranes by binding to syntrophin, PSD95/SAP90, or PSD93 (Brenman et al., 1996a, 1996b). These proteins are enriched in synaptic densities and interact with nNOS through PDZ domains, consensus sequences of about 100 amino acids that are found in proteins that tend to be associated with cell–cell junctions (Ponting and Phillips, 1995). The nNOS/PSD95 interaction involves a portion of nNOS that includes its sole PDZ domain and the second PDZ domain of PSD95. PSD95 was first isolated from postsynaptic densities (Cho et al., 1992) but also occurs in presynaptic nerve terminals (Kistner et al., 1993) and clusters neurotransmitter receptors and ion channels at synaptic sites (Kornau et al., 1997). For instance, the NMDA receptor and several potassium channels are associated with PSD95 at synapses (Kornau et al., 1995). The linking of NMDA receptors to nNOS by PSD95 may explain why calcium influx following NMDA receptor activation leads to a tightly coupled nNOS activation (Brenman et al., 1996a). Indeed, the effects of NO appear to be intimately tied to the NMDA receptor. For example, NMDA receptor-mediated neurotoxicity (Dawson and Dawson, 1996), neurotransmitter release (Schuman and Madison, 1994), and cGMP elevations (Bredt and Snyder, 1989; Garthwaite et al., 1989) each require nNOS and are blocked by nNOS-specific inhibitors. Moreover, NO can directly modulate NMDA receptors (Lipton and Stamler, 1994).

We wondered whether nNOS binding to other proteins might regulate its intracellular localization. Accordingly, we conducted a yeast two-hybrid screen in which we have identified a novel protein, which we designate CAPON (*ca*rboxy-terminal *P*DZ ligand *o*f *n*NOS). CAPON is a cytoplasmic protein whose C terminus binds to the PDZ domain of nNOS. CAPON competes with PSD95 and PSD93 for binding to nNOS and thus may participate in the translocation and impede the activation of this enzyme.

Results

Identification and Cloning of CAPON

We conducted a yeast two-hybrid screen employing the first 377 amino acids of nNOS, a region that includes the PDZ domain that comprises the first 100 amino acids of nNOS. Screening of six million clones resulted in the identification of three distinct cDNA inserts, one of which, PIN, has been previously reported (Jaffrey and Snyder, 1996), while the other two are overlapping cDNAs derived from a gene that is designated *CAPON*. The CAPON two-hybrid clones share a common C terminus and are predicted to translate into 125 and 327

*To whom correspondence should be addressed.

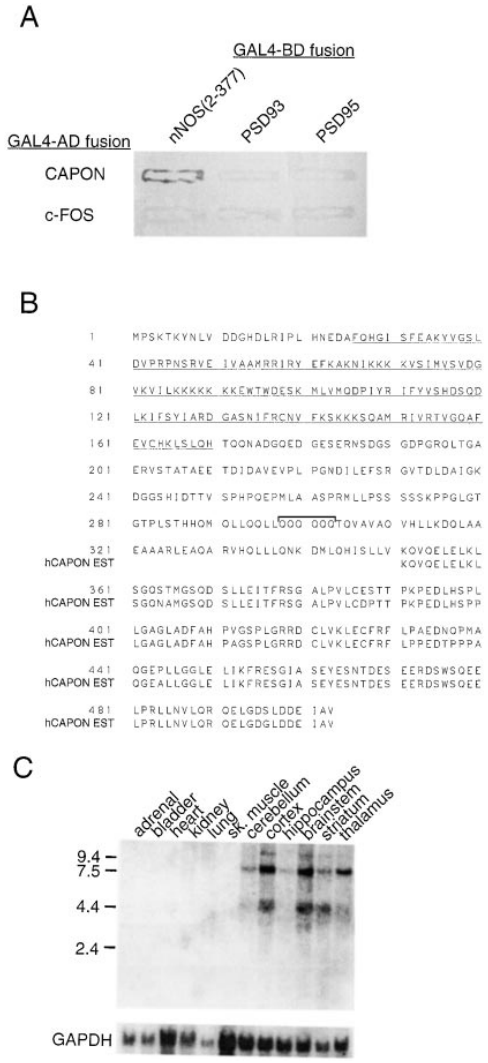

Figure 1. Cloning of the *CAPON* cDNA and Distribution of *CAPON* mRNA

(A) CAPON specifically interacts with nNOS in the yeast two-hybrid system. Yeast were transformed with the indicated GAL4 activation domain (AD) and GAL4 DNA-binding domain (BD) plasmids and grown on plates containing histidine. A typical filter lift is shown, in which β-galactosidase activity was detected by the appearance of a blue precipitate. pAD-CAPON1, comprising the last 125 amino acids of CAPON, activates *lacZ* transcription in the presence of pBD-nNOS(2–377) but not with plasmids comprising the first three PDZ domains of PSD95 (amino acids 20–364) or the second PDZ domain of PSD93 (amino acids 116–421).

(B) Amino acid sequence of rat CAPON and alignment with a partial human sequence. The underlined sequence corresponds to the putative PTB domain. The bracketed sequence is encoded by a CAG repeat. A human expressed sequence tag (EST) (accession number R19867) obtained from a library derived from infant brain contains a 459 bp open reading frame with homology to the C terminus of the *CAPON* cDNA ($p = 5.1 \times 10^{-17}$). The conceptual translation of this clone reveals a protein with 92% amino acid identity with the rat protein.

(C) *CAPON* is enriched in neuronal structures. Northern (RNA) blot analysis reveals that several *CAPON* transcripts are present, and these transcripts are enriched in neuronal tissues.

amino acid peptides followed by a stop codon. The 125 amino acid C-terminal fragment of CAPON specifically interacts with nNOS in the two-hybrid system as is evident from the failure of CAPON to interact with fragments of PSD93 and PSD95 containing PDZ domains (Figure 1A). Moreover, nNOS fails to interact with another control protein, c-fos. To obtain a full-length *CAPON* cDNA, we screened a rat brain cDNA library with the larger two-hybrid clone and isolated a 2100 bp cDNA that overlapped with the two-hybrid clone and was used to assemble a final 2820 bp cDNA (see Experimental Procedures). The conceptual translation of this cDNA produces a 503 amino acid protein (Figure 1B). The first ATG in the cDNA was 393 bp from the 5′ end of the cDNA and was situated in a context that conformed to the Kozak consensus sequence for an initiator methionine (Kozak, 1991).

CAPON displays no significant homology to any other known class of protein except for an N-terminal 145 amino acid stretch of amino acids that has residues suggestive of a phosphotyrosine-binding (PTB) domain (Zhou et al., 1995). CAPON's PTB domain most closely resembles the mouse numb protein's PTB domain (Zhong et al., 1996), with nearly 28% sequence identity on this region. The similarity between CAPON and numb is limited to this domain. PTB domains are targeted to phosphotyrosine-containing proteins such as growth factor receptors (reviewed in van der Geer and Pawson, 1995).

Outside of the PTB domain, *CAPON* lacks any well-known consensus sequences except for an 18 nucleotide stretch of CAG repeats that corresponds to six glutamines. Glutamine repeats occur in proteins whose expansion results in neurodegenerative diseases as exemplified by huntingtin, the protein that is altered in Huntington's disease (Ross, 1995). A BLAST search (Altschul et al., 1990) of an expressed sequence tag database, dBEST, reveals a human brain-derived EST with ~75% nucleotide identity to *CAPON*. The cDNA insert was 1.4 kb and corresponds to the C-terminal 156 amino acids of *CAPON* plus 1 kb of 3′ UTR. The conceptual translation of this portion of human *CAPON* has 92% amino acid identity with the rat protein (Figure 1B).

Northern (RNA) blotting reveals a predominant 7.5 kb transcript that is detected only in brain regions, with no expression evident in adrenal, bladder, heart, kidney, lung, and skeletal muscle (Figure 1C). Marked regional variations occur in the brain, with highest densities in the cerebral cortex and medulla-oblongata and lowest levels in the hippocampus.

CAPON and nNOS Interact In Vivo and In Vitro

To assess the specificity of interactions between CAPON and nNOS, we evaluated the binding of a GST–CAPON fusion protein, consisting of the C-terminal 125 amino acids of CAPON, with nNOS, eNOS, and inducible NOS (iNOS) (Figure 2A). Lysates of HEK-293 cells transfected with expression plasmids for each of the three forms of NOS were incubated with GST–CAPON. After extensive washing of the resin, bound nNOS was detected by Western blotting with the appropriate isoform-specific antibody. nNOS interacts strongly with GST–CAPON,

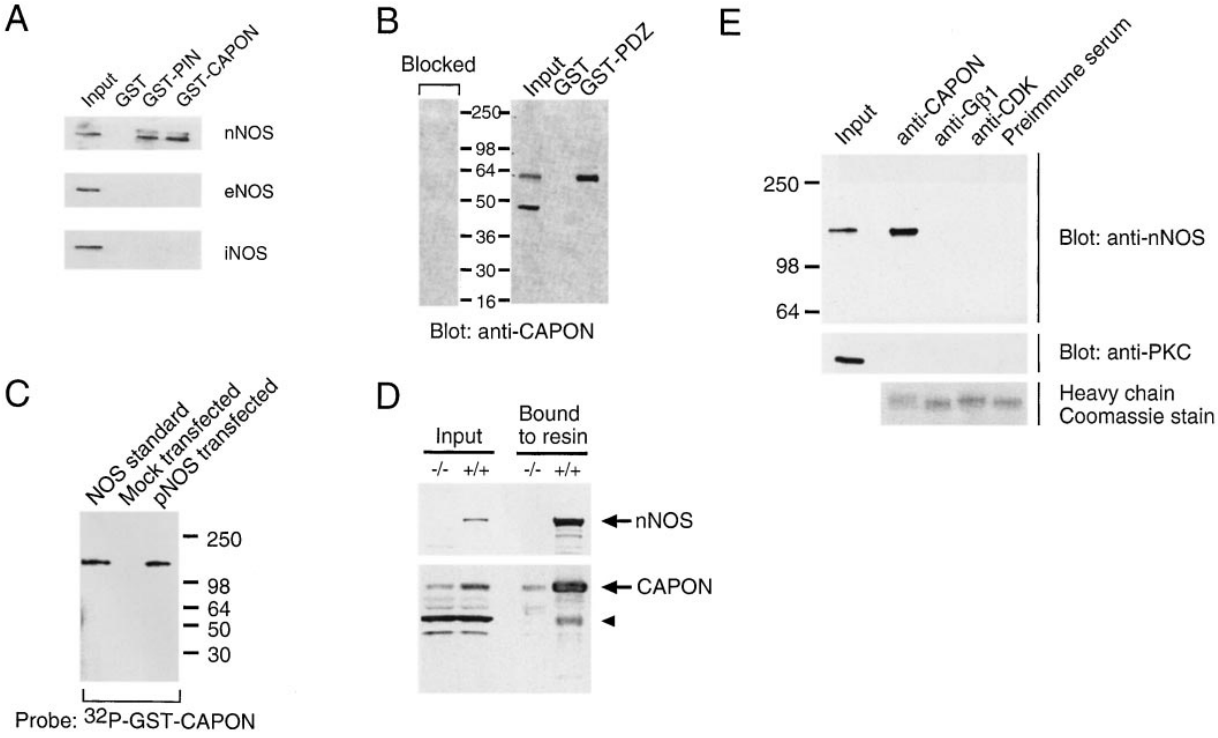

Figure 2. Interaction of CAPON and nNOS

(A) CAPON binds to nNOS but not eNOS or iNOS. Bacterially expressed GST, GST–PIN, and GST–CAPON were bound to glutathione agarose and then incubated with lysates of HEK-293 cells transfected with expression plasmids for the indicated isoforms of NOS. After extensive washing of the resins, bound NOS was detected with isoform-specific antibodies. While nNOS binds to both GST–PIN and GST–CAPON, neither eNOS or iNOS binds to either protein. Input = 10% of starting material applied to each resin.

(B) A GST–NOS fusion protein specifically binds to rat brain CAPON. A fusion protein consisting of GST and amino acids 1–100 of nNOS was bound to glutathione agarose and then incubated with cerebellar supernatants. After extensive washing, CAPON is detected on the NOS resin but not on the control GST resin. A second ~48 kDa band is also detected with this antibody but fails to interact with nNOS. This band may represent a cross-reactive protein, an alternatively spliced isoform of CAPON, or a degradation product.

(C) CAPON interacts with nNOS directly. HEK-293 lysates transfected with a expression plasmid containing the *nNOS* cDNA or empty vector were resolved by electrophoresis, transferred to nitrocellulose, and probed with radiolabeled CAPON (see Experimental Procedures). Purified nNOS is recognized with this probe, along with a comigrating band detected in NOS-transfected but not mock-transfected cells.

(D) CAPON and nNOS complexes are detectable in cerebellar lysates (I). Cerebellar supernatants were prepared from wild-type (+/+) and nNOS knockout (−/−) mice and incubated with 2′,5′-ADP-Sepharose, an nNOS affinity resin. Only CAPON derived from supernatants of wild-type and not knockout animals was capable of binding the resin, indicating that the presence of nNOS is required for CAPON to bind to the resin. CAPON levels were decreased in knockout animals, presumably due to decreased stability in the absence of the nNOS-binding partner. A lower molecular weight band (arrowhead), frequently detected with the anti-CAPON antibody, bound weakly to the resin in an nNOS-specific manner as well. Input = 20% of lysate used for binding.

(E) CAPON and nNOS complexes are detectable in cerebellar lysates (II). An antibody to CAPON (5 μg) specifically coprecipitates nNOS, while comparable amounts of antibody to the G protein subunit β1, cyclin-dependent kinase 2, and preimmune serum fail to coprecipitate nNOS. Enrichment of nNOS in CAPON immunoprecipitates was specific, as a control protein, protein kinase C-β I/II, did not display similar enrichment. Input = 10% of lysate used for immunoprecipitation.

while eNOS and iNOS do not interact. No interactions are evident with the GST control.

We also examined interactions of CAPON and nNOS by utilizing a GST fusion protein of the N-terminal 100 amino acids of nNOS, the PDZ domain. Following incubation with cerebellar lysates, the washed GST–nNOS resin was subjected to SDS-PAGE and Western blotted with a purified antibody directed against the C-terminal 125 amino acids of CAPON. A single band corresponding to CAPON is detected, while no CAPON is bound to the GST control. A lower molecular weight band in homogenates is occasionally detected using our anti-CAPON antibodies. This protein fails to interact with GST–nNOS fusion proteins (Figure 2B) and may represent an unrelated cross-reactive protein or the product of an alternatively spliced *CAPON* mRNA that fails to bind nNOS.

The protein–protein interactions we detected with cell lysates and GST fusion proteins may reflect a tertiary interaction between nNOS, an unidentified protein, and CAPON. To determine if CAPON directly binds to nNOS, we conducted blot overlay experiments (Figure 2C). Lysates of HEK-293 cells transfected with nNOS were resolved on a polyacrylamide gel, transferred to nitrocellulose, and then probed with [^{32}P]GST–CAPON. The radiolabeled CAPON probe binds to a single 160 kDa band

comigrating with an nNOS standard, while no binding is evident in mock transfected cells, demonstrating that CAPON physically interacts in a direct manner with nNOS.

To ascertain if complexes of CAPON and nNOS exist physiologically, we used two approaches. Our first approach took advantage of a NOS affinity resin consisting of 2′, 5′-ADP ribose crosslinked to an agarose matrix. This resin has been used previously to purify nNOS from cerebellar supernatants (Bredt and Snyder, 1990). Following incubation with cerebellar supernatants, the resin was washed extensively. As expected, a significant portion of the nNOS found in the starting material was bound to the resin (Figure 2D). To determine if nNOS and CAPON were physiologically associated, we next assayed for CAPON bound to the resin. Like nNOS, CAPON was substantially enriched in the bound fraction. As a control, we assayed for the resin-binding ability of CAPON from supernatants derived from mice with a genomic deletion of nNOS. These mice express a truncated version of nNOS that lacks the PDZ domain and is unable to bind CAPON. Substantially less CAPON in these supernatants bound to the 2′, 5′-ADP ribose resin, indicating that CAPON has negligible intrinsic affinity for the resin and that CAPON binding in wild-type supernatants was due to nNOS. The smaller cross-reactive band was also enriched on this resin, supporting the notion that it is in some manner related to CAPON by alternative splicing or proteolytic degradation. Interestingly, the total level of CAPON in knockout supernatants was approximately one half that in wild-type mice, possibly due to the absence of a stabilizing effect of nNOS.

As a second approach, we immunoprecipitated CAPON from cerebellar supernatants and assayed for nNOS by Western blot (Figure 2E). nNOS coprecipitates with anti-CAPON antibodies but is not detected in immunoprecipitates generated using preimmune serume, an antibody to the G protein subunit β1, or with an antibody to cyclin-dependent kinase 2. To determine if the enrichment observed in anti-CAPON immunoprecipitates was specific, we asked if a control protein, protein kinase C-β I/II, was similarly enriched in these fractions. We found that this protein was absent in all of the precipitates (Figure 2E), indicating that the coprecipitation of nNOS with CAPON was specific. These two approaches support the notion that CAPON and NOS exist as a complex in the rat cerebellum.

In other experiments, we metabolically labeled N1E-115 mouse neuroblastoma cells with [^{35}S]methionine and examined for the presence of proteins that would bind to GST–CAPON. The only protein that specifically interacted with CAPON is a 160 kDa protein that comigrates with an nNOS standard (unpublished data), suggesting that nNOS is the most abundant CAPON-binding protein in these cells.

CAPON and nNOS Are Colocalized in Rat Brain

To clarify further the relationship between nNOS and CAPON, we compared their distributions in the brain by immunohistochemistry (Figures 3A and 3B). Our localizations of nNOS are identical to those previously reported, with high levels of immunoreactivity in the accessory olfactory bulb, caudate-putamen, cerebellum, cerebral cortex, dentate gyrus of the hippocampus, Islands of Calleja, olfactory bulb, superior colliculus, and supraoptic nucleus (Figure 3A) (Bredt et al., 1990; Rodrigo et al., 1994). CAPON immunoreactivity displays similar distributions, with highest levels in the olfactory bulb and cerebellum like nNOS (Figure 3A). We also localized *CAPON* mRNA by in situ hybridization using a probe directed against the 3′ region of the *CAPON* open reading frame. *CAPON* mRNA densities were especially prominent in the accessory and main olfactory bulbs, as well as the hippocampus, cortex, and cerebellum.

High-power immunohistochemical studies reveal that CAPON and nNOS exhibit numerous colocalizations throughout the brain. The supraoptic nucleus stains intensely for both CAPON and nNOS (Figure 3B). The nucleus of the trapezoid body contains CAPON-positive and nNOS-positive cell bodies separated by unreactive fascicles of nerve fibers (Figure 3B). The adjacent pontine nucleus also exhibits CAPON and nNOS immunoreactivity of its small neuronal cell bodies (Figure 3B). In the cerebellum, Purkinje cells fail to stain for either CAPON or nNOS. The neuropil of the molecular cell layer stains intensely for both CAPON and nNOS. There are exceptions to CAPON–nNOS colocalizations. For example, the granular cell layer of the cerebellum exhibits strong nNOS staining but weak CAPON staining (Figure 3B).

Under higher magnification, we find that CAPON, like nNOS, is detected only in neurons, with labeling of both the soma and processes (Figure 3B). All immunoreactivity is abolished by preabsorption of the CAPON antiserum with the antigenic peptide overnight before incubation with brain sections (Figure 3A).

The C Terminus of CAPON Binds to the PDZ Domain of nNOS

To examine the region of nNOS that binds to CAPON, we conducted yeast two-hybrid experiments with various truncations of nNOS (Figure 4A). As little as the first 100 amino acids of nNOS binds to the C-terminal 125 amino acids of CAPON. This portion of nNOS contains the full PDZ domain as defined by MacKinnon and associates (Doyle et al., 1996), who identified the PDZ consensus domain in nNOS as amino acids 14–89. Deletion of the first 20 amino acids of nNOS, which include the first seven amino acids of the PDZ domain, does not abolish binding, but larger N-terminal deletions abolish binding, presumably because they result in a loss of important structural components of the PDZ domain. The nNOS construct comprising amino acids 163–245, which represents the PIN-binding domain of nNOS (Jaffrey and Snyder, 1996), shows no interaction with CAPON.

PDZ domains typically interact with a characteristic C-terminal motif in other proteins (Songyang et al., 1997). By contrast, the nNOS PDZ domain binds directly to other PDZ domains, such as those in PSD95, but has not previously been reported to interact with any known physiological C-terminal peptide motifs. Since we could not detect any PDZ domain motifs in the CAPON sequence, we sought to determine the region in CAPON that accounted for nNOS binding. We investigated the domain of CAPON that interacts with nNOS using GST–

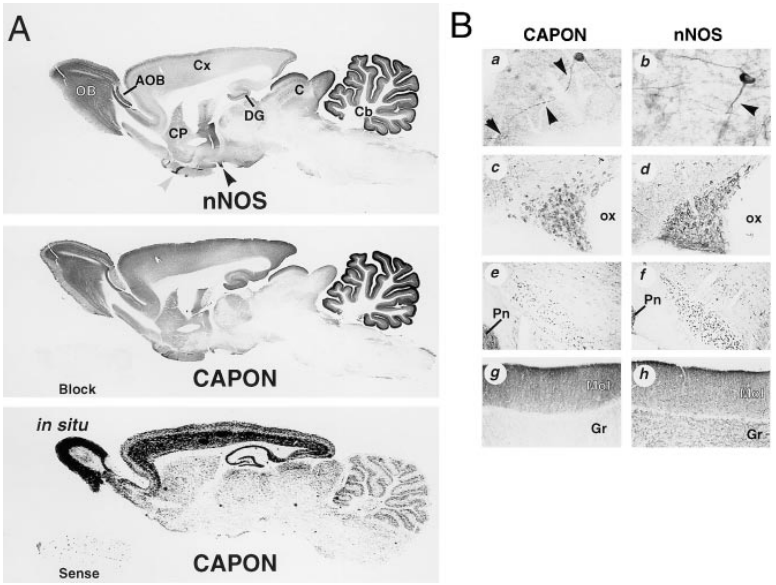

Figure 3. Immunohistochemical Localization of nNOS and CAPON

(A) Comparison of nNOS IR (immunoreactivity), CAPON IR, and *CAPON* in situ hybridization patterns in sagittal sections of adult rat. Islands of Calleja (solid arrowhead); supraoptic nucleus (open arrowhead); AOB, accessory olfactory bulb; C, colliculi; Cb, cerebellum; Cx, cerebral cortex; OB, olfactory bulb. Immunohistochemical nonspecific labeling (Block) was determined using a CAPON antibody preabsorbed with the antigenic peptide. Nonspecific hybridization (Sense) was detected using a sense probe.

(B) Comparison of cellular localization of nNOS IR and CAPON IR in adult rat brain. (a) CAPON IR and (b) nNOS IR hypothalamic neurons; solid arrowheads indicate IR dendritic processes. (c) CAPON IR and (d) nNOS IR of the supraoptic nucleus. (e) CAPON IR and (f) nNOS IR cell bodies of the nucleus of the trapezoid body separated by unreactive fascicles of nerve fibers. Adjacent is the pontine nucleus (Pn), which exhibits both CAPON and nNOS IR (e and f). (g) CAPON IR and (h) nNOS IR in the cerebellum. Mol, molecular cell layer; Gr, granular cell layer. Magnification: (e) and (f), 50×; (c), (d), (g), and (h), 100×; (a) and (b), 200×.

CAPON fusion proteins containing various deletions at the C terminus (Figure 4B). Immobilized fusion proteins were incubated with HEK-293 lysates containing nNOS. Bound nNOS was detected by Western blot. Robust interactions with nNOS are evident with constructs containing as little as the C-terminal 13 amino acids of CAPON. Deleting the C-terminal 20 amino acids of CAPON abolishes its interactions with nNOS. These data show that the C-terminal portion of CAPON is necessary and sufficient for nNOS binding.

Recently, Cantley and associates (Songyang et al., 1997) identified consensus sequences for binding to several PDZ domains. Binding of PDZ ligands involves the C terminus of proteins, with determinants of specificity lying within the eight or fewer C-terminal amino acids. A consistent requirement among all the PDZ domain ligands is a hydrophobic residue, such as valine or leucine, as the final amino acid. To determine if the binding of CAPON to nNOS exhibits similar sequence specificity, we examined the binding of mutagenized His_6–CAPON fusion proteins to immobilized GST–nNOS PDZ domain fusion proteins (Figure 4C). The C-terminal residue of CAPON is a valine, and conversion of this residue to alanine abolishes binding. Binding is also greatly reduced by changing the penultimate amino acid from alanine to aspartate. However, changing the n-2 amino acid from isoleucine to serine or alanine does not alter binding. These experiments indicate that the nNOS/CAPON interaction resembles that of other PDZ/C-terminal peptide ligand interactions. Specifically, C-terminal residues of CAPON are important for the specificity in CAPON binding to nNOS.

CAPON and PSD95 Compete for Binding to nNOS

Since the nNOS PDZ domain is capable of both PDZ–PDZ interactions and PDZ/C-terminal peptide interactions, we wondered whether CAPON and PSD95 can bind simultaneously to nNOS or whether their interactions with nNOS are mutually exclusive. To answer this question, we incubated lysates of HEK-293 cells containing nNOS with various concentrations of a His_6–CAPON fusion protein, comprising the last 125 amino acids of CAPON, and then added these lysates to GST–PSD95 immobilized on glutathione agarose resin (Figure 5A). After extensive washing of the resin, we assayed for nNOS bound to PSD95 by Western blot. As little as 5 nM His_6–CAPON causes a substantial reduction of nNOS binding to GST–PSD95. Half-maximal reduction of binding is evident between 5 and 50 nM His_6–CAPON (Figure 5B). Deletion of the C-terminal 20 amino acids of CAPON abolishes its ability to serve as a competitor for nNOS binding to GST–PSD95. As controls, we examined the effects of 5 μM His_6–PIN or 5 μM His_6–FK506 binding protein-12 (FKBP). Neither of these proteins competes for binding to nNOS. nNOS also binds to the second PDZ domain of PSD93, a protein that is highly homologous to PSD95 (Brenman et al., 1996b). His_6–CAPON is an effective competitor for nNOS binding to immobilized GST–PSD93 as well (Figures 5A and 5B). These effects of CAPON likely reflect its binding to nNOS rather than to the PDZ domains of either PSD95 or PSD93 because CAPON fails to interact with either PSD93 or PSD95 using a two-hybrid assay (see Figure 1A) and in vitro experiments utilizing immobilized GST–PSD95 and purified recombinant CAPON (data not shown).

We wanted to determine if CAPON and PSD95 compete for binding to nNOS in intact cells. Accordingly, we transfected HEK-293 cells with various mixtures of expression plasmids containing cDNAs of hemagglutinin antigen (HA)-tagged nNOS, *myc*-tagged PSD95, and/or full-length CAPON. Following immunoprecipitation with antibodies to HA, we examined which proteins

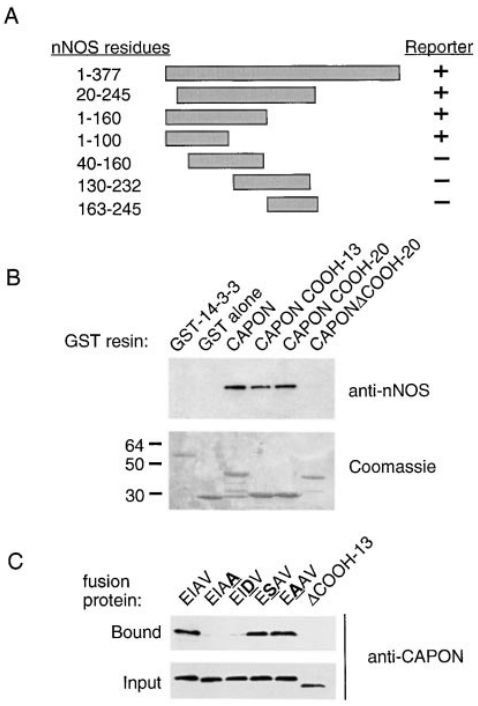

Figure 4. The PDZ Domain of nNOS Interacts with the C Terminus of CAPON

(A) The PDZ domain of nNOS (amino acids 20–100) is sufficient for binding to CAPON. Truncations of nNOS were subcloned into the GAL4 BD vector, and nNOS/CAPON interactions were detected by β-galactosidase assays.

(B) The C-terminal 13 amino acids of CAPON are sufficient for binding to nNOS. Various GST–CAPON fusion proteins were incubated with HEK-293 lysates containing nNOS. A CAPON fusion protein comprising the last 100 amino acids binds nNOS, as do fusion proteins comprising the last 13 or 20 amino acids of CAPON. A CAPON fusion protein with the last 20 amino acids deleted no longer binds nNOS. Neither of the control proteins GST or GST-14-3-3 is able to bind nNOS.

(C) Amino acid substitutions in the C terminus of CAPON prevent it from interacting with nNOS. His_6 fusion proteins of the last 100 amino acids of CAPON were generated and incubated with GST–NOS (1–100) immobilized on glutathione agarose. While the unmutagenized sequence binds (last four amino acids, EIAV), mutation of the terminal valine (EIAA) or the penultimate alanine (EIDV) prevents binding. Serine or alanine mutations are tolerated at the n-2 position (ESAV and EAAV), but truncation of the C-terminal 13 amino acids blocks binding altogether.

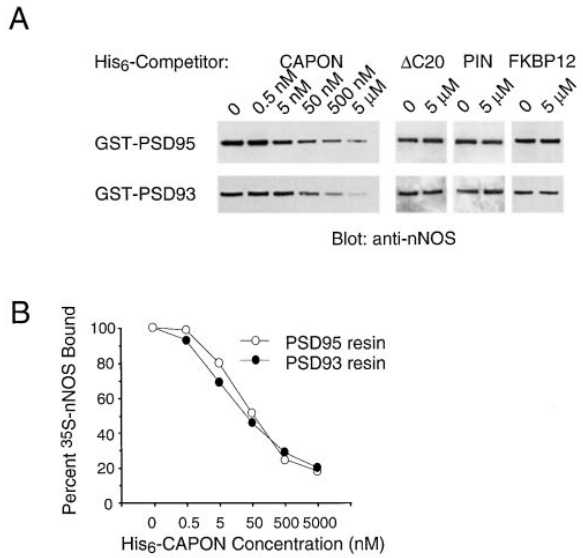

Figure 5. CAPON and PSD95 Compete for Binding to nNOS

(A) His_6–CAPON fusion proteins specifically block the nNOS/PSD95 interaction in vitro. The C-terminal 100 amino acids were fused to a His_6 tag and added to HEK-293 lysates transfected with nNOS expression plasmids at the indicated fusion protein concentration. This mixture was added to GST–PSD95 (amino acids 20–364) or GST–PSD93 (116–421), the regions of these proteins having been previously shown to interact with nNOS (Brenman et al., 1996). The disruption of the nNOS/PSD95 interaction required the C-terminal 13 amino acids, as this fusion protein (ΔC20) fails to block the interaction even at 5 μM. Other control proteins such as His_6-PIN or His_6-FKBP do not disrupt the nNOS/PSD95 interaction.

(B) Quantification of CAPON inhibition of the nNOS/PSD95 interaction. HEK-293 cells were transfected with nNOS expression plasmids and then metabolically labeled with [^{35}S]methionine. Radiolabeled nNOS was purified and mixed with His_6–CAPON as in (A), above. Bound nNOS was resolved by electrophoresis, and counts were determined on a PhosphoImager.

coprecipitated. In cells expressing HA-nNOS and *myc*-PSD95, antibodies to HA coprecipitate *myc*-PSD95 (Figure 6). When various amounts of *CAPON* cDNA containing expression plasmids are also transfected, HA immunoprecipitates contain CAPON but substantially less PSD95.

Discussion

The main finding of this study is the identification of a novel protein, CAPON, which interacts selectively with nNOS. The interaction of CAPON with nNOS is highly specific and has been verified by several methods of monitoring protein–protein interactions. The similarities in neuronal localizations of CAPON and nNOS imply that these proteins interact physiologically and that the principal biological function of CAPON may be to interact with nNOS. The apparent selectivity of this interaction contrasts with other nNOS-binding proteins, such as PSD95, PIN, and calmodulin, each of which binds to multiple other proteins.

The competitive binding for nNOS by CAPON and PSD95 suggests a model for regulating the translocation of nNOS between synaptic and nonsynaptic structures (Figure 7). Presumably, NMDA receptor-mediated NO release into the synaptic space must be preceded by the translocation of nNOS to synaptic structures by binding to PSD95. We propose that this process can be blocked by CAPON's removal of nNOS from PSD95 and translocating nNOS into the cytoplasm or some other cellular compartment. In this manner, CAPON could lead to effective nNOS inhibition. Although CAPON does not inhibit nNOS catalytic activity directly (data not shown), CAPON would reduce the accessibility of nNOS to NMDA receptor-mediated calcium influx, thus diminishing the capacity of nNOS to exert its physiologic or pathologic effects. Small molecules that specifically bind to nNOS in a manner similar to that of CAPON may be useful for blocking NO-mediated neuronal degeneration.

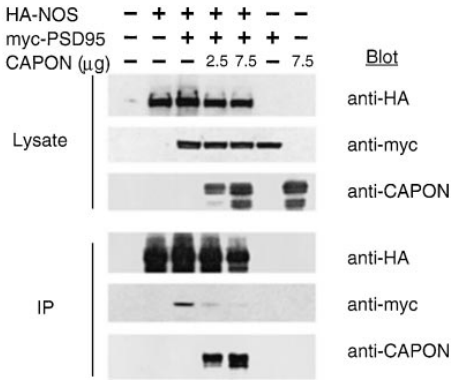

Figure 6. CAPON Expression Prevents the Interaction of PSD95 and nNOS

HEK-293 cells were transfected with various combinations of expression plasmids for HA-tagged nNOS (HA-NOS), *myc*-tagged PSD95, or CAPON. Following transfection, the lysates were immunoprecipitated with an anti-HA antibody, and bound proteins were detected with the appropriate antibodies. Following cotransfection of HA-NOS and *myc*-PSD95, *myc*-PSD95 is detected in anti-HA immunoprecipitates. Cotransfection of a full-length CAPON expression plasmid substantially reduces the amount of *myc*-PSD95 in anti-HA precipitates. In the absence of HA-nNOS transfection, neither *myc*-PSD95 or CAPON is immunoprecipitated by anti-HA antibodies.

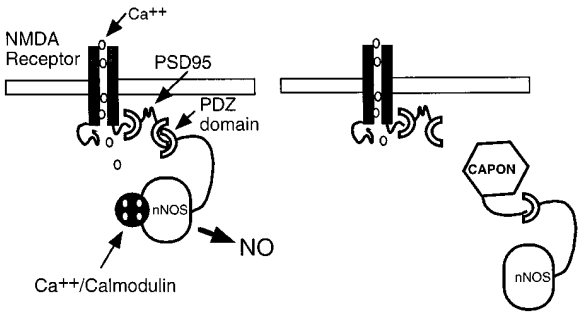

Figure 7. Model of PSD95/nNOS Regulation by CAPON

NMDA receptors are coupled to nNOS through a PSD95 multimer. These interactions are mediated by PDZ domains. In this complex, nNOS is situated close to NMDA receptor-modulated calcium influx (left). Binding of CAPON (right) results in a reduction of NMDA receptor/PSD95/nNOS complexes, leading to decreased access to NMDA receptor-gated calcium influx and a catalytically inactive enzyme.

We have explored potential mechanisms that might regulate the nNOS/CAPON interaction. For instance, we phosphorylated nNOS in transfected HEK-293 cells by treatment with forskolin, phorbol esters, dibutyryl cyclic AMP, and 8-bromo-cyclic GMP and in vitro with purified nNOS protein utilizing protein kinase C, protein kinase A, and calcium/calmodulin-dependent protein kinase using methods described previously (Bredt et al., 1992). We have been unable to alter CAPON/nNOS interactions by any of these treatments (unpublished data).

Conceivably, phosphorylation of CAPON regulates its interactions with nNOS. Recently, some of us showed that phosphorylation of the n-2 serine in the potassium channel BIRK-2 regulates its binding to a PDZ domain in PSD95 (Cohen et al., 1996). CAPON and several other PDZ domain ligands (Songyang et al., 1997) lack a serine in this position and so must be regulated in some other manner. One possible mechanism may be a regulation of the ligand's C-terminal secondary structure. A recent crystallographic study of a PDZ domain complexed with a short cognate peptide shows that the peptide binds in an antiparallel β-sheet conformation, with characteristic β-sheet contacts between the peptide and a strand of a β sheet within the PDZ domain (see Doyle et al., 1996). In a physiologic setting, the unbound cognate sequence may constitutively adopt a β-sheet conformation, with the other β strands coming from other, possibly distant, residues within the ligand protein's sequence. This β sheet might constitute an endogenous high-affinity ligand. This notion is supported by our observation that short, presumably unfolded, peptides comprising the C-terminal nine residues of CAPON bind nNOS weakly, while 16 residue peptides are more potent competitors (IC_{50} = 10 μM), although both are much less effective than 100 amino acid fusion proteins that are active in the nanomolar range (see Figure 5). Peptide competitors that interact with other PDZ domains have also been utilized at 10 and 500 μM concentrations (see Brenman et al., 1996a; Kornau et al., 1995). Conceivably, the nNOS/CAPON interaction would be disrupted simply by disrupting the β-sheet conformation of the C terminus, which might be achieved by phosphorylation at a distance.

The nNOS PDZ domain is an example of a PDZ domain that binds to other PDZ domains. The region of nNOS that possesses this property is the PDZ domain plus the adjacent ~50 amino acids on the C side of the PDZ domain (residues 1–150) (Brenman et al., 1996b). The additional amino acids in this super-sized PDZ domain may be required to accomodate larger ligands such as other PDZ domains. The finding in this report of another physiologic ligand for the PDZ domain, namely, the C-terminal region of CAPON, raises the question of whether the same or different portions of the nNOS PDZ domain account for the binding to two seemingly different ligands. Because these interactions are mutually exclusive, it is likely that the ligand-binding cleft in the PDZ domain mediates both interactions. Previously identified proteins that contain C-terminal PDZ-binding sequences have been membrane-associated. By contrast, CAPON is soluble. This demonstrates that PDZ domains may mediate purely cytosolic protein–protein interactions.

Stricker et al. (1997) recently characterized the specificity of the nNOS PDZ-binding domain. These researchers used a phage display method to identify NOS-binding peptides. Peptides ending in the sequence aspartate-X-valine were found to be high-affinity ligands. Interestingly, unlike CAPON, these peptides did not bind the canonical nNOS PDZ domain (amino acids 13–89) but bound the extended PDZ domain only (amino acids 1–150). This extended domain is the minimal sequence that mediates PDZ–PDZ interactions. Presumably, the differences in the binding sites in nNOS for the phage display peptide and CAPON account for the differences between the sequence specificity requirements for PDZ–PDZ interactions and PDZ–CAPON interactions.

Experimental Procedures

General Methods and Materials

Molecular biology reagents were from New England Biolabs (Beverly, MA) and all other reagents were from Sigma (St. Louis, MO) except as indicated. Protein concentrations were determined by Bradford assay.

Yeast Two-Hybrid Methods

Two-hybrid screens and the construction of the parent vectors pPC86, containing the GAL4 activation domain, and pPC97, containing the GAL4 DNA-binding domain, have been described (Jaffrey and Snyder, 1996). Plasmid pBD-NOS(2–377) was prepared by the insertion of an nNOS PCR product corresponding to amino acids 2–377 of rat nNOS into the SalI and BglII sites of pPC97, resulting in an open reading frame encoding a GAL4 BD-NOS fusion protein (Jaffrey and Snyder, 1996). The nNOS fragment was constructed by PCR using the following primers: 5′-GACTAGTCGACTGAAGAGAAC ACGTTTGGG-3′ (coding strand) and 5′-TCTGCAGATCTCAGTGGG CCTTGGAGCCAAA-3′ (noncoding strand).

A rat hippocampal cDNA library in pPC86 (Li et al., 1995) was amplified once in DH10B (GIBCO BRL) as described (Jaffrey and Snyder, 1996) and transformed into yeast containing the pBD-NOS(2–377) plasmid. pAD-CAPON1 and pAD-CAPON2 were identified as 0.8 kb and 1.9 kb clones, respectively, that activated *lacZ* transcription and conferred histidine prototrophy in the presence of pBD-NOS(2–377). Plasmids were sequenced by automated fluorescent sequencing of both strands. Yeast two-hybrid vectors containing the second PDZ domain of PSD93 (amino acids 116–421) and the three PDZ domains of PSD95 (amino acids 20–364) have been described previously (Brenman et al., 1996a).

Truncated NOS fragments comprising amino acids 2–165 and 2–284 were generated by restriction of the initial NOS (2–377) PCR fragment with NcoI and AvaI, respectively, followed by Klenow filling in of that end and ligation into pPC97. Other truncated NOS fragments were prepared by PCR and have been described (Jaffrey and Snyder, 1996).

Cloning of *CAPON* cDNA

A *CAPON* DNA probe was generated by the random hexamer method using the *pAD-CAPON2* cDNA as a template. This probe was used to screen a rat brain λZAPII cDNA library (Stratagene) using methods described by the manufacturer. A 2.1 kb cDNA was isolated that overlapped with the pAD-CAPON2 clone. The cDNAs were ligated at an overlapping XbaI site to produce the full-length 2,812 bp cDNA and subcloned into pCMV for eukaryotic expression. The human *CAPON* EST was the sole *CAPON* homolog identified in a BLAST seacrh (Altschul et al., 1990). I.M.A.G.E. consortium (http://www.bio.llnl.gov/bbrp/image/image.html/) clone 34183 (Lennon et al., 1996) was purchased from Research Genetics (Huntsville, AL).

RNA (Northern) Blotting

Thirty micrograms of whole RNA was isolated from rat tissues using the Triazol reagent (GIBCO BRL) and separated on agarose-formaldehyde gels. RNA was transferred to Hybond N+ membranes (Amersham), and a DNA probe, generated using the random hexamer method with the two-hybrid *CAPON* cDNA as a template, was hybridized in Rapid-hyb buffer (Amersham) overnight at 65°C. The blots were subsequently washed sequentially in 2× SSC with 0.1% SDS, once at room temperature for 15 min, 1× SSC with 1% SDS twice at 65°C, 0.1× SSC with 1% SDS twice at 65°C, and then with 0.1× SSC with 5% SDS twice at 65°C. The blot was apposed to film for 4 days at −80°C to visualize the bands.

GST Fusion Protein Binding Assays

GST fusion proteins were prepared in BL21(DE3) *Escherichia coli* (Novagen) with glutathione agarose as an affinity resin for purification (Smith and Johnson, 1988), except that bacterial pellets were sonicated in lysis buffer (50 mM Tris-HCl [pH 7.7], 100 mM NaCl, and 2 mM EDTA), supernatants were adjusted to 1% Triton X-100, washes were done in 50 mM Tris-HCl (pH 7.7), 500 mM NaCl, 2 mM EDTA, and 1% Triton X-100, and protein was purified with elution buffer (50 mM Tris-HCl [pH 7.7], 100 mM NaCl, 10 mM reduced glutathione, and 2 mM EDTA).

Transfections were performed with 10 μg of each plasmid using the calcium phosphate method. Following transfection, cells were sonicated in buffer A (50 mM Tris-HCl [pH 7.7], 100 mM NaCl, 2 mM EDTA, and 1% Triton X-100) and cleared by centrifugation. This cellular lysate was incubated with GST fusion protein immobilized on glutathione agarose for 1 hr at 4°C and washed extensively in HNTG buffer (20 mM HEPES [pH 7.4], 500 mM NaCl, 10% glycerol, and 0.1% Triton X-100) five times, for 10 min per wash at room temperature. A GST–CAPON fusion protein consisting of amino acids 379–503 was used for binding assays because it was more soluble when expressed in bacteria than larger CAPON fusion proteins.

For quantitative binding experiments, transfected cells were metabolically labeled overnight with 200 μCi [^{35}S]methionine, and nNOS was purified by NADPH elution of 2′,5′-ADP ribose as described previously (Bredt and Snyder, 1990).

The material remaining on the resin was eluted with SDS-PAGE sample buffer, and nNOS was detected by immunoblot using antibodies specific to each NOS isoform (Transduction Labs). A polyclonal antiserum to CAPON was generated in rabbits by using a His_6-tagged CAPON fusion protein. GST–CAPON was crosslinked to glutathione agarose with dimethylpimelimidate, and this resin was used to purify CAPON antibody. To confirm the specificity of the antibody, immune serum was incubated with His_6–CAPON, which results in the abolishment of the signal. Incubation with His_6-FKBP has no effect on the signal (data not shown).

For blot-overlay analysis, CAPON was inserted into pGEX-4T2, a derivative of PGEX4T2 in which two cyclic AMP-dependent protein kinase (PKA) sites were inserted between the GST moiety and the multiple cloning site (Jaffrey and Snyder, 1996). Kinase reactions and blot overlays were performed as described (Kavanaugh and Williams, 1994).

Immunoprecipitations

Immunoprecipitations were performed by homogenizing one rat cerebellum in 3 ml of lysis buffer followed by centrifugation at 100,000 × g for 30 min at 4°C. Two hundred microliters of the supernatant was incubated with 40 μl of protein A-agarose (Oncogene Sciences, Cambridge, MA) and 5 μg of the indicated antibody for 60 min at 4°C. The resins were then washed with IP wash buffer (50 mM Tris-HCl [pH 7.7], 400 mM NaCl, and 2 mM EDTA) six times and eluted in 60 μl of 1× SDS-PAGE sample buffer by boiling. Western blots were performed using an nNOS-specific monoclonal antibody (Transduction Labs) or a PKC-β I/II monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The immunoprecipitating antibodies used as controls were from Santa Cruz Biotechnology.

For experiments utilizing 2′,5′-ADP-Sepharose (Pharmacia), tissues were prepared identically as for immunoprecipitations, except mouse cerebella were used, and the homogenization volume was 400 μl per cerebellum. Supernatants were incubated with 100 μl of affinity resin. Incubations and washes were performed identically as for immunoprecipitations. The generation of mice with a targeted deletion of nNOS has been described previously (Huang et al., 1993).

Immunohistochemistry

Adult Sprague-Dawley rats (200–250 g) were obtained from Charles River and housed at the Johns Hopkins Animal Care Facility. A polyclonal antiserum to the C-terminal region of human nNOS (residues 1419–1433) was kindly provided by J. Spangenberg (IncStar, Stillwater, MN). The peroxidase Elite staining kit was from Vector Laboratories.

Anesthetized rats were perfused through the left ventricle with 50 ml of 0.9% NaCl followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4). The brains were removed, cut into saggital blocks, and postfixed in 4% paraformaldehyde in 0.1 M PB for 4 hr at room temperature. Blocks were cryoprotected for 2 days at 4°C in 50 mM sodium phosphate (pH 7.4)/0.1 M NaCl/20% (v/v) glycerol. Brain sections, 40 μm thick, were cut on a sliding microtome. Free-floating sections were incubated in PBS (10 mM [pH 7.4]/0.19 M NaCl) containing 4% normal goat serum (Jackson Labs) and 0.2% Triton X-100 for 45 min and then incubated overnight

at 4°C with the primary antiserum diluted 1:500 (CAPON) or 1:15,000 (nNOS) in phosphate-buffered saline (PBS) containing 2% goat serum and 0.1% Triton X-100. Immunoreactivity was visualized with the Vectastain ABC Elite kit following the nickel-enhanced diaminobenzidine procedure. To test immunohistochemical specificity of the CAPON antiserum, the antiserum was incubated overnight with 13.5 μg/ml of the antigenic fusion protein before incubation with brain sections.

In Situ Hybridization

In situ hybridization used DNA oligonucleotide probes corresponding to amino acids 478–503. Probes were end labeled with [α-^{32}P]dATP and terminal deoxynucleotidyl transferase to a specific activity of 800 μCi/μg, and in situ hybridization was performed as described previously (Jaffrey et al., 1994). Nonspecific hybridization was determined using the corresponding sense probe.

Acknowledgments

We thank M. Kennedy for kindly providing a *PSD95* cDNA; E. Fung, C. Lowenstein, T. Michel, J. Brenman, and D. Bredt for plasmids; E. Fung for yeast strains; and A. Lanahan and P. Worley for the rat hippocampal cDNA library. M. J. L. E. is a recipient of a Gustavus and Louise Pfeiffer Scholarship. S. R. J. is supported by training grant GM-07309. N. A. C. is supported by training grant MH-10341. This work was supported by USPHS grant DA00074 and a Research Scientist Award, MH-18501, to S. H. S.

Received October 27, 1997; revised December 11, 1997.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403–410.

Bredt, D.S. (1996). Targeting nitric oxide to its targets. Proc. Soc. Exp. Biol. Med. *211*, 41–48.

Bredt, D.S., and Snyder, S.H. (1989). Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. Proc. Natl. Acad. Sci. USA *86*, 9030–9033.

Bredt, D.S., and Snyder, S.H. (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proc. Natl. Acad. Sci. USA *87*, 682–685.

Bredt, D.S., Hwang, P.M., and Snyder, S.H. (1990). Localization of nitric oxide synthase indicating a neural role for nitric oxide. Nature *347*, 768–770.

Bredt, D.S., Ferris, C.D., and Snyder, S.H. (1992). Nitric oxide synthase regulatory sites. Phosphorylation by cyclic AMP-dependent protein kinase, protein kinase C, and calcium/calmodulin protein kinase; identification of flavin and calmodulin binding sites. J. Biol. Chem. *267*, 10976–10981.

Brenman, J.E., Chao, D.S., Gee, S.H., McGee, A.W., Craven, S.E., Santillano, D.R., Wu, Z., Huang, F., Xia, H., Peters, M.F., et al. (1996a). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and alpha1-syntrophin mediated by PDZ domains. Cell *84*, 757–767.

Brenman, J.E., Christopherson, K.S., Craven, S.E., McGee, A.W., and Bredt, D.S. (1996b). Cloning and characterization of postsynaptic density 93, a nitric oxide synthase interacting protein. J. Neurosci. *16*, 7407–7415.

Cho, K.O., Hunt, C.A., and Kennedy, M.B. (1992). The rat brain postsynaptic density fraction contains a homolog of the Drosophila discs-large tumor suppressor protein. Neuron *9*, 929–942.

Cohen, N.A., Brenman, J.E., Snyder, S.H., and Bredt, D.S. (1996). Binding of the inward rectifier K+ channel Kir 2.3 to PSD-95 is regulated by protein kinase A phosphorylation. Neuron *17*, 759–767.

Dawson, V.L., and Dawson, T.M. (1996). Nitric oxide in neuronal degeneration. Proc. Soc. Exp. Biol. Med. *211*, 33–40.

Dinerman, J.L., Dawson, T.M., Schell, M.J., Snowman, A., and Snyder, S.H. (1994). Endothelial nitric oxide synthase localized to hippocampal pyramidal cells: implications for synaptic plasticity. Proc. Natl. Acad. Sci. USA *91*, 4214–4218.

Doyle, D.A., Lee, A., Lewis, J., Kim, E., Sheng, M., and MacKinnon, R. (1996). Crystal structures of a complexed and peptide-free membrane protein-binding domain: molecular basis of peptide recognition by PDZ. Cell *85*, 1067–1076.

Garthwaite, J., Garthwaite, G., Palmer, R.M.J., and Moncada, S. (1989). NMDA receptor activation induces nitric oxide synthesis from arginine in rat brain slices. Eur. J. Pharmacol. *172*, 413–416.

Hecker, M., Mulsch, A., and Busse, R. (1994). Subcellular localization and characterization of neuronal nitric oxide synthase. J. Neurochem. *62*, 1524–1529.

Huang, E.P. (1997). Synaptic plasticity: a role for nitric oxide in LTP. Curr. Biol. *7*, R141–143.

Huang, P.L., Dawson, T.M., Bredt, D.S., Snyder, S.H., and Fishman, M.C. (1993). Targeted disruption of the neuronal nitric oxide synthase gene. Cell *75*, 1273–1286.

Huang, Z., Huang, P.L., Panahian, N., Dalkara, T., Fishman, M.C., and Moskowitz, M.A. (1994). Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. Science *265*, 1883–1885.

Jaffrey, S.R., and Snyder, S.H. (1996). PIN: an associated protein inhibitor of neuronal nitric oxide synthase. Science *274*, 774–777.

Jaffrey, S.R., Cohen, N.A., Rouault, T.A., Klausner, R.D., and Snyder, S.H. (1994). The iron-responsive element binding protein: a target for synaptic actions of nitric oxide. Proc. Natl. Acad. Sci. USA *91*, 12994–12998.

Kavanaugh, W.M., and Williams, L.T. (1994). An alternative to SH2 domains for binding tyrosine-phosphorylated proteins. Science *266*, 1862–1865.

Kistner, U., Wenzel, B.M., Veh, R.W., Cases-Langhoff, C., Garner, A.M., Appeltauer, U., Voss, B., Gundelfinger, E.D., and Garner, C.C. (1993). SAP90, a rat presynaptic protein related to the product of the *Drosophila* tumor suppressor gene *dlg*-A. J. Biol. Chem. *268*, 4580–4583.

Kornau, H.C., Schenker, L.T., Kennedy, M.B., and Seeburg, P.H. (1995). Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. Science *269*, 1737–1740.

Kornau, H.-C., Seeburg, P.H., and Kennedy, M.B. (1997). Interactions of ion channels and receptors with PDZ domain proteins. Curr. Opin. Neurobiol. *7*, 368–373.

Kozak, M. (1991). Structural features in eukaryotic mRNAs that modulate the initiation of translation. J. Biol. Chem. *266*, 19867–19870.

Lennon, G., Auffray, C., Polymeropoulous, M., and Soares, M.B. (1996). The I.M.A.G.E. consortium: an integrated analysis of genomes and their expression. Genomics *33*, 151–152.

Li, X.J., Li, S.H., Sharp, A.H., Nucifora, F.J., Schilling, G., Lanahan, A., Worley, P., Snyder, S.H., and Ross, C.A. (1995). A huntingtin-associated protein enriched in brain with implications for pathology. Nature *378*, 398–402.

Lipton, S.A., and Stamler, J.S. (1994). Actions of redox-related congeners of nitric oxide at the NMDA receptor. Neuropharmacology *33*, 1229–1233.

Moncada, S. (1994). Nitric oxide. J. Hypertens. Suppl. *12*, S35–S39.

O'Dell, T.J., Huang, P.L., Dawson, T.M., Dinerman, J.L., Snyder, S.H., Kandel, E.R., and Fishman, M.C. (1994). Endothelial NOS and the blockade of LTP by NOS inhibitors in mice lacking neuronal NOS. Science *265*, 542–546.

Ponting, C.P., and Phillips, C. (1995). DHR domains in syntrophins, neuronal NO synthases and other intracellular proteins. Trends Biochem Sci. *20*, 102–103.

Rodrigo, J., Springall, D.R., Uttenthal, O., Bentura, M.L., Abadia, M.F., Riveros, M.V., Martinez, M.R., Polak, J.M., and Moncada, S. (1994). Localization of nitric oxide synthase in the adult rat brain. Phil. Trans. R. Soc. (Lond.) Biol. *345*, 175–221.

Ross, C.A. (1995). When more is less: pathogenesis of glutamine repeat neurodegenerative diseases. Neuron *15*, 493–496.

Schuman, E.M., and Madison, D.V. (1994). Nitric oxide and synaptic function. Annu. Rev. Neurosci. *17*, 153–183.

Smith, D.B., and Johnson, K.S. (1988). Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene *67*, 31–40.

Songyang, Z., Fanning, A.S., Fu, C., Xu, J., Marfatia, S.M., Chishti, A.H., Crompton, A., Chan, A.C., Anderson, J.M., and Cantley, L.C. (1997). Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. Science *275*, 73–77.

Stricker, N.L., Christopherson, K.S., Yi, B.A., Schatz, P.J., Raab, R.W., Dawes, G., Bassett, D.J., Bredt, D.S., and Li, M. (1997). PDZ domain of neuronal nitric oxide synthase recognizes novel C-terminal peptide sequences. Nat. Biotechnol. *15*, 336–342.

van der Geer, P., and Pawson, T. (1995). The PTB domain: a new protein module implicated in signal transduction. Trends Biochem. Sci. *20*, 277–280.

Yun, H.Y., Dawson, V.L., and Dawson, T.M. (1996). Neurobiology of nitric oxide. Crit. Rev. Neurobiol. *10*, 291–316.

Zhong, W., Feder, J.N., Jiang, M.M., Jan, L.Y., and Jan, Y.N. (1996). Asymmetric localization of a mammalian numb homolog during mouse cortical neurogenesis. Neuron *17*, 43–53.

Zhou, M.M., Ravichandran, K.S., Olejniczak, E.F., Petros, A.M., Meadows, R.P., Sattler, M., Harlan, J.E., Wade, W.S., Burakoff, S.J., and Fesik, S.W. (1995). Structure and ligand recognition of the phosphotyrosine binding domain of Shc. Nature *378*, 584–592.

GenBank Accession Numbers

The rat and human CAPON sequences have been deposited in GenBank, accession numbers AF037071 and AF037070, respectively.