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PSD-95 and a bivalent nNOS PDZ domain assemble a signaling complex in neurons

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PSD-95 and a bivalent nNOS PDZ domain assemble a signaling complex in neurons

by

Karen S. Christopherson

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

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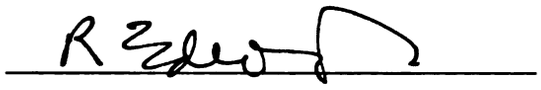

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Abstract

Nitric oxide (NO) biosynthesis in cerebellum is preferentially activated by calcium influx through *N*-methyl-D-aspartate (NMDA) type glutamate receptors, suggesting that there is a specific link between these receptors and neuronal NO synthase (nNOS). This thesis addresses the role of PSD-95 in assembling a postsynaptic protein complex containing nNOS and NMDA receptors. Formation of this complex is mediated by the PDZ domains of PSD-95, which is monomeric *in vitro* and can therefore use both PDZ1 and PDZ2 simultaneously to bind together nNOS and an NMDA receptor subunit. However, when expressed in heterologous cells PSD-95 can be coimmunoprecipitated with itself by a mechanism that appears to require only the palmitoyl groups that lie at the very N-terminus of the molecule. Our data suggests that PSD-95 *in vivo* is targeted to

particular detergent-insoluble lipid microdomains by the presence of these acyl groups, and that association with these detergent-insoluble lipids are responsible for the observed coimmunoprecipitation from cells. Since palmitoylation of PSD-95 is also required for the clustering activity of PSD-95 when coexpressed with specific membrane receptors and channels, targeting to these lipid microdomains may also be required for clustering by PSD-95 *in vivo*.

While PSD-95 binds to the C-termini of specific NMDA receptor subunits, nNOS is recruited to this complex by a novel PDZ / PDZ interaction in which PSD-95 recognizes an internal motif adjacent to the consensus nNOS PDZ domain. This internal motif is a structured "pseudo-peptide" extension of the nNOS PDZ that interacts with the peptide-binding pocket of PSD-95 PDZ2. This asymmetric interaction leaves the peptide-binding pocket of the nNOS PDZ domain available to interact with additional C-terminal PDZ ligands. Accordingly, we find that the nNOS PDZ domain can bind PSD-95 PDZ2 and a C-terminal peptide simultaneously. This bivalent nature of the nNOS PDZ domain further expands the scope for assembly of protein networks by PDZ domains.

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CHAPTER ONE

An Introduction to NOS

Summary

Nitric oxide (NO) is a diffusible chemical signaling molecule that plays a role in an overwhelming number of systems throughout the body. The first physiological functions attributed to NO were as a vasodilator of blood vessels and a tumoricidal and bactericidal agent produced by immune cells. Subsequently, it became apparent that NO also mediates many neuronal functions. In the peripheral nervous system, it acts as a neurotransmitter to regulate gastrointestinal motility, regional blood flow and neuroendocrine function. In the central nervous system, it functions as a neuromodulator, affecting complex phenomena such as memory formation and behavior. However, the inappropriate production of NO has toxic effects, which contribute to the permanent damage caused by stroke and other types of neuronal injury. Since NO has both physiological and pathophysiological effects in neurons, the regulation of NO synthesis and activity is critical. Clues to how this balance is achieved have been contributed by studies in skeletal muscle, which also contains high levels of the neuronal nitric oxide synthase (nNOS) isoform, suggesting that the specific intracellular localization of the enzyme is important for proper physiological function.

Nitric oxide synthases

In the early 1980's, it was known that the ability of acetylcholine and bradykinin to dilate blood vessels was mediated by a diffusible molecule secreted by endothelial cells that acted on the underlying smooth muscle to cause relaxation (Furchgott and Zawadzki, 1980). This substance was termed endothelial-derived relaxing factor (EDRF), and it was subsequently shown that nitric oxide (NO) was EDRF (Palmer et al., 1987) based on the convergence of several lines of evidence (Feelisch and Noack, 1987; Murad et al., 1978). Subsequently, Garthwaite and colleagues obtained the first evidence that NO is also involved in signaling in brain, demonstrating that NMDA receptor activation by glutamate leads to the release of NO in cerebellum (Garthwaite et al., 1988). Interestingly, NO was first identified as an agent responsible for tumoricidal and bactericidal actions of macrophages (Green et al., 1981; Hibbs et al., 1987; Stuehr et al., 1989), supplying evidence early on that this molecule can also be toxic to cells in certain circumstances.

The subsequent purification and cloning of neuronal nitric oxide synthase (nNOS) (Bredt et al., 1991; Bredt and Snyder, 1990) led to the cloning of two additional isoforms, endothelial NOS (eNOS) (Janssens et al., 1992; Lamas et al., 1992; Sessa et al., 1992) and inducible nNOS (iNOS) (Lowenstein et al., 1992; Lyons et al., 1992; Xie et al., 1992), which is expressed in macrophages (Figure 1-1). Both nNOS and eNOS are calcium-activated enzymes, activation being mediated by the direct binding of calmodulin in the presence of increased intracellular calcium levels. iNOS, however,

constitutively binds calmodulin and is therefore not regulated by intracellular calcium levels; instead it is regulated at the transcriptional level.

The biology of nNOS in excitable tissues

Although the nNOS isoform was originally purified from brain, it is also expressed in the peripheral nervous system and in skeletal muscle. Many of the biological actions of NO in various tissues are due to the activation of soluble guanylyl cyclase (GC). An increase in the concentration of intracellular calcium leads to the activation of nNOS by the direct binding of calcium/calmodulin (Bredt and Snyder, 1990), and the subsequent generation of NO. The direct binding of NO to GC leads to the activation of this enzyme and an increase in intracellular cGMP levels. For example, the robust increase in cGMP levels in cerebellum in response to glutamate is due to nNOS activation (Bredt and Snyder, 1989; Garthwaite et al., 1989). In skeletal muscle, cGMP mediates the effects of NO on contractile force (Kobzik et al., 1994). However, some effects of NO may be achieved through cGMP-independent mechanisms.

The first place that a physiological function for NO was demonstrated was in the gastrointestinal tract. Physiologists had long acknowledged an autonomic nervous system pathway (termed "NANC" for nonadrenergic, noncholinergic) in which the signaling agent was unknown. The NANC pathway also plays an important role in smooth muscle relaxation in the cerebral circulation, and in the respiratory and urogenital tracts. Several studies in the 1980's demonstrated that the NANC transmitter was the same as the EDRF described by Furchgott. NO is generated in myenteric neurons during

peristalsis and diffuses to adjacent smooth muscle cells to increase cGMP and cause relaxation, and NO inhibitors selectively block NANAC transmission (Boeckxstaens et al., 1991; Bult et al., 1990; Desai et al., 1991; Tøttrup et al., 1991). Furthermore, the most striking phenotype displayed by nNOS knockout mice is a grossly enlarged stomach resembling the human disease infantile pyloric stenosis (Huang et al., 1993).

Although the regulation of blood flow in endothelial cells by EDRF was originally attributed to the eNOS isoform, neuron-derived NO also plays major role in the regulation of blood flow in some tissues. High levels of nNOS are found in vasodilator nerves that innervate the large cerebral blood vessels (Bredt et al., 1990). Sumatripan constricts these vessels and alleviates both migraine headache and nitroglycerin-induced headache, suggesting that endogenous NO plays a role in migraine (Welch, 1993). nNOS also plays an important role in blood flow in the penis. The enzyme is enriched in the pelvic plexus (Burnett et al., 1993), and inhibitors of NO block relaxation of smooth muscle in strips human cavernosal tissue (Rajfer et al., 1992). nNOS enzyme levels seem to be regulated by androgens, as castration lease to a 60% decrease in nNOS protein levels which are restored to normal with testosterone replacement (Penson et al., 1996). Reduced levels of androgens may be a cause of the decrease in nNOS enzyme levels and impaired erectile function associated with aging (Carrier et al., 1997).

As mentioned above, nNOS is also found in skeletal muscle where it is localized beneath the sarcolemma (Kobzik et al., 1994). Muscle membrane depolarization leads to an increase in intracellular calcium and stimulation of nNOS activity. The subsequent generation of NO leads to an inhibition of contractile force in these fibers, which seems to be mediated by cGMP (Kobzik et al., 1994). During development, NO is involved in

the fusion of myocytes into myotubes (Lee et al., 1994), and in the regulation of myotube innervation by functioning as a retrograde messenger released from the postsynaptic muscle membrane (Wang et al., 1995). In the adult, nNOS is enriched in glycolytic fast twitch fibers, and NO regulates glucose uptake across the sarcolemma (Roberts et al., 1997). Evidence that mislocalization of nNOS may play a role in muscular dystrophies will be discussed later.

In the central nervous system (CNS), nNOS is expressed in discrete and highly conserved neuronal populations in all mammalian brains so far studied (Bredt et al., 1991; Hope et al., 1991). Because of the unique properties of NO as a diffusible messenger, it was proposed that NO could be a mediator of neuronal plasticity (Gally et al., 1990). NO signaling has been shown to be important for two cellular models of plasticity, long-term potentiation (LTP) in the hippocampus and long-term depression (LTD) in the cerebellum. LTP is blocked by non-specific NOS inhibitors (Böhme et al., 1991; Haley et al., 1992; O'Dell et al., 1991; Schuman and Madison, 1991), however the relevant NOS isoform was not clear from these studies since both eNOS and nNOS are expressed the hippocampus. It has now been shown that mice that are missing either isoform have essentially normal LTP, while mice missing both isoforms have significantly decreased LTP (Son et al., 1996).

LTD in the cerebellum refers to the effect of co-stimulation of two different fibers; the parallel and climbing fibers, on the transmission of signal from parallel fibers synapsed onto Purkinje neurons. The importance of NO in this mechanism is not so clear, as NOS inhibitors and the NO scavenger hemoglobin have been reported to either block LTD or to have no effect, depending on the experimental protocol used (Linden

and Connor, 1992; Shibuki and Okada, 1991). Recent results with caged NO in hippocampal slices have produced convincing results in support of the role of NO as a retrograde messenger in this phenomenon (Lev-Ram et al., 1997; Lev-Ram et al., 1995). However, it is possible that both NO-dependent and NO-independent pathways are involved in different neuronal population and under different conditions of stimulus.

NO also has effects on aspects of complex phenomena in the CNS such as brain development, memory formation and behavior. For instance, NO is involved in patterning of the visual system. During development, a refinement process occurs in which the initial ipsilateral retinotectal connections are lost, and inhibition of nNOS or blockade of NMDA receptor activity prevents this refinement process (Cramer et al., 1998; Ernst et al., 1999; Wu et al., 1994). NO has also been found to be important in several animal models of learning such as spatial learning, motor learning and passive avoidance [reviewed in (Hawkins, 1996)]. In addition, the formation of olfactory memories by mother sheep requires nNOS and GC activities, which also potentiate glutamate release in this system (Kendrick et al., 1997). However, the results of many studies of other models of learning have been contradictory, probably due the complex nature of this phenomenon and the differences in experimental protocols used. A general consensus is that NO is involved in some aspects of memory acquisition and retention of new memories, but not in retention or recall of already acquired memories.

Pathological effects of nitric oxide

Because endogenous NO produced by macrophages is cytotoxic to bacteria and tumors, it is not surprising that it can also be neurotoxic under some circumstances (Table 1-1). Many kinds of neuronal injury such as stroke, Alzheimer's disease and Huntignton's chorea are due to the excess release of glutamate, which leads to activation synaptic NMDA receptors and an increase in intracellular calcium. The first evidence that NO plays a role in NMDA-mediated glutamate toxicity derived from studies in cultured cortical neurons, where inhibition of NOS attenuates glutamate toxicity (Choi, 1994; Dawson et al., 1991). This work was controversial because other studies generated contradictory results due to the use of different neuronal cell populations and culture conditions, which can have a large effect on nNOS protein levels. Mice lacking nNOS have helped clarify these discrepancies. Cultured neurons from knockout mice are resistant to glutamate toxicity, proving that NO generated by nNOS activation in response to NMDA receptor calcium can be toxic (Dawson et al., 1996).

In animal models of stroke, NOS inhibitors were found to be protective in many experimental systems, due to the role of NO in mediating toxicity associated with excess glutamate release [reviewed in (Choi, 1991; Samdani et al., 1997)]. However, again there were conflicting reports due to the use of non-specific inhibitors. Knockout mice have clarified the specific actions of the different NOS isoforms (Iadecola, 1997). nNOS knockout mice have smaller lesions but show similar changes in regional blood flow as wild-type mice, confirming that NO generated by this isoform is usually toxic in this model (Huang et al., 1994). eNOS knockouts show decreased blood flow at the

periphery where NO-mediated toxicity is most prevalent, and an increased lesion size (Huang et al., 1996), therefore NO produced by this isoform is usually protective during stroke due to the effects on blood flow. The expression of iNOS is induced in later stages of ischemia and iNOS knockouts also show a decreased lesion size, suggesting that post-ischemic inflammation leads to recruitment of macrophages, which contributes to a late phase of neuronal death (Iadecola et al., 1997).

The role of NO in neurodegenerative disorders has been more difficult to study due to the slow progression of these diseases. However, histopathological studies have shown that neurons with NADPH-diaphorase activity, which is now known to be same as nNOS activity, are selectively spared in Huntington's disease (Ferrante et al., 1985). nNOS is found in a small percentage (1-2%) of striatal neurons, and this selective pathology can be replicated in striatal culture models and *in vivo* after lesion with glutamate agonists (Koh et al., 1986; Uemura et al., 1990). The spared NADPH diaphorase neurons are also uniquely endowed with high levels of SOD (Inagaki et al., 1991), which may protect cells from damage cause by the powerful oxidant peroxynitrate generated by the reaction of NO with superoxide.

A more causal role for NO and peroxynitrate toxicity has been demonstrated in some forms of Parkinson's disease (Przedborski and Jackson-Lewis, 1998). MPTP, which contaminated batches of illicit drugs in the 1970's, produces Parkinsonian-like symptoms in humans. MPTP causes this pathology by targeting the destruction of nigrostriatal dopaminergic neurons the same cells that are selectively lost in idiopathic Parkinson's, and inhibition of NOS prevents MPTP toxicity (Hantraye et al., 1996).

Furthermore, both nNOS knockout mice and mice that overexpress Cu/Zn SOD are resistant to MPTP toxicity (Przedborski et al., 1996).

Cellular mechanisms of NOS regulation

NO is a unique messenger molecule. The nature of the biological actions of NO require that significant amounts of NO are generated in a short amount of time. However, the physical properties of NO as a free radical gas prevents storage in vesicles, and the rate of degradation cannot be controlled by degratory enzymes. Further more, excessive NO production is toxic to neurons and other cells when inappropriately generated, as discussed above. Therefore, it is important to understand how the activity of NOS and the effects of NO are spatially and temporally regulated.

One level of regulation is the activation of nNOS by distinct calcium influx pathways in various tissues (Figure 1-2). In the myenteric nervous system, NO functions as a neurotransmitter, and its activity is primarily regulated by calcium influx through voltage-dependent calcium channels. Intestinal relaxation mediated by NO is suppressed by the N-type calcium channel antagonist, ω-conotoxin (Daniel et al., 1994). In the brain, NO biosynthesis is predominantly regulated by calcium influx at the synapse. Glutamate, the major excitatory neurotransmitter in the brain, is the most effective activator of NOS activity in most brain regions (Garthwaite et al., 1988; Kiedrowski et al., 1992).

This specific activation may be achieved by localization of the NOS enzyme to specific plasma membrane regions. Interestingly, the eNOS isoform associates with the

plasma membrane of endothelial cells due to N-terminal dual acylation (Robinson and Michel, 1995) and is further localized to caveolae, plasma membrane subdomains enriched in cholesterol and signaling molecules (Garciacardena et al., 1996; Shaul et al., 1996). This is mediated by the direct binding of eNOS to the protein caveolin (Feron et al., 1996; Feron et al., 1998), which results in inhibition of enzyme activity (Michel et al., 1997). Stimulation of eNOS activity by agonists such as bradykinin leads to the dissociation of eNOS from caveolin upon calmodulin binding and subsequent translocation to the cytoplasm (Feron et al., 1998). This association of eNOS with caveolae may facilitate the specific activation by appropriate receptors and efficient release of NO to nearby target cells, with minimal exposure of the generator cell to this potentially toxic molecule. In addition, the subsequent cytoplasmic translocation and phosphorylation of eNOS may be a way to quickly inhibit NO release once the signal has been transmitted.

In terms of the localization of nNOS, studies in skeletal muscle have been the most informative. nNOS is quantitatively associated with the sarcolemma due to interaction with the dystrophin complex (Brenman et al., 1995). Unlike eNOS, nNOS is not acetylated; instead localization is mediated by direct binding of nNOS to a component of the dystrophin complex, α-syntrophin. Biochemical studies have shown that the N-terminus of nNOS contains a PDZ protein-protein interaction motif, which binds to a similar PDZ motif at the N-terminus of α-syntrophin (Brenman et al., 1995). Genetic support of these studies can be seen in patients with Duchenne muscular dystrophy and *mdx* mice which lack dystrophin (Ervasti et al., 1990). In both cases,

syntrophin and nNOS are lost from the sarcolemma (Brenman et al., 1995; Ervasti et al., 1990).

The loss of nNOS in human patients and mouse models of muscular dystrophies suggests that the loss of NOS activity from the sarcolemma may play a role in the pathology of these diseases. For instance, it has been proposed that the loss of dystrophin disrupts the normal link between the extracellular matrix and the myofiber skeleton (Campbell, 1995), resulting in sarcolemmal damage and myofiber necrosis. The loss of sarcolemmal nNOS signaling may contribute to the failed muscle regeneration in Duchenne dystrophy. Another model is that disruption of intracellular calcium homeostasis and subsequent free radical-induced oxidative damage contributes to muscle pathology in Duchenne dystrophy (Brown, 1995). Improperly localized (and therefore regulated) nNOS enzyme activity could be responsible for the generation of these damaging free radicals.

In neurons, the localization of nNOS to specific plasma membrane regions is beginning to be understood. As mentioned above, NO generation in brain is predominantly stimulated by glutamate, and nNOS is preferentially activated by calcium influx through the NMDA receptor versus other sources calcium. Along these lines, a detailed study of the primate visual cortex revealed that nearly all of the dendritic spine and shaft labeling of nNOS clustered over post-synaptic densities, and these immunoreactive profiles were often dually-labeled with the obligate NMDA receptor subunit NR1 (Aoki et al., 1993; Aoki et al., 1997). This suggest that in neurons also the specific activation of nNOS, in this case by calcium influx through the NMDA receptor, is mediated by the specific localization of the enzyme to regions of the highest

concentration of the appropriate calcium source. Recently, the PDZ domain of nNOS has been found to bind to synaptic proteins, PSD-95 and related protein PSD-93, in a PDZ / PDZ interaction analogous to the interaction of nNOS with syntrophin in muscle (Brenman et al., 1996; Brenman et al., 1996). This suggests a possible mechanism for the specific localization of nNOS in the brain, and this hypothesis will be explored in the chapter to follow.

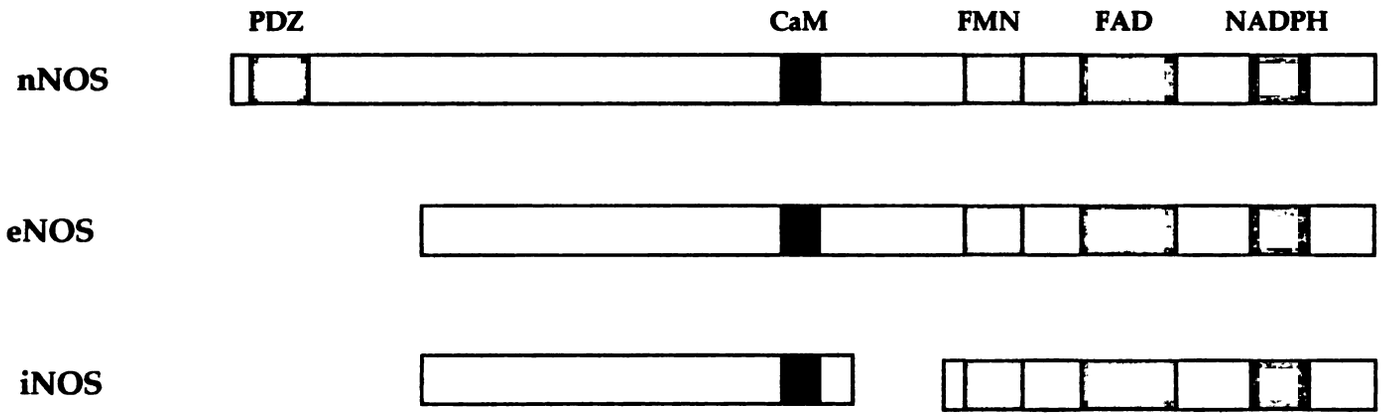
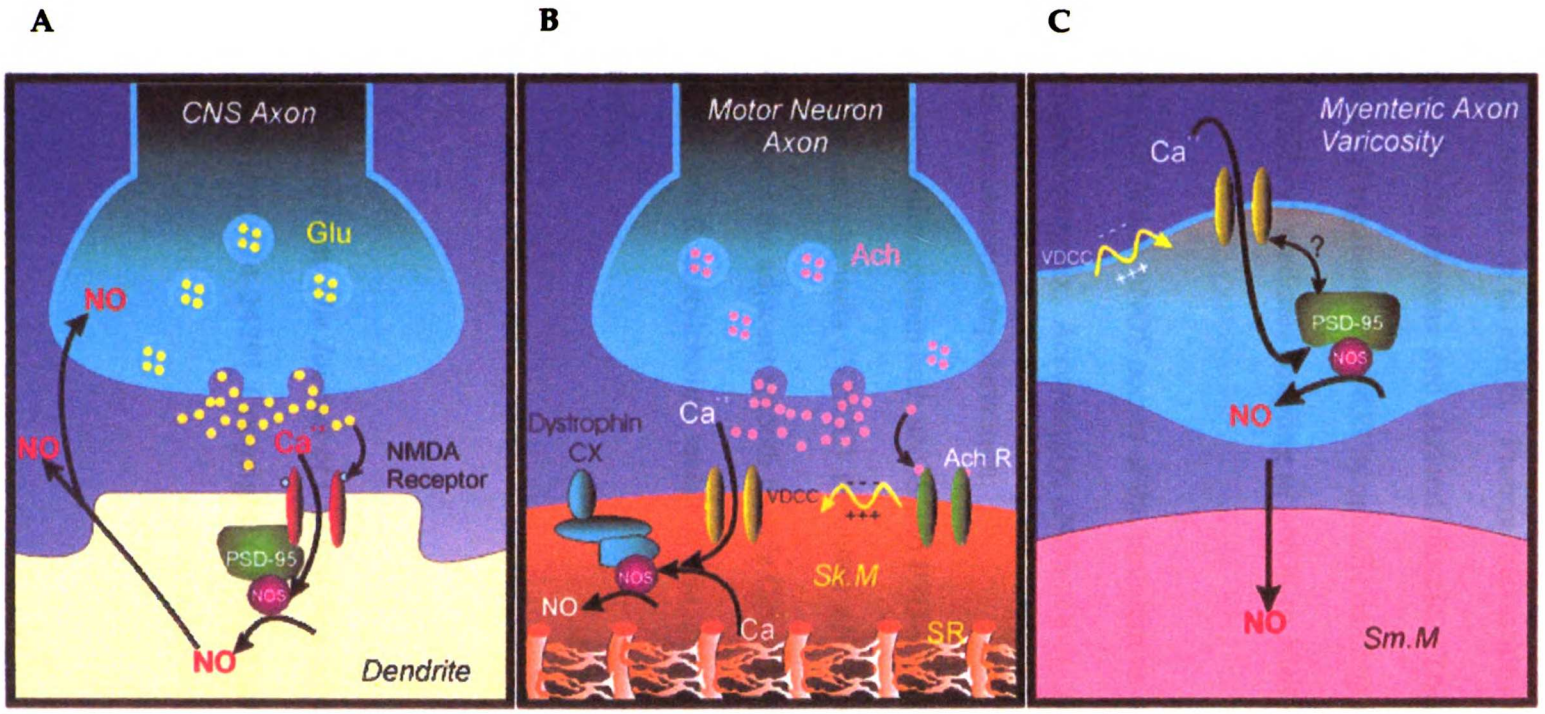


Figure 1-1

Figure 1-1 Schematic alignment of the three NOS isoforms. All three require calmodulin binding for activity (CaM), however the site on iNOS is constitutively bound. The nNOS isoform has a unique N-terminal region that contains a PDZ domain. The abbreviations used are: nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; CaM, calmodulin; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate.

DISEASE	EXPRESSION/REGULATION OF NOS IN DISEASED TISSUE	ROLE OF NO IN DISEASE PATHOGENESIS
STROKE	nNOS activity acutely increases during cerebral ischemia. iNOS expression and activity are upregulated 12-48 hours following ischemia.	Excessive NO causes free radial injury. Mice treated with NOS inhibitors and nNOS or iNOS mutant mice have decreased infarct volume.
PARKINSON'S	Axons from the degenerating substantia nigra project to the corpus striatum, which contains a rich density of nNOS neurons.	NO mediates nigral injury in the MPTP model of Parkinson's disease. NO inhibitors and nNOS mutant mice have decreased neuronal loss after MPTP.
HUNTINGTON'S	nNOS neurons are selectively spared in the otherwise degenerating corpus striatum.	NO may mediate neurodegeneration as NO inhibitors prevent glutamate toxicity in cultures of striatal neurons.
HYPERTROPHIC PYLORIC STENOSIS	nNOS-positive neurons are absent from the pylorus in diseased infants.	Absence of NO results in increased tone of pyloric sphincter. nNOS is a susceptibility locus for infantile pyloric stenosis
MUSCULAR DYSTROPHY	nNOS is absent form myofiber membranes in Duchenne and Becker dystrophies.	Role of NO in pathophysiology unclear.

Table 1-1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B
C
CNS Axon
Glu
NO
NO
Ca
NMDA
Receptor
PSD-95
NOS
NO
Dendrite
Motor Neuron
Axon
Ach
Dystrophin
CX
Ca
Ach R
VDCC
Sk.M
NOS
NO
Ca
SR
Myenteric Axon
Varicosity
Ca
VDCC
?
PSD-95
NOS
NO
NO
Sm.M

Figure 1-2

Figure 1-2* Synaptic regulation of nNOS. Protein interactions with nNOS target the synthase to discrete sites in neurons and skeletal muscle. These interactions likely account for the differential regulation of nNOS by specific calcium influx pathways.

A. Association with PSD-95 mediates coupling of nNOS to NMDA receptor activity in the CNS.

B. In skeletal muscle (*Sk. Mus.*), nNOS occurs at the sarcolemma owing to interaction of nNOS with the dystrophin complex. Accordingly, nNOS activity is regulated by calcium influx associated with sarcolemmal depolarization.

C. Myenteric axon varicosities contain both nNOS and PSD-95. Calcium influx through voltage-dependent calcium channels (VDCC) regulates nNOS activity in myenteric neurons, and the NO relaxes the adjacent smooth muscle (*Sm. Mus.).* Abbreviations used are: Ach, acetylcholine; Ach R, acetylcholine receptor; Glu, glutamate; SR, sarcoplasmic reticulum; CX, complex.

CHAPTER TWO

MAGUKS and Other PDZ Proteins

Summary

PSD-95 was originally identified as an abundant protein component of neuronal synapses, although its function in neurons is not yet clear. PSD-95, along with the related PSD-93 / chapsyn 110, SAP 102 and SAP 97, is a member of a larger family of proteins called MAGUKs. MAGUK proteins are expressed in many cell types and a variety of different organisms, and generally function at sites of cell-cell contact to facilitate cell polarization and signaling. In neurons, the PSD-95 family also appears to be involved in these kinds of activities. PSD-95 binds to and clusters ion channels and receptors in heterologous cells, suggesting that it functions *in vivo* to specifically localize proteins to synapses. These interactions occur through the direct binding of the concatamerized PDZ domains in PSD-95 to the C-termini of specific proteins. PDZ domains are protein-protein interaction motifs, and the tandem array of PDZ repeats in PSD-95 is thought to facilitate clustering. In addition, these repeats also bind to an increasing number of intracellular proteins. Therefore, PSD-95 could also function as a molecular scaffold to link plasma membrane signaling events to second messenger pathways.

MAGUK proteins

A large class of proteins has recently been identified that functions in many cell types to organize signaling and adhesion molecules at various plasma membrane locations, particularly sites of cell-cell contact (Figure 2-1). This protein family has been designated MAGUKs for "membrane-associated guanylate kinases" because they contain a C-terminal domain homologous to guanylate kinases, although no enzymatic activity has been identified (Kim, 1995). These proteins also share a Src homology domain 3 (SH3) domain, and one or three PDZ domains at their N-termini. PDZ domains are protein-protein interaction motifs that are named for the three original proteins in which this motif was identified (PSD-95, Dlg and ZO-1) (Ponting and Phillips, 1995; Saras and Heldin, 1996).

PSD-95 is neuronal MAGUK that was originally purified in an effort to identify proteins present at high levels in synapses (Cho et al., 1992), and is now known be located either pre- or post-synaptically, depending on the brain region (Cho et al., 1992; Hunt et al., 1996; Kistner et al., 1993). The first clues to the biological role for PSD-95 at synapses came from yeast two-hybrid screens using the C-terminal domains of the NR2 subunit of the NMDA receptor and the potassium channel $K_V1.4$ as bait (Kim et al., 1995; Kornau et al., 1995). In both cases, PSD-95 was isolated and shown to bind to the very COOH-termini of these proteins through the first or second PDZ repeats. Subsequent analysis in heterologous cells demonstrated that PSD-95 can for large clusters with $K_V1.4$ in heterologous cells but not when either protein is expressed alone. Furthermore, it has been reported that PSD-95 can homomultimerize and

heteromultimerize with the closely related protein PSD-93, suggesting that this multimerization is involved in receptor clustering (discussed below).

Although evidence for an *in vivo* function of PSD-95 family members in receptor clustering in mammalian neurons is lacking, studies of another MAGUK present in *Drosophila*, DLG, have been wonderfully informative. DLG binds to Shaker K^+ channels, and these two proteins colocalize at the larval neuromuscular junction (NMJ) (Tejedor et al., 1997). Furthermore, clustering of Shaker K^+ channels is abolished in *dlg* mutant or in flies that express a truncated DLG protein that is lacks PDZ domains (Tejedor et al., 1997). As expected, the C-terminus of the Shaker K^+ channel is required for its localization but not for the localization of DLG. It has also been demonstrated that the intracellular C-terminus of Shaker K^+ amino acids is sufficient to target a chimeric transmembrane protein (CD8) to the NMJ synapse (Zito et al., 1997). Thus, genetic experiments in *Drosophila* have provided overwhelming evidence that MAGUKs lay a role in receptor targeting.

In contrast, developmental studies in cultured mammalian neurons suggest that PSD-95 is not involved in the formation of receptor clusters, but may play a role at a later stage in either targeting preformed clusters to the synapse or maintaining proteins at synaptic locations (Rao and Craig, 1997; Rao et al., 1998). Knockout mice of PSD-95 have been generated but the phenotype is not as one might expect based on the *Drosophila* studies (Migaud et al., 1998). One explanation is that the major role of PSD-95 at synapses is not in clustering, but to bring second messengers and other effector proteins, such as nNOS, to the vicinity of synaptic receptors and channels. A potential complication in the interpretation of the knockout phenotype is the presence of at least

three highly related post-synaptic proteins (PSD-95 / SAP 90, PSD-93 chapsyn-110 and SAP 102) that have nearly identical binding specificities, at least *in vitro*.

PSD-95 multimerization

As mentioned above, in addition to the direct binding of receptors and ion channels, it has been suggested that the PSD-95 family multimerizes *in vivo*. In heterologous cells, PSD-95 has been shown to multimerize with itself and with the related PSD-93, and both associate into the same clusters with either $K_v1.4$ or the NMDA receptor (Hsueh et al., 1997; Kim et al., 1996). In addition, PSD-95, PSD-93 and the highly related SAP 102 are expressed in some of the same neuronal populations, suggesting that these family members could heteromultimerize *in vivo* (Brenman et al., 1996; Cho et al., 1992; Kim et al., 1996; Muller et al., 1996; Rao et al., 1998). Because some PDZ domains can bind to each other, one model for the clustering activity of PSD-95 is that multimerization occurs through the binding of one PDZ domain to another on a separate PSD-95 molecule, leaving the other two PDZ domains free to associate with other proteins. However, multimerization of these proteins requires only one PDZ domain; these surprising results are explained by the finding that multimerization occurs at the very N-terminus, requiring only the first 64 amino acids that are N-terminal to the start of the first PDZ domain (Hsueh et al., 1997).

The role of the N-terminus in the function of PSD-95 was determined based on the observation that there are two critical cysteines in this region, and that when these residues are mutated to alanines, PSD-95 can no longer multimerize or perform its

clustering function in heterologous cells (Hsueh et al., 1997). This was proposed to be due to intracellular disulfide bond-formation between cysteines on different molecules, leading to the formation of tetrameric complexes. However, the Bredt lab has found that these cysteines in PSD-95 are palmitoylated in brain and heterologous cells (Topinka and Bredt, 1998), suggesting an alternative explanation for their importance in the function of PSD-95. Although it is formally possible that these two events both occur *in vivo*, evidence will be presented in Chapter 5 indicating that disulfide bond-formation does not play a role *in vivo* and that multimerization occurs through an alternative mechanism involving palmitoylation.

Coordination of signaling pathways by PDZ domains

In addition to the proposed clustering function of PSD-95 family members in mammalian neurons, they may also function to localize membrane receptors and link them to downstream signaling molecules. The best examples of PDZ proteins that are involved in signal transduction come from genetic studies. For example, in *C. elegans*, vulval precursor cell development is dependent on PDZ proteins to properly target at least one signaling component to the basolateral membrane. The LET-23 protein is an EGF-like receptor that must be localized to the basolateral side of the vulval precursor cell to receive the secreted ligand signal from the opposing anchor cell (Simske et al., 1996). A mutation in any of three PDZ-containing proteins, LIN-2, LIN-7 or LIN-10, results in mislocalization of the receptor and a vulvaless phenotype [reviewed in (Kim, 1995)]. One of these proteins, LIN-7, directly binds to the LET-23 receptor, and LIN-2

binds to both LIN-7 and LIN-10 to form a ternary complex *in vitro* (Kaech et al., 1998), suggesting that this complex is important for LET-23 receptor localization *in vivo*. In addition to the role of these PDZ proteins in epithelial cell signaling, LIN-10 also appears to be involved in the proper postsynaptic targeting of the glutamate receptor GLR-1 (Rongo et al., 1998).

In *Drosophila*, the multiple PDZ domain-containing protein INAD is involved not only in receptor localization but also in the recruitment of downstream signaling components. In photoreceptor cells, proper phototransduction is mediated by a G-protein signaling cascade coupled to the rhodopisin receptor, in which the TRP (transient receptor potential) Ca^{2+} channel is activated by the PLC-β (phospholipase C-β) pathway and subsequently quickly deactivated by PKC (protein kinase C) [reviewed in (Fanning and Anderson, 1999; Ranganathan and Ross, 1997)]. INAD, which contains five PDZ domains, is required both for the localization of these components to the rhabdomere and effective phototransduction (Chevesich et al., 1997; Shieh and Niemeyer, 1995; Shieh and Zhu, 1996). Many genetic and biochemical studies have shown that rhodopsin, PKC, PLC- β,and calmodulin all bind to different PDZ domains of INAD (Tsunoda et al., 1997; Xu et al., 1998). This arrangement facilitates not only speed and efficiency of pathway activation and deactivation, but also specificity of the response, as many of these components are used in other pathways.

In a final example of how PDZ proteins can be involved in signaling, the epithelial PDZ protein NHERF is involved in coordinating the activities of two transmembrane proteins. NHERF (Na^+ / H^+ exchanger regulatory factor) was first identified as a protein required for the regulation of NHE-3 (Na^+ / H^+ exchanger 3)

(Weinman et al., 1995). NHE-3 is activated by the β_2-adrenergic receptor and is responsible for the modulation of intracellular pH by adrenaline and noradrenaline. NHERF has two PDZ domains and binds to the C-terminus of the β_2-adrenergic receptor in an agonist-dependent manner and also to NHE-3 (Hall et al., 1998). Mutation of the final residue in the β_2-adrenergic receptor prevents agonist-dependent association of NHERF with the β_2-adrenergic and abolishes the regulation of NHE-3 by NHERF. This is the first example of a mammalian PDZ-containing protein that has been shown to regulate the activity of a transmembrane protein.

PSD-95 binding proteins

In order to understand further the function of PSD-95 in mammalian neurons, many groups have identified proteins that interact with PSD-95. Although in many cases the biological relevance of the interaction is not yet clear, in some cases the identified proteins have suggested interesting possibilities. The protein GKAP (guanylate kinase-associated protein) binds to the guanylate kinase-homology domain of PSD-95 (Kim et al., 1997). Although not homologous to any known proteins, GKAP can bind to multiple GK domains on different PSD-95 molecules, suggesting that it could contribute to the clustering function of PSD-95. A developmental study in cultured neurons demonstrated that NMDA receptor clusters first form non-synaptically. In contrast, both PSD-95 and GKAP form clusters opposite presynaptic terminals several days before NMDA receptors are present at the synapse (Rao et al., 1998). These observations are consistent with a

role for PSD-95 and GKAP in the formation of a synaptic scaffold that later directs the attachment of the NMDA receptor and other proteins to their final synaptic sites.

A protein called p135 SynGAP, independently identified by two different groups, binds to the PDZ domains of PSD-95 through its COOH-terminal sequence (Chen et al., 1998; Kim et al., 1998). p135 SynGAP is a synaptically localized protein with RasGAP-like enzymatic activity; the GAP domain can stimulate the GTPase activity of Ras. In addition, GAP activity of p135 SynGAP is inhibited by phosphorylation by CaM kinase II (CaMKII) (Chen et al., 1998), which is activated by the influx of Ca^{2+} through NMDA receptors. Taken together, these experiments present a model in which PSD-95 acts to bring p135 SynGAP to synaptic NMDA receptors. Activation of CaMKII in response to glutamate could result in the phosphorylation of p135 SynGAP and inhibition of GAP activity, leading to elongation of the mitogen-activated protein (MAP) kinase pathway signal.

Another PSD-95-interacting protein with a potentially interesting function in neurons is CRIPT (cysteine-rich interactor of PDZ three) which has a COOH-terminus that interacts with PDZ3 of PSD-95 (Niethammer et al., 1998). CRIPT is also colocalized with PSD-95 at synapses. When coexpressed in heterologous cells, CRIPT causes a dramatic redistribution of PSD-95 to the microtubule cytoskeleton. This may also be relevant in neurons, as CRIPT, PSD-95 and tubulin can be coimmunoprecipitated from brain, although it is not clear from these experiments if this interaction with microtubules is direct through PSD-95 or CRIPT, or indirect through another protein(s). These observations provide evidence for a link between PSD-95 and the cytoskeletal network *in vivo*.

There is also evidence that PSD-95 provides a link between the intracellular domains of adjacent cells. PSD-95 binds to the COOH-termini of neuroligins, post-synaptic cell adhesion molecules (Ichtchenko et al., 1995; Irie et al., 1997). Neuroligins interact with a subset of β-neurexins (Ushkaryov et al., 1992), presynaptic cell adhesion proteins, to form intercellular junctions between heterologous cells (Ichtchenko et al., 1995; Nguyen and Sudhof, 1997). Interestingly, the mammalian homologue of the *C. elegans* protein LIN-2 (see above), CASK, binds to the intracellular domain of β-neurexins, and homologues of other *C. elegans* proteins, LIN-7 (Veli) and LIN-10 (Mint1) are also found presynaptically in mammalian neurons (Butz et al., 1998). It will be interesting to see whether these proteins have any postsynaptic role in receptor localization in mammals as they do in *C. elegans*.

PDZ domain binding

As mentioned above, binding partners for PSD-95 were first characterized in screens to identify proteins that interact with the intracellular COOH-terminal domains of transmembrane proteins (Kim et al., 1995; Kornau et al., 1995). The binding interactions were mapped to PDZ domains 1 and 2 of PSD-95 and to the very C-terminal nine residues of the interacting proteins. Subsequently, binding specificities for other PDZ domains were identified based primarily on peptide library screens, all requiring a carboxyl-terminus (Saras and Heldin, 1996; Songyang et al., 1997).

All PDZ domains so far studied have a preference for a hydrophobic residue (Val, Ile, Met, Ala) at the very COOH-terminus of the peptide (Songyang et al., 1997).

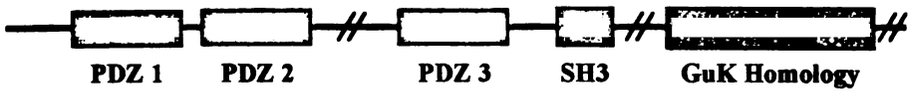
However, they have been divided into two categories according to amino acid preference for the –2 position of the peptide (the third residue from the end; Table 2-1). Type I PDZ domains recognize COOH-terminal peptides with a hydroxyl group (Ser or Thr) at the –2 position, while Type II PDZ domains recognize COOH-terminal peptides with a hydrophobic amino acids at his position (Phe or Tyr) (Songyang et al., 1997).

The crystal structure of a PDZ domain bound to peptide has been published, and the basis of this difference in PDZ recognition can be understood (Doyle et al., 1996). A PDZ domain consists of two α-helices and six β-strands. The peptide inserts itself between one of the β-strands (βB) and one of the α-helices (αB) and the final residue of the peptide fits into a hydrophobic pocket that is highly conserved in most PDZ domains. However, the first residue in the αB helix (αB1) of the PDZ makes contact with the side chain of the amino acid in the –2 position of the peptide (Doyle et al., 1996). This residue is a histidine in Type I PDZs, while the Type II PDZs lack a basic residue at this position and are therefore unable to coordinate the hydroxyl.

In addition to binding to C-terminal peptides, there have are examples of PDZ domains that bind to internal sequences, which is surprising based on the data with peptide libraries showing that PDZs do not bind internal sequences of peptides in these libraries (Songyang et al., 1997). In one case, the internal sequence has been identified and it corresponds to the COOH-terminal peptide binding motif (Shieh and Zhu, 1996). However, in most other cases no such motif has been identified (Bhat et al., 1999; Brenman et al., 1996; Cuppen et al., 1998; Srivastava et al., 1998; vanHuizen et al., 1998; Xia et al., 1997; Xu et al., 1998). Many of these interaction are homotypic PDZ / PDZ interactions between the PDZ domains of related molecules (Bhat et al., 1999; Srivastava

et al., 1998; Xu et al., 1998). The nNOS PDZ domain, however, can participate in heterotypic PDZ / PDZ interactions with the PDZ domains of PSD-95 and α-syntrophin. The mechanism for this internal sequence recognition will be presented in Chapter 5.

A



B

PSD-95 family of MAGUKs	**MAGUK superfamily members**
PSD-95 / SAP 90 (vertebrate)	ZO-1 (vertebrate)
PSD-93 / chapsyn 110 (vertebrate)	ZO-2 (vertebrate)
SAP 102 (vertebrate)	p55 (vertebrate)
SAP 97 / hdlg (vertebrate)	CASK (vertebrate)
dlg (*Drosophila*)	LIN 2 (*C. elegans*)

Figure 2-1

Figure 2-1 MAGUK proteins.

A. Schematic diagram of the shared domains in MAGUK proteins. Most MAGUKs contain three PDZ domains, a region of Src homology (SH3) and a domain homologous to guanylate kinases (GuK). The double vertical slashes indicate regions where the distance between the domains varies considerably.

B. Selected MAGUK family members. All members listed share the domain layout shown in (A), except that P55 and CASK / LIN-2 have only one PDZ, and CASK / LIN-2 has an additional N-terminal domain homologous to calmodulin-dependent protein kinase II.

	PDZ-containing examples	C-terminal binding partners	Peptide -2 position	PDZ domain αβ1 amino acid
Type I PDZ	PSD-95 family syntrophins dlg	NMDA NR2; Kv1.4 VGSCs Shaker; Fasciclin II	Hydroxyl (Ser / Thr)	His
Type II PDZ	p 55 LIN-2 PTPbas-6	glycophorin C β-neurexin ???	Hydrophobic (Phe / Tyr)	Val / Leu
Type III PDZ	nNOS	(see chapter 4)	(see chapter 4)	Tyr

Table 2-1

Table 2-1 The structural basis for Type I versus Type II PDZ domain specificity. Representative family members from each PDZ group are listed along with known examples of C-terminal (peptide) binding partners. The preferred amino acid for the –2 position of the peptide for each PDZ protein class is given, as is PDZ domain amino acid responsible for coordinating the side chain of the -2 amino acid in the peptide ($\alpha\beta1$).

CHAPTER THREE

Materials and Methods

Immunoprecipitation

Rat forebrain was homogenized in 20 volumes (w/v) TEE (25 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM EGTA) containing 1 mM phenylmethylsulfonylfluoride (PMSF). Na-deoxycholate was then added to 1% (w/v), and the homogenate was incubated at 4°C for 30 minutes. The lysate was cleared by centrifugation at 17,000 x g for 15 minutes, and the supernatant was incubated at 4°C for 1 hour with 6 μg/ml of rabbit nNOS antibody (Transduction Labs), rabbit NR2B antibody (Zymed) or a control rabbit antibody to phosphorylated CREB (Upstate Biotechnology). Immune complexes were precipitated with 20 μl protein A Sepharose (Sigma), washed 3 times with 1 ml lysis buffer, eluted with SDS and analyzed by Western blotting.

Transfected HEK293 cells (Chapter 5) were incubated in lysis buffer [TEE with 150 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol (DTT), 1 mM PMSF] at 4°C for 30 minutes. Lysates were cleared by centrifugation at 17,000 x g for 15 minutes and incubated for 1 hour at 4°C with 5 μg of rabbit nNOS antibody (Zymed), rabbit PSD-95 antibody (Brenman et al., 1996) or rabbit GFP antibody (Clontech). Immunoprecipitated complexes were washed and detected as above.

Transfected COS7 cells (Chapter 6) were lysed in RIPA buffer (25 mM Tris-HCl 7.4, 150 mM NaCl, 1% Triton X-100, 0.5 % deoxycholate, 0.1% SDS) and lysates cleared by centrifugation at 100,000 X g for 30 minutes. Lysates were incubated with 5 μg of rabbit GFP antibody (Clontech) or FLAG M2 monoclonal antibody (Scientific Imaging Systems) for 3 hours and the immunocomplexes bound to 10 μl Protein A

Sepharose (Sigma) or Protein G Sepharose (Sigma), respectively. Immunoprecipitated complexes were washed and detected as above.

Western blotting analysis

Protein samples were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and processed for western blotting as described (Brenman et al., 1996). The following antisera or antibodies were used: guinea pig PSD-93 antiserum (1:1000; (4)), rabbit PSD-95 antiserum (1:500; (4)), monoclonal NR2B antibody (1:250, Transduction Labs), monoclonal nNOS antibody (1:250, Transduction Labs), monoclonal PSD-95 antibodies MA1-045 and MA1-046 (1:500, Affinity Bioreagents), rabbit α1-syntrophin antiserum (Peters et al., 1994), monoclonal α-CaM kinase II antibody (1:1000, Zymed) and monoclonal GST antibody B-14 (1:200, Santa Cruz Biotechnology)

Bacterial and mammalian expression vectors

A bacterial expression vector encoding GST-NR2B was generated by cloning the following annealed oligos encoding the last nine amino acids of NR2B into pGEX-4T-1 (Pharmacia) digested with BamHI and EcoRI: 5'-GATCCAAGCTTTCTAGTATTG-AGTCTGATGTCTGAG-3' and 5'-AATTCTCAGACATCAGACTCAATACTAGAAA-GCTTG-3'. A bacterial expression vector encoding GST-GDAV was generated by cloning the following annealed oligos encoding the nNOS PDZ-binding peptide LSSYYGDAV* into pGEX-4T-1 (Pharmacia) digested with BamHI and EcoRI: 5'-

GATCCCTGAGCTCTTACTACGGAGACGCAGTATGAG-3' and 5'-AATTCTCATA-CTGCGTCTCCGTAGTAAGAGCTCAGG-3'. GST-nNOS fusion protein constructs were generated by PCR of the appropriate nNOS coding region to incorporate flanking BamHI and EcoRI sites followed by ligation into pGEX-4T-1. GST-PSD-95 included amino acids 1-386 of PSD-95 in pGEX-2T (Brenman et al., 1995). Full-length PSD-95 or nNOS 1-130 coding sequences were inserted into the pRSETA bacterial expression vector (Invitrogen) to generate hexahistidine-tagged fusion proteins. Full-length nNOS was expressed in the pCWori bacterial expression vector (Gerber and Demontellano, 1995). Maltose binding fusion proteins were generated by cloning the appropriate nucleotide sequences into pELM3 (Schatz et al., 1996). This allows expression of the corresponding maltose binding protein fused to the appropriate peptide sequence. The procedure for expression of maltose binding proteins was identical to that for GST fusion proteins except that the LB medium was supplemented with 2% glucose.

Mammalian expression vector PSD-95-GW1-CMV was a generous gift from Dr. Morgan Sheng (Kim et al., 1995) and pRK-NR2B was kindly provided by Dr. Lynn Raymond (Raymond et al., 1996). Full-length nNOS was expressed in the pCIS2 vector (Bredt et al., 1991).

Expression and purification of recombinant proteins

GST fusion proteins were expressed and purified as described (Brenman et al., 1995). Histidine-tagged PSD-95 or nNOS 1-130 were expressed in the bacterial strain BL21(DE3)pLysS (Novagen), solubilized by sonication in 50 mM sodium phosphate

buffer (pH 8.0) containing 6 M guanidine HCl / 300 mM NaCl / 10% glycerol and purified by cobalt-iminodiacetic acid Sepharose chromatography (Sigma). Full-length nNOS in pCWori was expressed in BL21(DE)pLysS and purified by 2',5'-ADP Sepharose chromatography as described (Gerber and Demontellano, 1995).

GST fusion protein chromatography

For binding assays using brain extracts, adult rat brain was homogenized in 10 volumes (w/v) TEE containing 1 mM PMSF and centrifuged at 15,000 x g for 15 minutes. The pellet was solubilized in TEE / 1% Triton X-100, 100 mM NaCl, 1 mM PMSF and cleared by centrifugation at 20,000 x g for 20 minutes. Purified GST-fusion proteins (100 μg) bound to glutathione Sepharose (Pharmacia) were incubated in 1 ml TEE / 0.5% Triton X-100 containing 2 mg rat brain extract for 1 hour at 4° C. Beads were loaded onto disposable columns, washed with 50 volumes solubilization buffer, and bound proteins eluted with 100 μl TEE containing 0.2% SDS. For the peptide competition experiment, 100 μM of peptide (KLSSIESDV, VSPDFGDAV or KLSSIEADA; Research Genetics) was added.

For binding assays using purified components, 10 μg of each GST fusion protein or control GST was bound to glutathione Sepharose and incubated with 2 μg recombinant native nNOS or recombinant His_6-PSD-95 in the absence or presence of 5 or 20 nM His_6-PSD-95 or 35 or 140 nM recombinant nNOS 1-130, respectively.

Maltose binding protein chromatography

Rat whole brain was homogenized in 10 volumes (w/v) Tris-HCl, pH 7.4 and centrifuged at 32,000 X g for 20 min. Membranes were solubilized for 2 h at 4^0C in buffer containing 200 mM NaCl and 1% Triton X-100, and insoluble material pelleted by centrifugation at 100,000 X g for 30 min. Extract were incubated with control amylose beads or amylose beads saturated with the indicated maltose binding protein fusion proteins. Samples were loaded onto disposable columns, washed with 50 volumes buffer containing 1% Triton X-100 and 300 mM NaCl. Retained proteins were eluted with SDS sample buffer and resolved by SDS-PAGE. Blots were analyzed by western blotting with a monoclonal antibody to nNOS (Transduction Labs).

Cell culture

HEK293 cells were transfected using the Lipofectamine Plus method (GIBCO/BRL) in 6-well dishes. A total of 2 μg of DNA was used for each well, and cotransfected wells received 1 μg of each expression vector. Cells were harvested 2 days post-transfection.

Circular dichroism (CD) and guanidine hydrochloride denaturation

CD measurements were taken on an AVIV 62DS spectropolarimeter with protein (cleaved from GST by thrombin) at a concentration of 5 mM in 50 mM Na phosphate

(pH 7.0) / 50 mM NaCl, in 1 cm path length cell. Protein unfolding by guanidine hydrochloride was analyzed by following the decrease in CD ellipticity at 222 nm. For proteins that showed a cooperative unfolding transition, CD ellipticity was fit to a two-state model by nonlinear least-squares analysis using Sigma Plot (Jandel Scientific). The equation used was:

$$q = ((a_N[GuHCl] + b_N)+(a_D[GuHCl] + b_D)\exp(-\Delta G'/RT))/(1+\exp(-\Delta G'/RT)$$

where q is the ellipticity at 222 nm, b_N is the intensity of the native state at 0 M GuHCl, a_N is the slope of the native baseline, b_D is the (extrapolated) intensity of the denatured state at 0 M GuHCl, a_D is the slope of the denatured state baseline, and $\Delta G'$ is the free energy change upon unfolding at the given concentration of GuHCl. $\Delta G'$ is assumed to be a linear function of denaturant concentration, as described by the equation:

$$\Delta G' = \Delta G_{H2O} - m\,[GuHCl]$$

where ΔG_{H2O} is the free energy of unfolding at 0 M GuHCl calculated by extrapolation (Santoro and Bolen, 1992).

Yeast two-hybrid analysis

Yeast transformations, filter lift assays and quantitative liquid culture assays were performed as described in the Matchmaker Library protocol (Clontech). One β-galactosidase unit = 1000 x OD_{420} / (t in min x V in ml x OD_{600}. Yeast strain SFY526

was cotransformed with the appropriate plasmids and cotransformants were selected on plates lacking tryptophan and leucine. nNOS constructs encoding fusion proteins with the Gal4 DNA binding domain were generated by PCR of the appropriate coding sequence to incorporate EcoRI and BamHI flanking sequences (nNOS 1-100; 1-111; 1-130; 1-130 Y77H, D78E; 1-159; 1-159 V93K) and ligation into pGBT9 (Clontech). Expression vectors encoding the Gal4 DNA binding domain fused to a nine residue peptide ending GESV was generated by cloning the following annealed oligos into pGBT9 digested with EcoRI and BamHI: 5'-AATTCTACGCCGGCCAGTGGGGC-GAGTCCGTGTAAG-3' and 5'-GATCCTTACACGGACTCGCCCCACTGGCCGGC-GTAG-3'. PSD-95 and PSD-93 constructs encoding fusion proteins with the Gal4 activation domain were generated by PCR of the appropriate coding sequence to incorporate EcoRI and BamHI flanking sequences (PSD-93 PDZ2, amino acids 117-371; PSD-93 PDZ2 H258V; PSD-95, amino acids 113-364; PSD-95 L241K) and ligation into pGAD424 (Clontech). An expression vector encoding the Gal4 activation domain fused to a nine residue peptide ending ESDV was generated by cloning the following annealed oligo into pGAD424 digested with EcoRI and BamHI: 5'-AATTCAAGCTTTCTAGTAT-TGAGTCTGATGTCTGAG-3' and 5'-GATCCTCAGACATCAGACTCAATACTAGA-AAGCTTG-3'.

Native PSD-95 Analysis

Adult rat brain was homogenized in 10 volumes (w/v) TEE containing 1 mM PMSF. Two 100 µl aliquots of crude homogenate were pipetted immediately into either

non-reducing or reducing sample buffer (containing 50 mM BME) and frozen in liquid nitrogen. The remaining extract was incubated at 4°C. After 20 hours, two 100 µl aliquots of the incubated sample were pipetted into either non-reducing or reducing sample buffer. These samples were analyzed by SDS-PAGE in the absence of additional reducing agents. Western blotting for PSD-95 was performed as described above.

Gel Filtration Chromatography

Adult rat brain was homogenized in 10 volumes (w/v) TEE buffer containing 10 mM BME, and insoluble material was removed by centrifugation at 130,000 x g for 20 minutes. Extract was prepared from HEK293 cells expressing PSD-95 in the same manner, except that 1 mM DTT was used as the reducing agent. Cleared extracts (200 µl) were loaded onto a Superose 6 HR 10/30 gel filtration column (Pharmacia) equilibrated with TBS containing 10 mM BME. Fractions (0.5 ml) were collected and analyzed for the presence of PSD-95 by western blotting. Gel filtration standards (Bio-Rad; thyroglobulin, 670 kD; gamma globulin, 158 kD; ovalbumin, 44 kD; myoglobin, 17 kD; and vitamin b-12, 1.35 kD) were separated on a Superose 6 column eluted with TBS. Nine residue peptides included in some samples were synthesized by Research Genetics (Huntsville, AL).

Cell Fractionation

Transfected COS7 cells were lysed in 1 ml of lysis buffer (25 mM Tris 7.4, 150 mM NaCl) by homogenization and lysates cleared by centrifugation at 100,000 X g for 30 min. Supernatants (cytosolic fraction) were saved for analysis, and pellets were resuspended 1 ml lysis buffer containing 1% Triton X-100 and incubated for 30 min at 4^0C with rocking. Samples were again cleared by centrifugation at 100,000 X g for 30 min, supernatants were saved for analysis (Triton-soluble fraction) and the pellets resuspended in 1 ml RIPA buffer and incubated for 30 min at 4^0C with rocking. Samples were again cleared by centrifugation at 100,000 X g for 30 min, supernatants were saved for analysis (0.5% deoxycholate, 0.1% SDS-soluble fraction). Samples were analyzed by western blotting as above.

CHAPTER FOUR

The nNOS PDZ Domain is Bivalent

Summary

PDZ domains are protein-protein interaction motifs that bind the C-termini of specific binding partners, as well as to small peptides corresponding to these C-terminal sequences. For instance, a peptide library screen determined that the PDZ domain of nNOS binds to peptides terminating Asp / Glu-X-Val-COOH. However, in addition to binding C-terminal peptides, the PDZ domain of nNOS can also bind to other PDZ domains, and we were interested in how the nNOS PDZ can recognize both types of binding partners. We initially hypothesized that a linear epitope in one of the PDZ partners mimics a small peptide, but we find that the tertiary structure of both PDZ domains is important. In addition, the nNOS PDZ domain extends approximately 30 residues beyond the C-terminal boundary of the consensus PDZ motif, and these additional sequences form a second binding site which sits in the peptide binding pocket of its PDZ partner. The crystal structure of the nNOS PDZ complexed with the PDZ of α-syntrophin was determined. This structure indicates that the sequences in this extended region do mimic peptide contacts, but that the entire PDZ domain is required to maintain these contact residues in the proper conformation for binding. Finally, we show that the nNOS PDZ domain can utilize both of its binding sites simultaneously, suggesting that *in vivo* it can function as a molecular brings to link together two proteins.

Results

PDZ / PDZ interactions require the tertiary structure of both binding partners

The "classical" binding partners for PDZ domains are small C-terminal motifs. However, the PDZ domain of nNOS also binds to the PDZ domain of α-syntrophin, and to PDZ repeats 1 and 2 of PSD-95 and the highly related family member PSD-93. While the classical mode of PDZ-peptide binding is well characterized (Doyle et al., 1996), the mechanism for PDZ / PDZ associations like these is less clear. To understand how the nNOS PDZ domain can associate with two such disparate types of binding partners, we first addressed the simple hypothesis that a linear epitope in one of the PDZ partners mimics a C-terminal peptide ligand. If this were the case, the ternary structure of such a "ligand PDZ" domain would presumably not be crucial for this interaction. We therefore sought a mutation that disrupts PDZ domain tertiary structure. A previous study showed that a Met to Lys mutation in one of the PDZ domains of the *Drosophila* inactivation no afterpotential D (INAD) protein disrupts binding of INAD to the C-terminal tail of the transient receptor potential (TRP) calcium channel (Shieh and Zhu, 1996). The corresponding amino acid in the crystal structure of the third PDZ domain of PSD-95 (Ile 388) occurs within a hydrophobic core (Doyle et al., 1996), indicating that insertion of a charged residue could generally affect domain structure and function. We made the corresponding mutation in the PDZ domain of nNOS (Val93Lys) and in PDZ2 of PSD-95 (Leu241Lys). When this mutation is introduced into the nNOS PDZ domain, it completely disrupts the tertiary structure as determined by CD spectroscopy. The mutant

nNOS protein shows a significantly reduced CD ellipticity at 222 nm, corresponding to a loss of structure (Figure 4-1*A*). Additionally, no cooperative unfolding transition is observed with chemical denaturation, indicating that the mutant protein is largely unfolded (Figure 4-1*B*). We analyzed the effect of this mutation on PDZ binding in the yeast two hybrid assay, and found that when this point mutation is introduced into the PDZ domain of nNOS, it completely eliminates binding to a PDZ partner, PDZ2 of PSD-95 (Figure 4-2). In addition, introducing this mutation into PDZ2 of PSD-95 also blocks the PDZ / PDZ interaction with nNOS, suggesting that the tertiary structure of both partners is critical for recognition.

PDZ / PDZ binding requires an extended nNOS PDZ domain

In our initial observations of the binding of the nNOS PDZ domain to its partners, we observed that the canonical nNOS PDZ domain is not sufficient for this interaction (Brenman et al., 1996; Stricker et al., 1997); the binding required additional residues C-terminal to the nNOS PDZ consensus motif (Figure 4-3). We therefore defined the minimal region of the nNOS PDZ necessary to bind to partner PDZ domains. We constructed a set of 8 GST fusion proteins containing fragments of the N-terminus of nNOS and evaluated the binding of each of these constructs to PSD-95, PSD-93 and α1-syntrophin proteins from rat brain extracts by the "pull down" assay. We consistently found that a fusion protein encompassing the canonical PDZ domain of nNOS (amino acids 1-99) is not competent for binding. Instead, binding requires a core region of nNOS amino acids 16-130 that contains approximately 30 additional residues C-terminal

to the consensus PDZ domain (Fig. 4-4). Deletion of 17 amino acids from the N-terminal side or 14 amino acids from the C-terminal side of this core completely eliminates binding. As a control we confirmed that a fusion protein containing the final 9 residues of NR2B binds to both PSD-95 and PSD-93 but not to α1-syntrophin. The bottom panel in Fig. 4-4 verifies that equal amounts of GST-nNOS fusion proteins were present in the binding assays.

We also evaluated binding of these nNOS fragments to fusion proteins containing PSD-93, amino acids 117-371 (PDZ2) or PSD-95, amino acids 20-364 (PDZ1-3) by the yeast two-hybrid system. The yeast results were consistent with the fusion protein binding experiments (Table 4-1). Again, amino acids 16-130 were implicated as the minimal PDZ / PDZ interaction domain of nNOS. Therefore, the interactions between nNOS and other PDZ domains require: (1) intact tertiary structures in both partners, and (2) an additional 16-30 residues following the canonical nNOS PDZ in addition to the entire PDZ domain itself.

nNOS PDZ/ peptide binding

Although the C-terminal peptide sequences bound by PSD-95/93 and α-syntrophin were known (Glu-Ser / Thr-X-Val-COOH), it was not known what C-terminal peptide sequence the nNOS PDZ domain recognizes. We therefore screened a random peptide library to find peptide binding partners for the nNOS PDZ domain (Stricker et al., 1997), and found that the nNOS PDZ binds short linear peptides with an affinity in the low nanomolar range that terminate with the consensus Gly-Asp-X-Val-COOH (Figure

4-5). Although the nNOS PDZ and the PDZ domains of PSD-93 and α-syntrophin both share a preference for a valine at the final ("0") position, the nNOS PDZ shows a distinct binding specificity for the amino acid at the –2 position, preferring an Asp rather than a Ser or Thr. To test whether this peptide can bind to native nNOS protein, we used affinity chromatography with a maltose binding protein (MBP)-peptide fusion protein bound to amylose resin. nNOS protein from crude rat brain homogenate binds to the MBP-Gly-Asp-Ala-Val-COOH matrix, while it does not bind to an analogous column in which the Asp at the –2 position was changed to a Thr (Figure 4-6).

Based on the crystal structure of PDZ3 of PSD-95 bound to peptide, the side chain of His 372 forms a critical sequence-specific hydrogen bond with the Thr at the –2 position of the bound peptide (Doyle et al., 1996). Interestingly, the amino acid at the corresponding position of the nNOS PDZ is a Tyr, suggesting that a His to Tyr substitution at this position is responsible for the difference in binding specificity. Amino acid sequence comparison of a number of PDZ domains present in Genbank shows that the residue after the His or Tyr is also conserved. To test whether these residues are responsible for the peptide binding specificity we observed, we mutated Tyr77,Asp78 in the nNOS PDZ to H77,E78 to correspond to the PSD-95 amino acids. When binding is analyzed by ELISA, this nNOS mutant no longer binds to a Gly-Asp-X-Val-COOH peptide but now is able to bind a Glu-Ser-X-Val-COOH peptide (Figure 4-7).

Since we had found that an extended nNOS PDZ domain was required to bind to other PDZ domains, we were interested in determining whether this was also true for nNOS PDZ / peptide binding. When assayed by ELISA, we found that the extended nNOS PDZ domain (nNOS 1-130) is also required to bind C-terminal peptides (Figure 4-

8). However, others used an nNOS construct containing only the canonical PDZ domain (amino acids 1-111) in the yeast two hybrid assay to find peptide binding partners for the nNOS PDZ (Schepens et al., 1997), and obtained the same results for the peptide consensus motif (Gly-Asp / Glu-X-Val-COOH). We wondered whether this discrepancy in the size of the nNOS PDZ required to bind peptide could be explained by the different methods used (i.e. *in vitro* binding vs. yeast two hybrid). To test this, we generated peptide fusion proteins for use in the yeast two hybrid system and found that the nNOS PDZ alone does bind C-terminal peptides, in contrast to our *in vitro* results (Fig. 4-9). However, a smaller construct that also contains the consensus PDZ domain (nNOS 1-100) binds more weakly than nNOS 1-111, whereas a larger construct (nNOS 1-130) binds more strongly than nNOS 1-111.

The progressive attenuation in binding observed with nNOS 1-111 and nNOS 1-100, together with our inability to detect binding with purified nNOS 1-111 *in vitro*, suggested that the PDZ domain may become less structurally stable as the C-terminal extension is truncated. To test this, we evaluated the thermodynamic stability of bacterially-expressed nNOS PDZ domain proteins using a guanidine hydrochloride denaturation assay. Protein unfolding by guanidine hydrochloride was analyzed by following the decrease in ellipticity at 222 nm (Lim et al., 1994), which presumably corresponds to the loss of the two α-helices observed in known PDZ domain structures. Using this assay we find that the canonical nNOS PDZ domain (amino acids 1-99) is significantly less stable than larger nNOS constructs which contain the entire extended domain (amino acids 1-130 and 1-159; Figure 4-10). Furthermore, nNOS 1-116 shows an unfolding transition that is intermediate between nNOS 1-99 and 1-130 (data not

shown), indicating that residues adjacent to the canonical nNOS PDZ (1-99) increase stability. Therefore, the canonical nNOS PDZ is sufficient to bind C-terminal ligands, though the instability of this minimal construct makes it more difficult to assay.

The nNOS PDZ domain has two binding sites

The above results demonstrate that residues 100-130 of nNOS are necessary for PDZ-PDZ interactions but not for PDZ-peptide interactions. This difference suggests that either: (1) the increased stability of the larger nNOS constructs is required for PDZ domain but not peptide binding, or (2) there are two distinct binding interfaces on nNOS, and only the PDZ / PDZ interface incorporates residues 100-130. To evaluate this latter possibility, we tested whether a C-terminal nNOS PDZ-binding peptide, Gly-Asp-Ala-Val*, competes with PSD-95 for binding to nNOS. We find that this Gly-Asp-Ala-Val* peptide does not disrupt the nNOS / PSD-95 interaction (Fig. 4-11), providing evidence that the PDZ / PDZ and PDZ / peptide binding interfaces of nNOS are distinct. By contrast, a C-terminal PSD-95-binding peptide that terminates Glu-Ser-Asp-Val* does disrupt the nNOS / PSD-95 interaction.

Taken together, these data suggest that the nNOS PDZ domain has distinct binding sites for C-terminal peptides and for other PDZ domains, whereas these interfaces are overlapping or identical in PDZ2 from PSD-95. To confirm these results, we sought a point mutation in the nNOS PDZ that would differentiate between the two modes of binding. As mentioned above, in the co-crystal structure of PSD-95 PDZ3 bound to a C-terminal peptide, the critical threonine at the -2 position of the peptide

makes a hydrogen bond with His 372 (Doyle et al., 1996). The equivalent residue in the nNOS PDZ is Tyr 77, and we have previously shown that changing this residue to His converts the nNOS peptide-binding specificity from Asp-X-Val* to Ser-X-Val*. Whereas this mutation in nNOS alters PDZ-peptide binding, it does not disrupt PDZ / PDZ associations (nNOS 1-130 Y77H, D78E; Fig. 4-12*A*). In contrast, mutating the analogous residue in PSD-93 PDZ2 (H258V) disrupts interactions with both C-terminal peptides and with the nNOS PDZ (Fig. 4-12*B*), consistent with the idea that PDZ2 of PSD-93 and PSD-95 use overlapping or identical binding interfaces to contact the nNOS PDZ and C-terminal peptides.

Very recently, we determined the crystal structure of nNOS (amino acids 1-130) complexed with the PDZ domain of α-syntrophin (Hillier et al., 1999). This structure demonstrates that the 30 additional amino acids following the PDZ domain of nNOS that are specifically required for PDZ / PDZ binding form a β-hairpin "finger" (Figure 4-13). This finger contains the tetrapeptide Glu-Thr-Thr-Phe (amino acids 108-111), which inserts into the peptide binding groove of the α-syntrophin PDZ domain. These residues in the nNOS finger are nearly identical to the consensus residues for peptides that bind to α-syntrophin and PSD-95 thus allowing the structured β-hairpin "finger" of nNOS to mimic a typical C-terminal peptide ligand (Figure 4-14*A*).

Guided by this structure, we made alanine mutations of specific residues in the nNOS finger. Mutations of either Glu 108 (E108A) or Thr 109 (T109A), disrupt PDZ / PDZ binding but do not affect PDZ / peptide binding (Figure 14*B*). In contrast, mutation of Thr 110 (T110A), which is not predicted to be essential for PDZ binding, does not affect either interaction. Mutations of the critical hydrophobic residue, Phe 111 (F111A

and F111S), disrupt both PDZ / PDZ and PDZ / peptide binding, suggesting that mutations of this residue may perturb the structure of the nNOS PDZ domain.

These data clearly demonstrate that the nNOS PDZ domain contains distinct binding interfaces for PDZ and C-terminal peptide binding partners. We therefore asked whether both binding sites can be simultaneously occupied. For this experiment, we used purified protein components and tested whether the nNOS PDZ can form a ternary complex with both PSD-95 and a C-terminal peptide ligand. We first verified that PSD-95 does not directly interact with a GST-fusion protein terminating Gly-Asp-Ala-Val*. However, when we titrate in the extended nNOS PDZ domain (amino acids 1-130) at 35 to 140 nM, we readily detect formation of a ternary complex (Fig. 4-15).

Discussion

nNOS binds to its PDZ partners through a unique PDZ-PDZ interaction in which an internal domain adjacent to the nNOS PDZ peptide-binding pocket is recognized, instead of a classical C-terminal motif that has been thought to be required for PDZ binding. The mechanism for this interaction requires that one of the PDZ partners (the "ligand PDZ") contains an extended PDZ domain that contains a second binding interface, followed by a sharp turn, that can interact with the peptide binding pocket in the "receptor" PDZ. A few other examples of PDZ-PDZ interactions have been identified. The neuronal proteins ABP (AMPA receptor-binding protein) and the related GRIP (glutamate-receptor interacting protein) contain six PDZ domains and can form homomultimers and heteromultimers with each other through a subset of these repeats (Srivastava et al., 1998). In *Drosophila*, the proteins INAD and the recently identified DLT (Discs Lost) also form homomultimers (Bhat et al., 1999; Xu et al., 1998). Interestingly, homomultimerization of PDZ3 of INAD requires that one of the PDZ3 domain partners includes additional amino acids C-terminal to the consensus PDZ domain (Xu et al., 1998). The mechanism for association of these proteins may therefore be similar to the nNOS / PSD-95 interaction. It is worth noting that no other examples of PDZ / PDZ associations between non-homologous proteins, such as nNOS and its binding partners, have yet been identified.

Our finding that the nNOS PDZ is actually composed of a second binding interface has interesting implications for protein-protein interactions involving other PDZ-containing proteins. It suggests that a PDZ domain can theoretically interact with

any protein that presents an internal peptide sequence in the correct conformation for binding followed by a sharp to allow immediate exit from the binding pocket. Supporting this idea, the PDZ domain of α-syntrophin can bind to specific cyclic peptides that are similar in sequence to the consensus (Arg/Lys)-Glu-(Ser/Thr)-X-Val* (Gee et al., 1998). Therefore, conformational restriction by cyclization of a linear sequence can be sufficient to present the contact residues in the proper structural context.

Examples of other PDZ proteins that appear to participate in PDZ-internal peptide motif interactions have been identified. The third PDZ domain of INAD binds to TRP at an internal Ser-Thr-Val motif, a known consensus sequence for C-terminal peptides that bind to many PDZ domains (Shieh and Zhu, 1996), while PDZ5 of INAD binds an internal motif in PLC (phopholipase C-β) (vanHuizen et al., 1998). In addition, the second PDZ domain of PTP-BL (protein tyrosine phosphastase) and the PDZ domain of RIL (reversion-induced LIM gene) both bind to internal sequences in the LIM domain of RIL (Cuppen et al., 1998). It will be interesting to see whether any of these interactions are indeed similar to the binding of the extended nNOS PDZ to its partners.

The nNOS PDZ also binds linear C-terminal peptides (Glu-Asp/Glu-X-Val*) originally identified in peptide library screens (Schepens et al., 1997; Stricker et al., 1997). Evidence that it binds peptide using the same binding pocket as other PDZ domains comes from experiments with peptide specificity mutants. The crystal structure of PSD-95 PDZ3 bound to peptide demonstrated that His 372 in the αβ1 position was responsible for coordinating the hydroxyl group in the –2 position of the peptide (Doyle et al., 1996). When the equivalent residue in the nNOS PDZ is changed from Tyr to His, it changes the peptide specificity to Ser-X-Val* (Stricker et al., 1997). Interestingly,

although the nNOS peptide binding interface is contained within the consensus PDZ domain, we found that this domain by itself is less stable than the full domain that includes both binding sites, suggesting that the two sites form one structured domain with two interfaces. Candidates for ligands of the peptide-binding site *in vivo* include CAPON in neurons (Jaffrey et al., 1998) and the glycolytic enzyme phosphofructokinase (PFK) (Firestein and Bredt, 1999). In muscle, nNOS inhibits contractile force and increases blood flow during exercise, and interaction with PFK may be a way to coordinate nNOS activity with the metabolic rate.

Multiple tandem PDZ domains are found in many PDZ-containing proteins including the PSD-95 family, and this concatamerization is thought to be necessary for these molecules to perform their bridging and clustering functions. The existence of two binding interfaces on the single nNOS PDZ suggests that it can also function as a molecular bridge similar to other PDZ proteins that contain multiple domains. To test this possibility, we have shown that the nNOS PDZ can bind a peptide ligand and a PDZ domain simultaneously. The fact that two binding sites exist on nNOS may explain previous observations in Becker's dystrophy, which results from deletions of the central rod-like domain of the dystrophin protein. In human patients and mouse models of the disease, nNOS is absent from the motor endplate even though α-syntrophin is still properly localized (Chao et al., 1996). Our data suggests that the proper localization of nNOS could require interaction with another component of the dystrophin complex though the second binding interface on the extended PDZ domain. In addition to the two binding sites on the nNOS PDZ, the observation that the nNOS enzyme is a stable dimer in solution enhances the possibilities for the assembly of multiple proteins together into

one complex (Klatt et al., 1995; Schmidt et al., 1991). These findings have important implications for the function of other proteins that contain only a single PDZ domain, since they could contribute to the formation of multiprotein complexes and signaling networks that have been previously thought to require concatamerized PDZ domains.

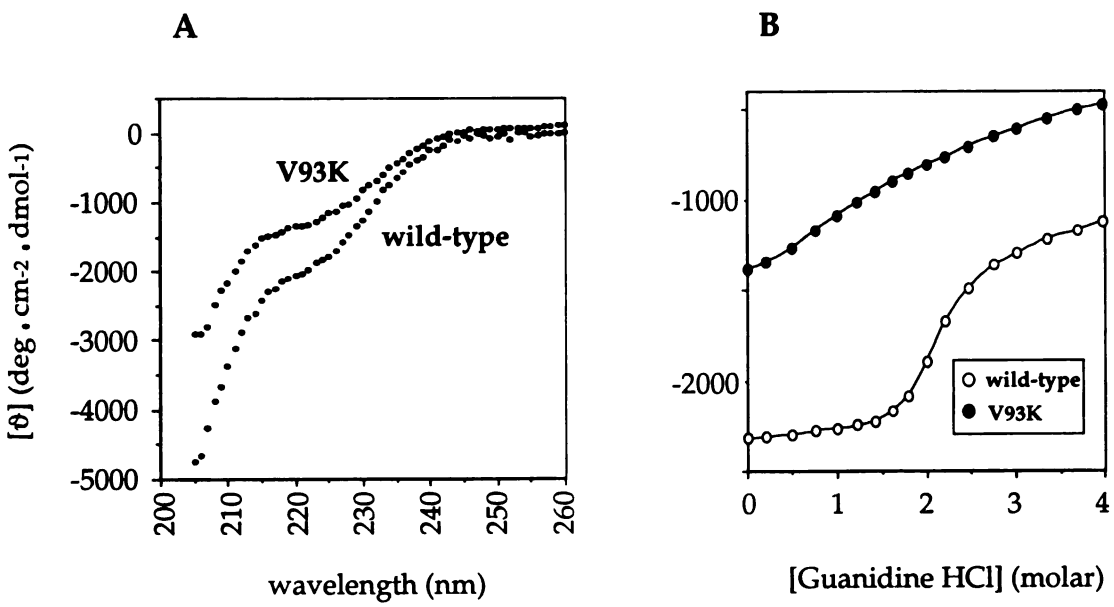

A
B
0
-1000
-2000
-3000
-4000
-5000
V93K
wild-type
[θ] (deg . cm-2 . dmol-1)
200
210
220
230
240
250
260
wavelength (nm)
-1000
-2000
wild-type
V93K
0
1
2
3
4
[Guanidine HCl] (molar)

Figure 4-1

Figure 4-1 A point mutant homologous to an INAD PDZ mutation disrupts tertiary structure of the nNOS PDZ domain.

A. CD spectra of wild-type nNOS PDZ domain (amino acids 1-159) and a point mutant (V93K). The mutant protein shows dramatically reduced ellipticity at 222 nm, indicative of a loss of helical structure.

B. Guanidine HCl denaturation of wild-type and V93K nNOS, amino acids 1-159. The mutant protein does not show a cooperative unfolding transition, indicating that it is already largely unfolded at 0 M guanidine HCl.

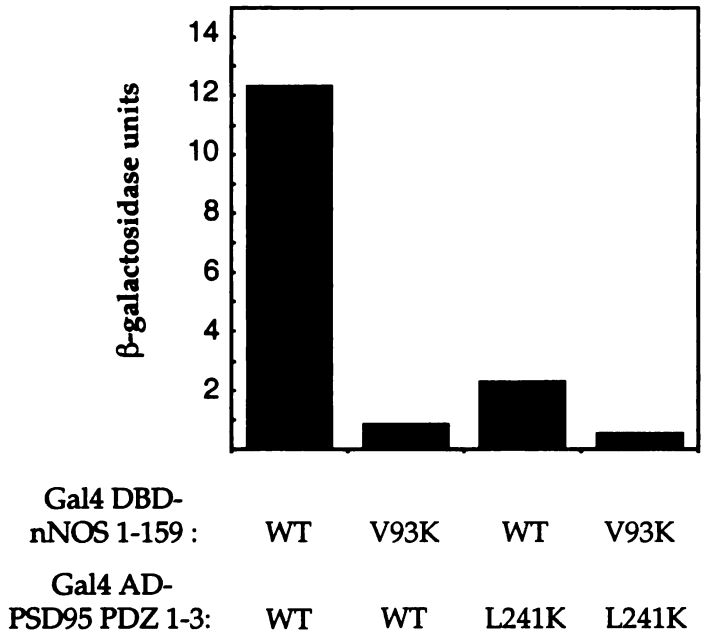

β-galactosidase units
14
12
10
8
6
4
2
Gal4 DBD-
nNOS 1-159 :
WT
V93K
WT
V93K
Gal4 AD-
PSD95 PDZ 1-3:
WT
WT
L241K
L241K

Figure 4-2

Figure 4-2 The INAD PDZ mutation blocks PDZ / PDZ binding when present in either nNOS or PSD-95 PDZ 2.

Yeast constructs containing nNOS 1-159 or PSD-95 PDZ 1-3 were generated that contained a mutation homologous to the INAD PDZ mutant described in the text (V93K and L241K, respectively). Yeast strain SFY526 was cotransformed with the indicated wild-type or mutant constructs, and β-galactosidase expression was assayed by quantitative liquid culture assay. Values shown are averages of duplicates that varied by <10%. Assays were repeated with similar results.

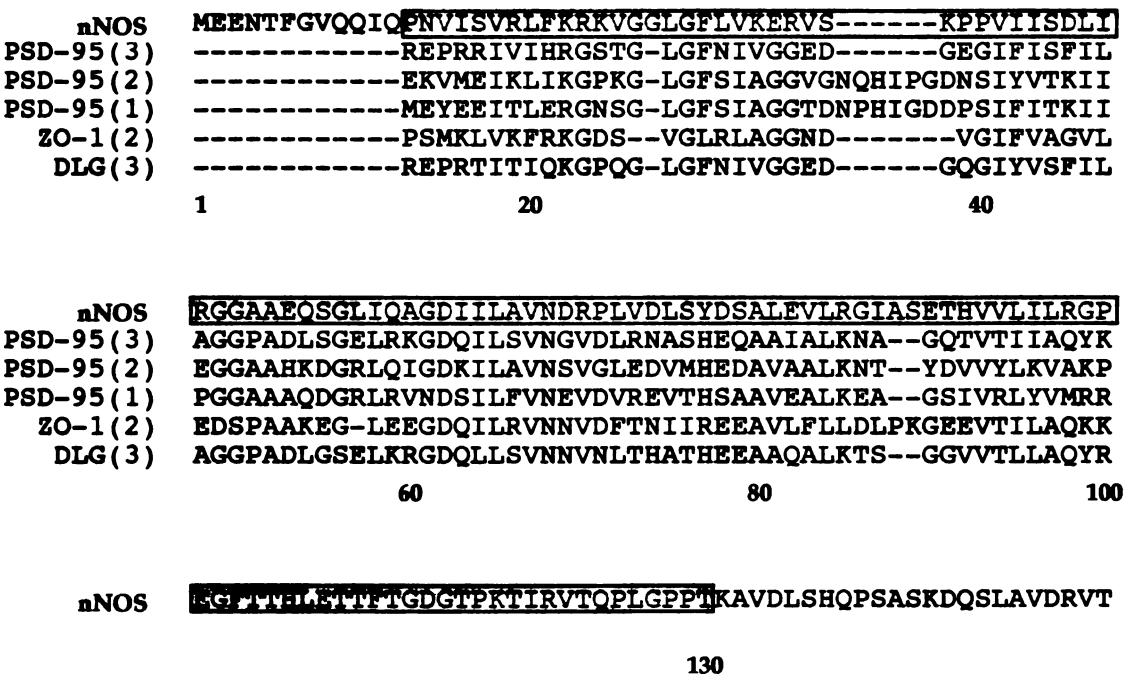

nNOS MEENTFGVQQIQPNVISVRLFKRKVGGLGFLVKERVS------KPPVIISDLI
PSD-95(3) ----------REPRRIVIHRGSTG-LGFNIVGGED------GEGIFISFIL
PSD-95(2) ----------EKVMEIKLIKGPKG-LGFSIAGGVGNQHIPGDNSIYVTKII
PSD-95(1) ----------MEYEEITLERGNSG-LGFSIAGGTDNPHIGDDPSIFITKII
ZO-1(2) ----------PSMKLVKFRKGDS--VGLRLAGGND-------VGIFVAGVL
DLG(3) ----------REPRTITIQKGPQG-LGFNIVGGED------GQGIYVSFIL
1 20 40
nNOS RGGAAEQSGLIQAGDIILAVNDRPLVDLSYDSALEVLRGIASETHVVLILRGP
PSD-95(3) AGGPADLSGELRKGDQILSVNGVDLRNASHEQAAIALKNA--GQTVTIIAQYK
PSD-95(2) EGGAAHKDGRLQIGDKILAVNSVGLEDVMHEDAVAALKNT--YDVVYLKVAKP
PSD-95(1) PGGAAAQDGRLRVNDSILFVNEVDVREVTHSAAVEALKEA--GSIVRLYVMRR
ZO-1(2) EDSPAAKEG-LEEGDQILRVNNVDFTNIIREEAVLFLLDLPKGEEVTILAQKK
DLG(3) AGGPADLGSELKRGDQLLSVNNVNLTHATHEEAAQALKTS--GGVVTLLAQYR
60 80 100
nNOS EGPTTHLETTFTGDGTPKTIRVTQPLGPPTKAVDLSHQPSASKDQSLAVDRVT
130

Figure 4-3

Figure 4-3 Alignment of the nNOS PDZ domain with PDZ domains of the original defining PDZ family members.

The sequences shown are as follows: rat PSD-95, PDZ-3 (amino acids 309-393), PDZ 2 (amino acids 156-246), PDZ 1 (amino acids 61-151); human ZO-1, PDZ 2(amino acids 408-491); *Drosophila* DLG, PDZ 3 (amino acids 482-566); rat nNOS (amino acids 1-153). The numbers correspond to amino acids of nNOS. Blue shaded area identifies the nNOS PDZ consensus motif, green shaded area identifies the extended region required to participate in PDZ / PDZ binding.

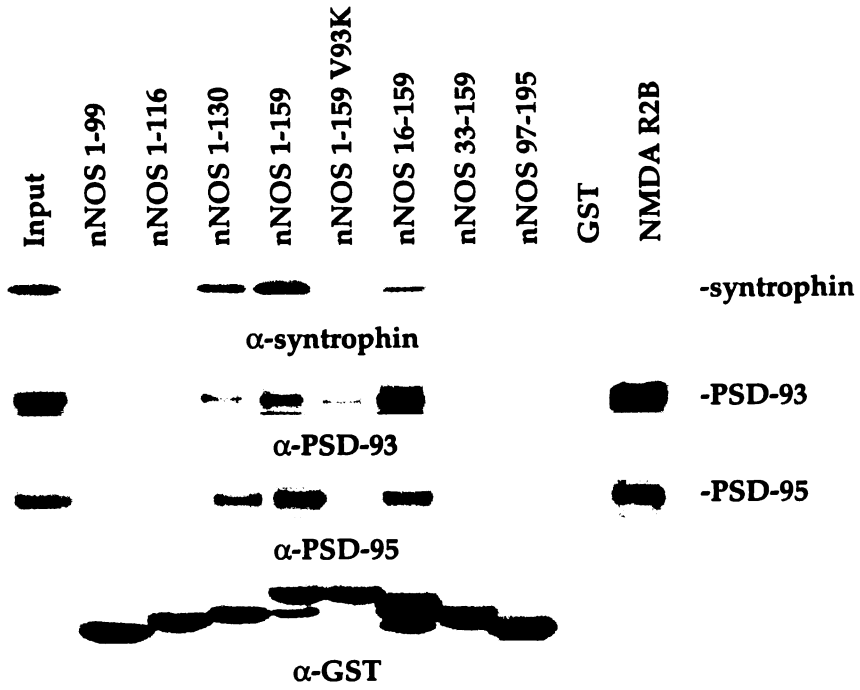

Input
nNOS 1-99
nNOS 1-116
nNOS 1-130
nNOS 1-159
nNOS 1-159 V93K
nNOS 16-159
nNOS 33-159
nNOS 97-195
GST
NMDA R2B
-syntrophin
α-syntrophin
-PSD-93
α-PSD-93
-PSD-95
α-PSD-95
α-GST

Figure 4-4

Figure 4-4 PDZ / PDZ binding requires additional residues adjacent to the nNOS PDZ consensus motif.

Bacterial GST fusion constructs containing the indicated nNOS residues or the final nine residues from NR2B were generated, and the proteins encoded were expressed and purified. Fifty micrograms each of GST-nNOS or GST-NR2B fusion protein bound to glutathione Sepharose was incubated with solubilized rat brain extract. After extensive washing, bound proteins were eluted with SDS and analyzed by western blotting with an antibody to either α-syntrophin, PSD-93, PSD-95 or GST. Input = 10% of protein present in each binding reaction.

Table 4-1 Deletional analysis of the interactions of nNOS with PSD-95 and PSD-93

	PSD-95 PDZ1-3 (β-gal units)	**PSD-93 PDZ2** (β-gal units)
nNOS 1-99	0.409	0.210
nNOS 1-116	0.892	0.761
nNOS 1-130	34.8	64.0
nNOS1-159	28.1	41.7
nNOS 16-159	27.2	38.4
nNOS 33-159	5.37	0.273
nNOS 97-197	7.17	0.565

Table 4-1 Deletional analysis of the interactions of nNOS with PSD-95 and PSD-93.

Yeast strain SFY526 was cotransformed with pGBT9 and pGAD424 (Clontech) constructs expressing the appropriate Gal4 fusion proteins. The Gal4 DNA binding domain was fused to domains of nNOS containing the indicated amino acids. The Gal4 activation domain was fused to PSD-95 PDZ 1-3 (amino acids 20-364) or PSD-93 PDZ 2 (amino acids 117-371). Transformants were selected on plates lacking tryptophan and leucine, and assayed for β-galactosidase expression using a quantitative liquid culture assay as described in the Matchmaker Library protocol (Clontech). β-galactosidase units = 1000 x OD_{420} / (t in min x V in ml x OD_{600}) and numbers shown are averages of duplicates that varied by <10%.

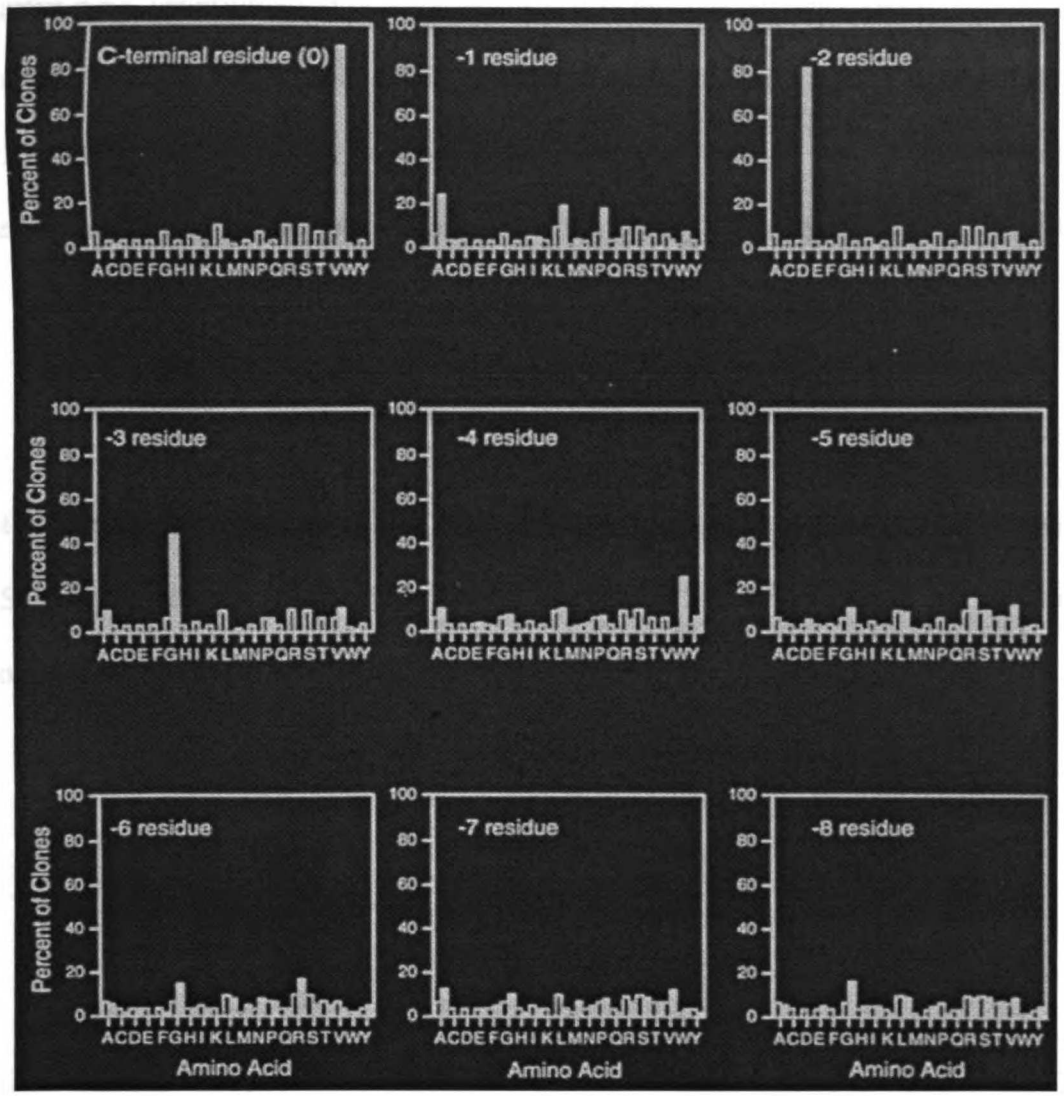

C-terminal residue (0)
-1 residue
-2 residue
-3 residue
-4 residue
-5 residue
-6 residue
-7 residue
-8 residue
Percent of Clones
Amino Acid
A C D E F G H I K L M N P Q R S T V W Y
0
20
40
60
80
100

Figure 4-5

Figure 4-5* Determination of a consensus nNOS binding peptide (NBP).

Normalized amino acid abundance of the final nine residues from a population of 96 independent nNOS binding peptides (black bars) is compared with codon frequency in the original library (white bars). Residues in the library linker region are not included.

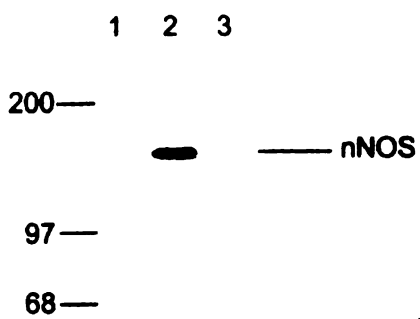


Figure 4-6

Figure 4-6* Peptides terminating Asp-X-Val-COOH bind specifically to nNOS protein from rat brain.

Solubilized rat brain extracts were incubated with amylose resin alone (lane 1), amylose resin saturated with a fusion protein containing maltose binding protein (MBP) fused to a C-terminal Asp-Ala-Val-COOH sequence (lane 2) or with the same fusion protein in which the –2 aspartate was changed to a threonine (lane 3). The beads were washed and retention of nNOS was detected by Western blot analysis.

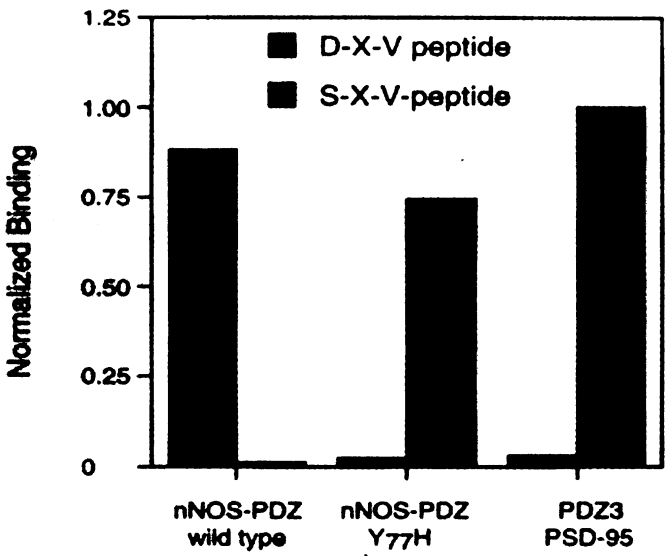
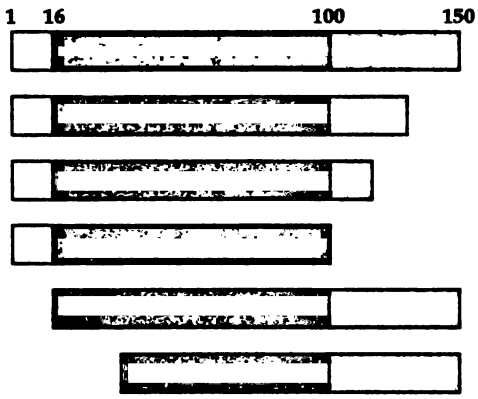

1.25
1.00
0.75
0.50
0.25
0
Normalized Binding
D-X-V peptide
S-X-V-peptide
nNOS-PDZ wild type
nNOS-PDZ Y77H
PDZ3 PSD-95

Figure 4-7

Figure 4-7* nNOS PDZ binding specificity for the amino acid at the -2 position in the peptide can be altered by mutating the amino acid in the αβ1 position of the nNOS PDZ. Mutating $Tyr_{77}Asp_{78}$ to $His_{77}Glu_{78}$ changes the nNOS PDZ binding specificity from Asp-X-Val to Thr-X-Val. ELISA results of two high affinity peptides made as maltose binding protein (MBP) fusion proteins are shown. mNBP-161 is an MBP fusion with a peptide terminating Asp-Ala-Val-COOH and mPD-325 is an MBP fusion with a peptide terminating Thr-Ser-Val-COOH. The EC_{50} of nNOS for mNBP-161 was approximately 8 nM and the EC_{50} for mPD-325 was approximately 2 nM.



	Peptide binding	PSD-93 PDZ 2 binding
	ELISA (norm)	β-gal units
nNOS 1-150	0.67	43.8
nNOS 1-130	1.00	65.1
nNOS 1-116	0.05	0.43
nNOS 1-00	0.03	0.00
nNOS 16-150	0.55	48.6
nNOS 40-150	0.05	0.28

Figure 4-8

Figure 4-8 The interaction of nNOS with peptide requires amino acids 16-130 in the ELISA.

Interaction of five different nNOS binding peptides generated as fusion proteins with maltose binding protein and different nNOS domains generated as fusion proteins with GST were evaluated by ELISA and binding is expressed as normalized OD_{405}. The five COOH-terminal peptide sequences were: Asp-Ala-Val, Asp-Pro-Val, Asp-Leu-Val, Asp-Ile-Val and Asp-Trp-Val. For comparison, the association of the same nNOS domains with PSD-93 evaluated by quantitative yeast two hybrid assay are shown.

Gal DBD Fusions	Gal AD Fusions				
		GDAV		GESV	
		Filter assay	β-gal units	Filter assay	β-gal units
	nNOS 1-100	0	0.205	0	0.344
	nNOS 1-111	++	2.24	++	2.34
	nNOS 1-130	++	3.30	++	4.48

Figure 4-9

Figure 4-9 The canonical nNOS PDZ domain is sufficient to interact with C-terminal peptides.

Yeast strain SFY526 was cotransformed with a construct encoding the GAL4 DNA binding domain fused to domains of nNOS and a construct encoding the Gal4 activation domain fused to nine residue peptides terminating GDAV or GESV. β-galactosidase expression was assayed by both filter lift assay (++ 90-180 min; + 180-360 min; -/+ >360 min) and quantitative liquid culture assay (values shown are averages of duplicates that varied by <10%). Assays were repeated with similar results.

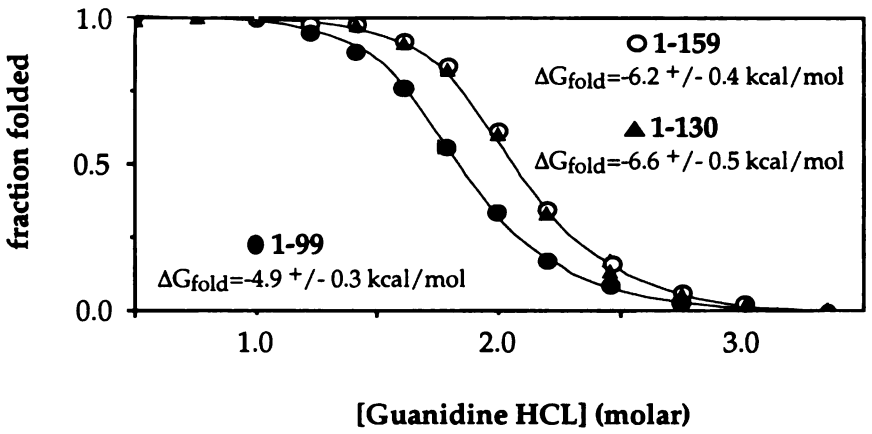

1.0
0.5
0.0
fraction folded
1.0
2.0
3.0
[Guanidine HCL] (molar)
1-159
ΔGfold=-6.2 +/- 0.4 kcal/mol
1-130
ΔGfold=-6.6 +/- 0.5 kcal/mol
1-99
ΔGfold=-4.9 +/- 0.3 kcal/mol

Figure 4-10

Figure 4-10 The canonical nNOS PDZ domain is less stable than the extended PDZ binding domain.

Stability of constructs *in vitro* were analyzed by guanidine HCl denaturation. nNOS domains 1-130 and 1-159, which are both completely functional *in vitro*, have a stability of ~6.5 kcal / mol. A construct that contains the entire canonical nNOS PDZ domain and is able to bind peptide in yeast but not *in vitro* is destabilized by ~2 kcal / mol.

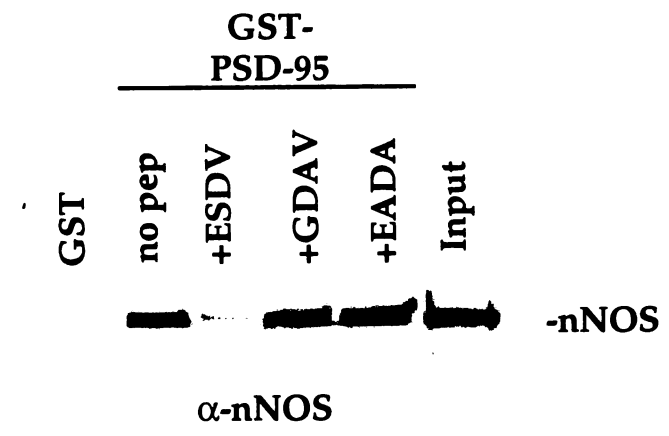


Figure 4-11

Figure 4-11 Peptides terminating in ESDV but not GDAV block nNOS / PSD-95 binding.

Glutathione Sepharose-bound GST-PSD-95 was incubated with rat brain extract in the presence or absence of the indicated nine residue peptides (100 μM). Peptide ESDV is derived from the C-terminus of NR2B and binds PDZ1 and 2 of PSD-95 / 93, GDAV is an nNOS PDZ-binding peptide, and EADA is a control peptide. After extensive washing, bound proteins were eluted with SDS and analyzed by western blotting with an antibody to nNOS. Input = 10% of protein present in each binding reaction.

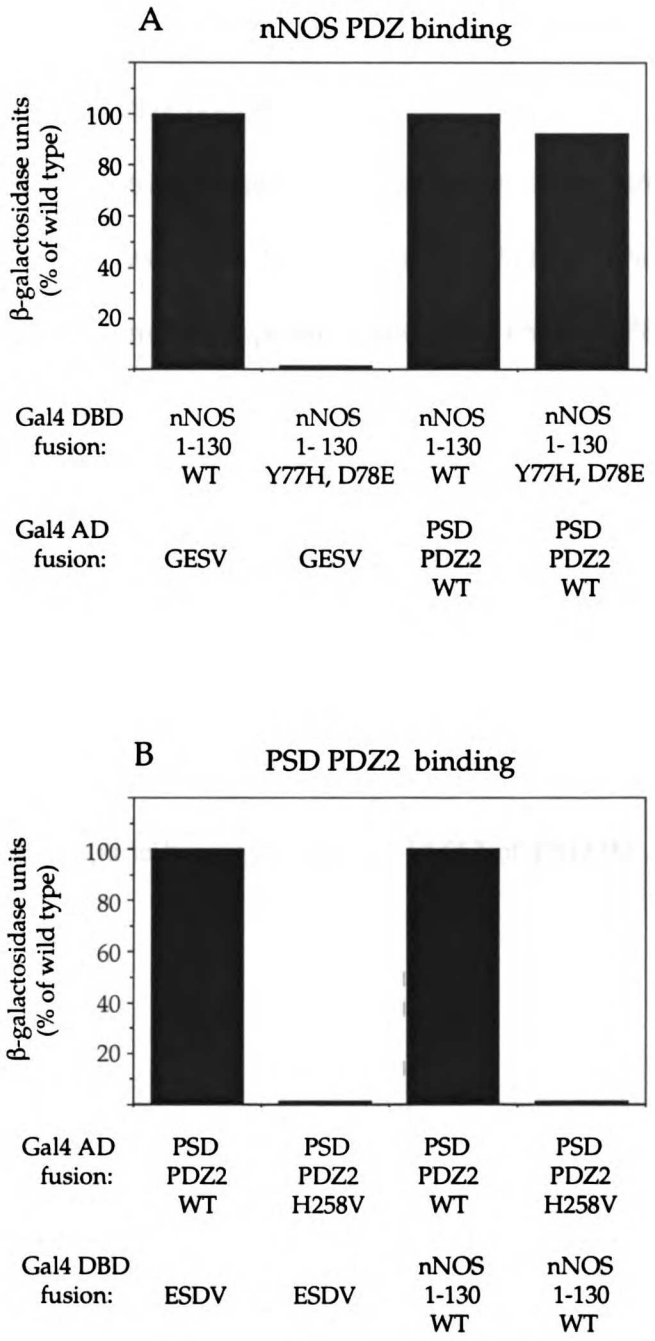

A
nNOS PDZ binding
β-galactosidase units (% of wild type)
100
80
60
40
20
Gal4 DBD fusion:
nNOS 1-130 WT
nNOS 1- 130 Y77H, D78E
nNOS 1-130 WT
nNOS 1- 130 Y77H, D78E
Gal4 AD fusion:
GESV
GESV
PSD PDZ2 WT
PSD PDZ2 WT
B
PSD PDZ2 binding
β-galactosidase units (% of wild type)
100
80
60
40
20
Gal4 AD fusion:
PSD PDZ2 WT
PSD PDZ2 H258V
PSD PDZ2 WT
PSD PDZ2 H258V
Gal4 DBD fusion:
ESDV
ESDV
nNOS 1-130 WT
nNOS 1-130 WT

Figure 4-12

Figure 4-12 The nNOS PDZ domain contains two separate interfaces for binding C-terminal peptides and other PDZ domains.

Yeast strain SFY526 was cotransformed with a plasmid encoding nNOS 1-130, nNOS 1-130 Y77H, D78E or a nine residue peptide terminating ESDV as a fusion protein with the GAL4 DNA binding domain and a plasmid encoding PSD-93 PDZ2, PSD-93 PDZ2 H258V or a nine residue peptide terminating GESV as a fusion protein with the Gal4 activation domain. β-galactosidase expression was quantitated by liquid culture assay and values shown are averages of duplicates that varied by <10%. Assays were repeated with similar results.

A. A mutation in the peptide-binding pocket of nNOS does not affect association with other PDZ domains.

B. A mutation in the peptide-binding pocket of PDZ2 of PSD-93 abolishes interaction with the nNOS PDZ.

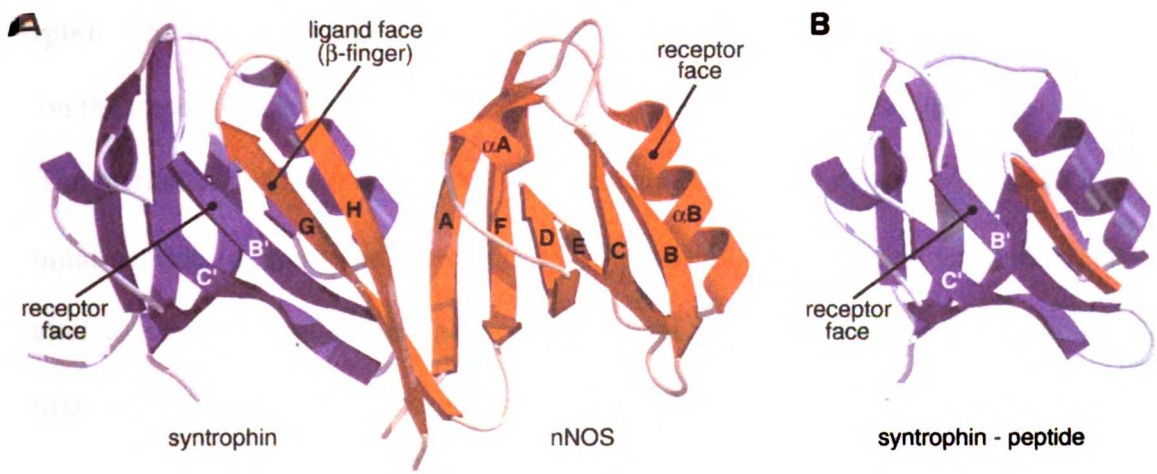

A
ligand face
(β-finger)
receptor
face
G
H
B'
C'
receptor
face
αA
A
F
D
E
C
αB
B
syntrophin
nNOS
B
B'
C'
receptor
face
syntrophin - peptide

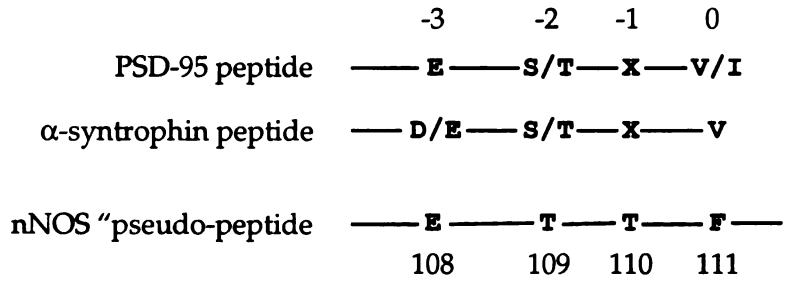
Figure 4-13

Figure 4-13* Ribbon diagram of the nNOS PDZ complexed with α-syntrophin.

A. The additional amino acids C-terminal to the nNOS PDZ domain (orange) form a β-hairpin finger, one side of which docks into the peptide binding groove of α-syntrophin (purple). The nNOS PDZ domain is polarized, such that the peptide-binding interface sits on the opposite side of the structured domain from the PDZ-binding interface.

B. The orientation of the α-syntrophin PDZ domain complexed with its cognate COOH-terminal peptide (orange) and the nNOS PDZ extension. The peptide is oriented anti-parallel to strand βB and contributes to a β sheet in the α-syntrophin PDZ domain. Amino acids of the nNOS extension sit in binding groove of α-syntrophin in an analogous manner, but the nNOS extension provides two strands that contribute to the structured β-sheet. This figure was generated with the program MOLSCRIPT.

A



B

	PSD PDZ2		GESV	
	β-gal units	Filter assay	β-gal units	Filter assay
nNOS WT	44.9	++++	5.12	++
E108A	1.41	+	6.00	++
T109A	0.940	+	4.12	++
T110A	44.8	++++	4.61	++
F111A	0.481	+/-	1.41	+
F111S	0.610	+/-	1.45	+

Figure 4-14

Figure 4-14 Amino acids in the nNOS PDZ extension mimic a C-terminal peptide.

A. The terminal four amino acids in the peptides that bind to the PDZ domains of PSD-95 and a-syntrophin are compared to the internal sequence in the nNOS PDZ domain extension. The critical residues are conserved.

B. Yeast strain SFY526 was cotransformed with pGBT9 and pGAD424 (Clontech) constructs expressing the appropriate Gal4 fusion proteins. The Gal4 DNA binding domain was fused to nNOS amino acids 1-130 wild-type or 1-130 containing one of the above mutations. The Gal4 activation domain was fused to PSD-93 (amino acids 117-371) or to the nine residues peptide LSSYYGESV*. Transformants were selected on plates lacking tryptophan and leucine, and assayed for β-galactosidase expression by both filter lift assay (+++ <30 min; ++ 90-180 min; + 180-360 min) and quantitative liquid culture assay as described in the Matchmaker Library protocol (Clontech). β-galactosidase units = 1000 x OD_{420} / (t in min x V in ml x OD_{600}) and numbers shown are averages of duplicates that varied by <10%.

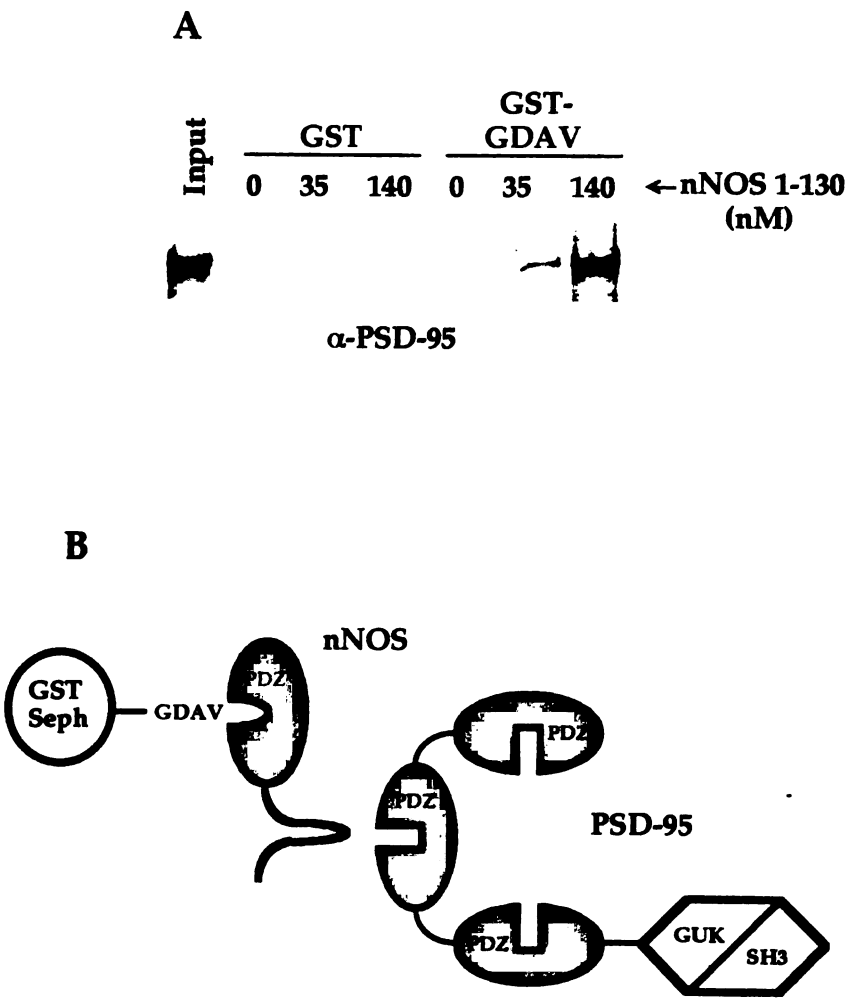

A
Input
GST
GST-
GDAV
0 35 140 0 35 140
←nNOS 1-130
(nM)
α-PSD-95
B
nNOS
GST
Seph
GDAV
PDZ
PDZ
PDZ
PDZ
PSD-95
GUK
SH3

Figure 4-15

Figure 4-15 The nNOS PDZ domain can bind two different ligands simultaneously.

A. The extended nNOS PDZ domain can simultaneously interact with a heterologous PDZ protein and a C-terminal peptide ligand. A GST-fusion protein containing a nine residue nNOS PDZ-binding peptide (GST-GDAV) or GST control was bound to glutathione Sepharose and incubated with full-length purified His_6-PSD-95 in the absence or presence of increasing amounts of purified nNOS 1-130. After extensive washing, bound proteins were eluted with SDS and analyzed by immunoblotting. Input = 10% of His_6-PSD-95 present in each binding reaction.

B. Schematic of the experiment showing nNOS bridging its two binding partners. nNOS is associating with the immobilized GST-GDAV beads through its peptide-binding pocket. Purified PSD-95 is able to bind to the β-finger on the PDZ extension that lies on the other side of the nNOS domain.

CHAPTER FIVE

NR2B, PSD-95 and nNOS Ternary Complex Formation

Summary

Efficiency and specificity in cellular signaling cascades is often mediated by assembly of multi-protein transduction networks. Signaling by the calcium / calmodulin regulated neuronal nitric oxide synthase (nNOS) in cerebellar neurons is activated by calcium influx through *N*-methyl-D-aspartic acid (NMDA) receptors (Bredt and Snyder, 1990; Garthwaite et al., 1988) but nNOS is not efficiently stimulated by activation of non-NMDA receptors that also generate calcium influx (Kiedrowski et al., 1992). We were interested in whether PSD-95 could act as a molecular bridge to specifically couple NMDA receptor-mediated calcium influx to nNOS. PSD-95 binds directly to nNOS through a PDZ-PDZ interaction that involves the second PDZ domain of PSD-95 (Brenman et al., 1996). Moreover, PSD-95 is a member of a larger family of postsynaptic density proteins that bind to and cluster NMDA receptors (Brenman et al., 1996; Hunt et al., 1996; Kim et al., 1996; Kornau et al., 1995; Muller et al., 1996). Accordingly, we find that PSD-95 can mediate formation of a ternary complex containing the NMDA receptor, PSD-95 and nNOS, which *in vitro* can be accomplished by one molecule of PSD-95. In brain, we find that the three proteins can be coimmunoprecipitated together, suggesting that *in vivo* similar binding interactions occur that are responsible for the specific activation of nNOS by NMDA receptor calcium.

Results

nNOS, PSD-95 and NR2B are specifically coimmunoprecipitated from brain

We were interested in testing the hypothesis that PSD-95 could function as a molecular link between nNOS and the NMDA receptor in brain. The concatenated PDZ domains in PSD-95 bind to both C-terminal peptides derived from NR2B and to the PDZ domain of nNOS (Brenman et al., 1996; Kornau et al., 1995), but it is not known whether these interactions can occur simultaneously to form a macromolecular signaling complex. To investigate whether PSD-95 can act as a scaffolding protein to link nNOS and NR2B, we performed immunoprecipitation experiments from brain. Solubilized rat brain extracts were immunoprecipitated with an antibody to nNOS, NR2B, or a control antibody and duplicate immunoblots were analyzed for NR2B, PSD-95, nNOS or the alpha subunit of CaM kinase II (CaMKII). Both PSD-95 and NR2B specifically coimmunoprecipitate with nNOS (Figure 5-1*A*), and both PSD-95 and nNOS specifically coimmunoprecipitate with NR2B (Figure 5-1*B*). However, the alpha subunit of CaMKII, which is also enriched in the postsynaptic density (Cho et al., 1992), does not coimmunoprecipitate with either nNOS or NR2B.

PSD-95 can organize a ternary complex with nNOS and NR2B

Although the above results show that these three protein exist in a complex in brain, it does not address whether PSD-95 is required to recruit nNOS into a complex

with PSD-95. We next transfected heterologous cells to ask whether PSD-95 is sufficient to link nNOS and NR2B. HEK293 cells were transfected with expression constructs encoding either NR2B alone, NR2B and nNOS, or NR2B, nNOS and PSD-95. Cell lysates were immunoprecipitated with an antibody to nNOS and coimmunoprecipitated proteins were analyzed by western analysis (Figure 5-2). NR2B does not directly associate with nNOS, but does coimmunoprecipitate when PSD-95 is also expressed.

To determine whether the PSD-95 protein alone is sufficient to mediate complex formation, we expressed and purified the three recombinant proteins from *E. coli.* The final 9 amino acids of NR2B was expressed as a GST-fusion protein, full length PSD-95 was expressed as a histidine-tagged fusion protein, and nNOS was expressed as the native protein (Figure 5-3*A*). As expected, purified nNOS does not bind to the tail of NR2B (Figure 5-3*B*). However, when PSD-95 is added to the mixture in nanomolar concentrations, the three proteins form a tight ternary complex.

PSD-95 can utilize both PDZ 1 and PDZ 2 simultaneously

PSD-95 contains three PDZ repeats (see Figure 2-1), but it is not know whether it can utilize more than one repeat at a time to bind together two proteins simultaneously. In the binding assay with purified components, we found that PSD-95 can bridge nNOS and NR2B, and we sought to ascertain the stoichiometry of this *in vitro* complex. PSD-95 contains three PDZ repeats, and although nNOS only binds PDZ2, NR2B can bind either PDZ1 or PDZ2. This suggests a model in which a PSD-95 monomer could

mediate complex formation such that PDZ1 is bound to NR2B and PDZ2 is bound to nNOS.

However, it has been reported in the literature that PSD-95 can form multimers in heterologous cells, and that this multimerization is required for clustering of ion channels (Hsueh et al., 1997; Kim et al., 1996). If the mechanism of this multimerization was such that this could also happen *in vitro*, this would suggest a model in which nNOS and NR2B may bind to each other through different multimerized PSD-95 molecules. In this study (Hsueh et al., 1997), is was concluded that this multimerization is occurring through disulfide bond-formation. Therefore, we treated our purified PSD-95 protein with 10 mM DTT and ensured it was monomeric by non-reducing SDS-PAGE analysis. When we repeated the experiment in Figure 5 exactly as before, but with the 10 mM DTT-treated PSD-95 in the continuing presence of 1 mM DTT, we obtained identical results (data not shown). Therefore, this data confirms that at least *in vitro* disulfide-bonding is not the mechanism of formation of this ternary complex.

The other possible mechanism for multimerization of PSD-95 is by non-covalent associations. For instance, PDZ/ PDZ interactions could theoretically be responsible, since there have been reports that other multiple PDZ-containing proteins can bind to each other through these repeats (Bhat et al., 1999; Srivastava et al., 1998; Xu et al., 1998). Although PDZ/ PDZ binding has not been shown to occur between PDZ domains of PSD-95, we evaluated binding of bacterially expressed histidine-tagged PSD-95 to a GST-PSD-95 fusion protein. We included reducing agents in this and the next experiment to prevent oxidation *in vitro*. Under our conditions we did not observe detectable interaction between these bacterially expressed PSD-95 proteins (Figure 5-4).

Although the above results suggest that the ternary complex we observed using bacterially expressed proteins is not dependent on PSD-95 multimerization, we were interested in whether PSD-95 has any inherent ability to self-associate when two molecules are co-expressed in the same cell. We transfected HEK293 cells with a PSD-95 expression vector and analyzed the cell lysates by gel filtration. PSD-95 eluted from the column as a broad peak centered at ~100 kD, consistent with PSD-95 being inherently monomeric (Figure 5-5*A*). To determine the inherent oligomeric structure of PSD-95 *in vivo*, we performed gel filtration chromatography on rat brain extract. Most of PSD-95 in brain is insoluble and membrane-associated due to interactions with receptors and other proteins (Cho et al., 1992; Kim et al., 1995; Kornau et al., 1995; Wyszynski et al., 1997). Because this membrane-associated population of PSD-95 is known to exist as part of a larger heterogeneous complex, it is not possible to use this material to determine the intrinsic multimeric structure of PSD-95. We therefore used soluble PSD-95 isolated from brain as a source of native protein. Rat brain extract was applied to the gel filtration column, and fractions were analyzed by western blotting. Again, PSD-95 eluted from the column in a peak centered ~100 kD, confirming that PSD-95 is inherently a monomeric protein (Figure 5-5*B*).

These results show that *in vitro* one molecule of PSD-95 can use two PDZ domains simultaneously to link together nNOS and an NMDA receptor subunit. This may also happen *in vivo*, as we were unable to detect any non-covalent association between molecules of PSD-95. Although we showed that disulfide-bonding also does not play a role in our *in vitro* assay, we have not ruled out in these experiments the possibility that this may occur *in vivo* at the synapse. We will explore this possibility in Chapter 6.

Our data in this chapter, combined with our results from the study of the PDZ domain of nNOS in the last chapter, suggest the model depicted in Figure 5-6. Future studies of the stoichiometry and composition of PSD-95 and nNOS-containing complexes from brain synaptic densities will determine the accuracy of this model.

Discussion

The demonstration that a ternary complex is formed between nNOS, PSD-95 and an NMDA receptor subunit has important implications for the biology of nNOS. nNOS is selectively stimulated by NMDA receptor calcium but not by other receptors that also generate calcium influx (Bredt and Snyder, 1990; Garthwaite et al., 1988; Kiedrowski et al., 1992). The hypothesis that the specific intracellular localization of nNOS protein might be responsible for this specific coupling was substantiated by ultrastructural studies, which found that NMDA receptor subunits and a subpopulation of nNOS are colocalized at the postsynaptic density of synaptic spines (Aoki et al., 1993; Aoki et al., 1997; Huntley et al., 1994; Petralia et al., 1994). We investigated PSD-95 as candidate protein that localizes nNOS to the NMDA receptor based on biochemical studies demonstrating that both nNOS and NR2B coimmunoprecipitate with PSD-95 in solubilized brain extracts (Brenman et al., 1996; Wyszynski et al., 1997).

Our studies suggest that PSD-95 can function in vertebrate neurons to link plasma membrane receptors to second messenger pathways, in addition to its proposed role in ion channel clustering. The only PDZ-containing protein that has been shown to function in a similar manner is the INAD protein (inactivation no afterpotential D) in *Drosophila* (Shieh and Niemeyer, 1995). This protein contains five PDZ domains that all display unique binding specificities for components of the visual signal transduction machinery, and brings these signaling components together in close proximity to the TRP (transient receptor potential) ion channel and to each other (Chevesich et al., 1997; Shieh and Zhu, 1996; Tsunoda et al., 1997). This arrangement allows for the efficient and specific

activation and deactivation of the pathway (Scott and Zuker, 1998; Tsunoda et al., 1997), which one would also expect to be important for nNOS as it can be toxic to cells when produced inappropriately (Choi, 1994; Dawson et al., 1996; Samdani et al., 1997).

The stoichiometry of this ternary complex at synaptic densities is not yet clear. Although there are examples of PDZ-mediated hetero- and homomultimerization (Bhat et al., 1999; Brenman et al., 1996; Srivastava et al., 1998; Xu et al., 1998), our data suggest that PSD-95 is inherently a monomeric protein. Affinity chromatography studies failed to detect reversible binding between PSD-95 monomers. Also, PSD-95 expressed in heterologous cells or contained in the soluble fraction of brain extracts migrates predominately as a monomer by gel filtration fractionation. These studies show that at least *in vitro*, one molecule of PSD-95 is sufficient to bridge together two proteins, and that it can therefore utilize both of its PDZ domains simultaneously. This mechanism may also function *in vivo*, since all NR2 subunits can interact with either PDZ1 or PDZ2 of PSD-95. Future studies of the stoichiometry and composition of the PSD-95 protein complexes from brain synaptic densities are necessary to clarify these issues.

Although the experiments proposed in this chapter address the inherent ability of PSD-95 to multimerize, work from other labs has suggested that multimerization of PSD-95 occurs in when PSD-95 is expressed in heterologous cells, and that this multimerization is required for PSD-95 to perform its receptor clustering function *in vivo* (Hsueh et al., 1997). This observation adds another level of complexity to protein network assembly at the synapse, and this phenomenon and its possible mechanism will be addressed in Chapter 6. It is also possible that interactions with other proteins allow synaptic PSD-95 to be incorporated into large protein complexes wherein individual

monomers associate with each other indirectly. Indeed, GKAP proteins have concatenated domains that bind the guanylate kinase domain of PSD-95 (Kim et al., 1997; Naisbitt et al., 1997).

The experiments presented in this chapter, along with the studies of the nNOS PDZ domain presented Chapter 4, suggest the model shown in Figure 5-6. The bivalent nNOS domain is depicted with its possible peptide-binding pocket partners *in vivo*. We have shown in this chapter that indeed PSD-95 can act as a molecular bridge between nNOS and the NMDA receptor. Also, we have demonstrated that this can be achieved *in vitro* by a single molecule of PSD-95, such that PDZ1 of PSD-95 binds to an NMDA receptor subunit while PDZ2 of PSD-95 binds to the nNOS PDZ domain. This may therefore also occur *in vivo*, which this gives us an important understanding of how other proteins containing concatamerized PDZ domains can function. In addition, there is evidence for PSD-95 association in cells by a mechanism other than the affinity of protein domains for each other, which may also contribute to protein complex formation at the synapse.

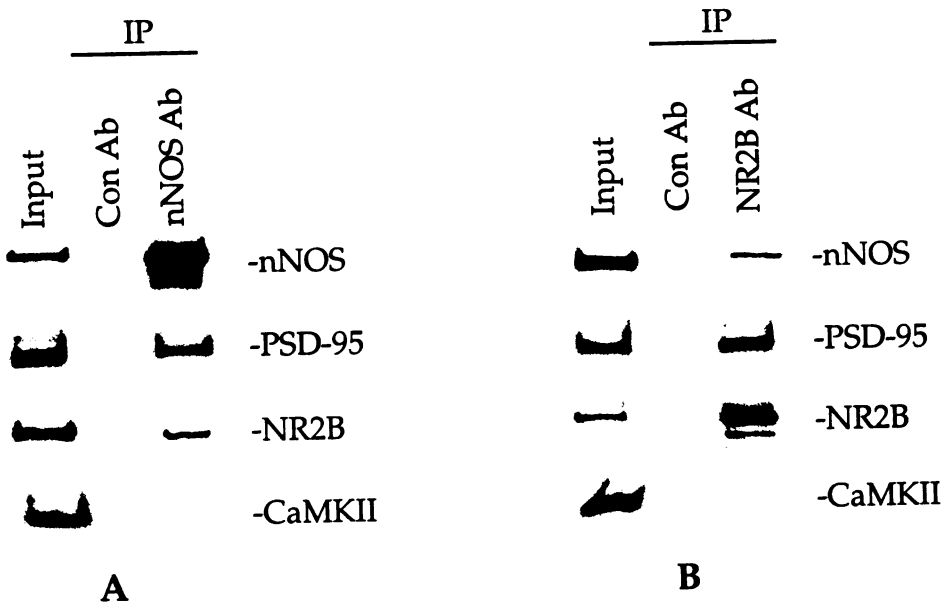

IP
Input
Con Ab
nNOS Ab
-nNOS
-PSD-95
-NR2B
-CaMKII
A
IP
Input
Con Ab
NR2B Ab
-nNOS
-PSD-95
-NR2B
-CaMKII
B

Figure 5-1

Figure 5-1 nNOS, PSD95 and NR2B specifically coimmunoprecipitate from brain. Solubilized rat forebrain homogenates were immunoprecipitated with an antibody to nNOS (A) NR2B (B) or a control antibody to phosphorylated CREB ("Con Ab"; A and B). Identical samples were analyzed by immunoblotting for nNOS, PSD-95, NR2B or the alpha subunit of CaM kinase II. PSD-95 and NR2B or nNOS coimmunoprecipitate with nNOS or NR2B respectively.

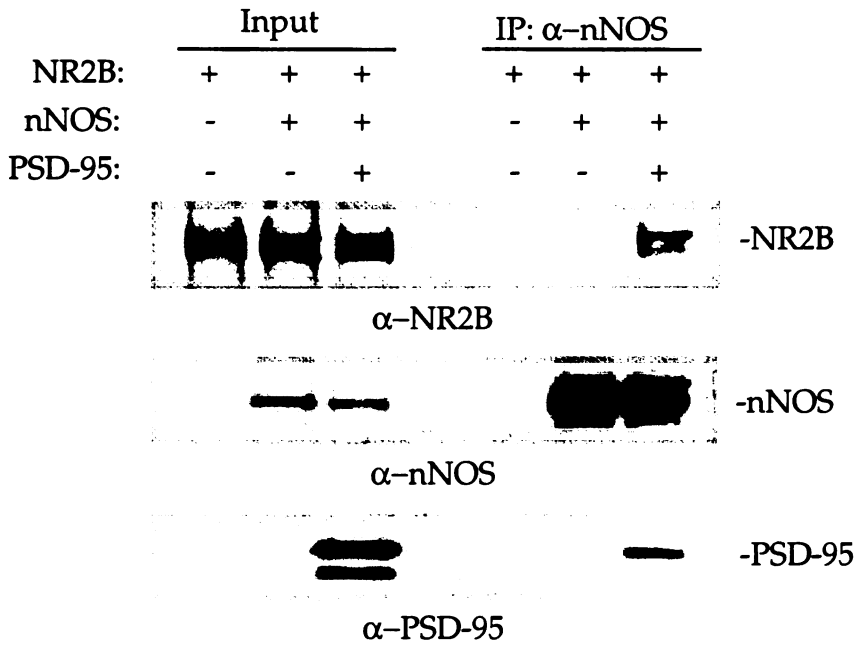

Input
IP: α–nNOS
NR2B: + + + + + +
nNOS: - + + - + +
PSD-95: - - + - - +
-NR2B
α–NR2B
-nNOS
α–nNOS
-PSD-95
α–PSD-95

Figure 5-2

Figure 5-2 Coimmunoprecipitation of NR2B with nNOS is dependent on PSD-95. Lysates of HEK293 cells expressing NR2B alone, NR2B and nNOS, or NR2B, PSD-95 and nNOS were immunoprecipitated with an antibody to nNOS. Identical samples were analyzed by immunoblotting for nNOS, PSD-95, or NR2B. NR2B immunoprecipitates with nNOS only when PSD-95 is coexpressed.

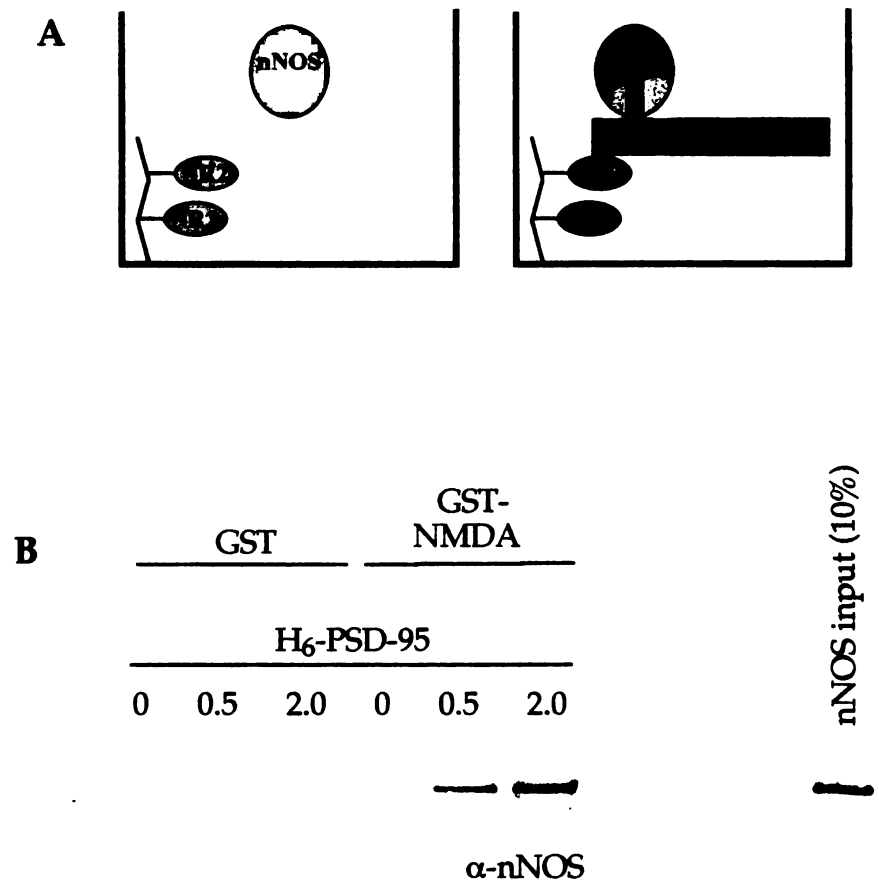


Figure 5-3

Figure 5-3 PSD-95 is sufficient to link the C-terminus of NR2B to nNOS.

A. Schematic diagram of experiment. "NR2" represents a GST fusion protein with the last nine residues of the NMDA NR2B subunit immobilized on glutathione Sepharose. Small vertical rectangles represent PDZ domains.

B. Purified GST or GST-NR2B containing the last nine amino acids of NR2B were bound to glutathione Sepharose and incubated with recombinant nNOS in the absence or presence of increasing amounts of purified full-length His_6-PSD-95. After extensive washing, bound proteins were eluted with SDS and analyzed by immunoblotting with an antibody to nNOS. nNOS does not bind to NR2B directly but associates in a complex when PSD-95 is present.

Figure 5-4

Figure 5-4 Bacterially expressed PSD-95 does not form reversible multimers.

Purified GST or GST-PSD-95 bound to glutathione Sepharose was incubated with full-length His_6-PSD-95. Beads were washed and SDS eluates analyzed by western blotting with an antibody recognizing the His_6 fusion protein. No detectable binding was observed between GST-PSD-95 and His_6-PSD-95 even when film was overexposed (data not shown).

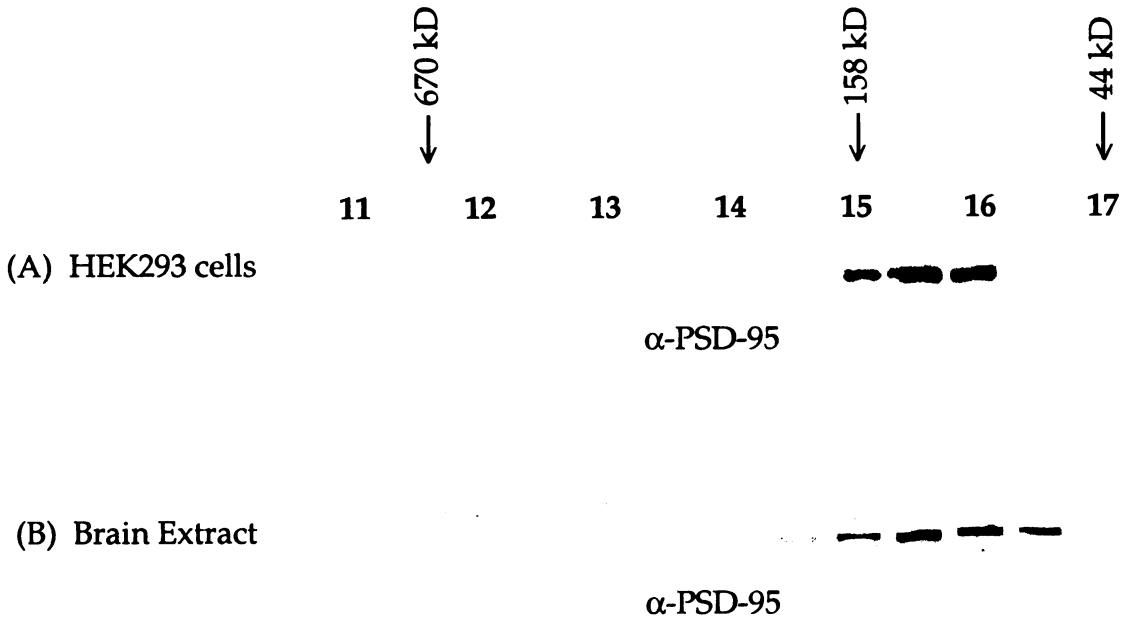

670 kD
158 kD
44 kD
11
12
13
14
15
16
17
(A) HEK293 cells
α-PSD-95
(B) Brain Extract
α-PSD-95

Figure 5-5

Figure 5-5 PSD-95 is predominantly monomeric

Soluble fraction of lysates from transfected HEK293 cells (A) or brain extract (B) were separated by gel filtration chromatography on a Superose 6R 10/30 column (Pharmacia) and collected fractions analyzed by western blotting for PSD-95. In both cases PSD-95 eluted as a peak centered at ~100 kD.

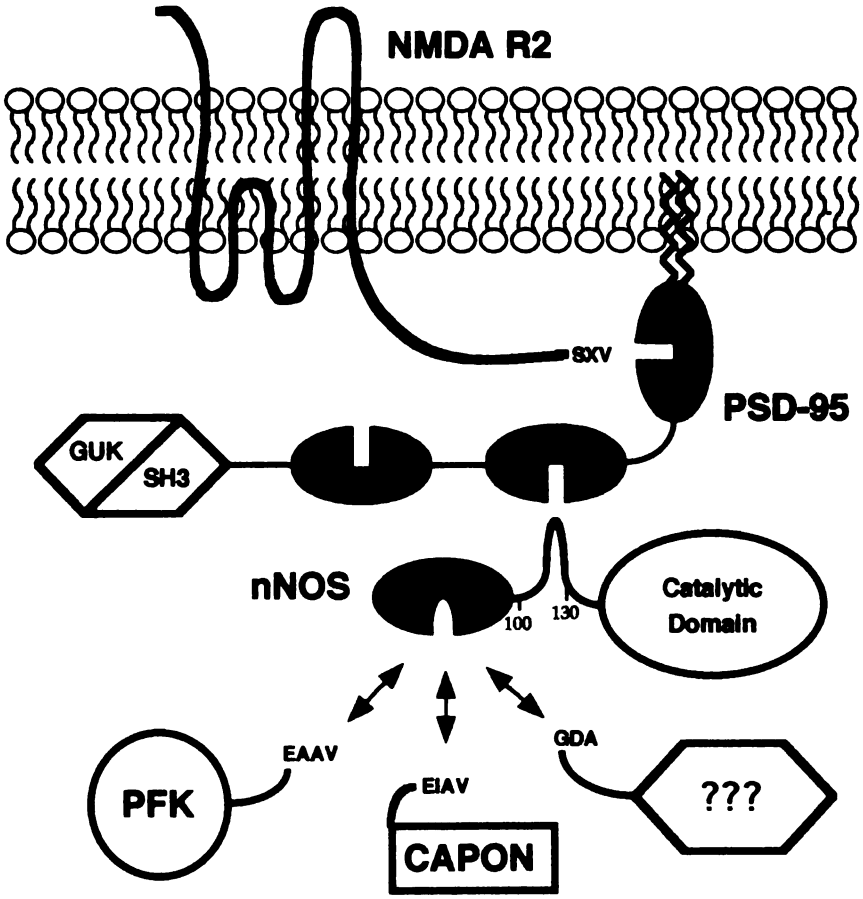

NMDA R2
SXV
PSD-95
GUK
SH3
nNOS
100
130
Catalytic
Domain
EAAV
PFK
EIAV
CAPON
GDA
???

Figure 5-6

Figure 5-6 Schematic model for protein complex assembly at the synaptic membrane. PSD-95 functions to bring nNOS to the NMDA receptor, allowing specific activation of nNOS in response to glutamate-induced calcium influx. This may be accomplished by one molecule of PSD-95. Binding of nNOS to PSD-95 leaves the nNOS PDZ domain peptide-binding pocket free to interact with other proteins such as CAPON (Jaffrey et al., 1998) and PFK (Firestein and Bredt, 1999). nNOS exists as a stable dimer (not pictured) which further increases the intricacy of molecules that can be brought into this complex through the nNOS PDZ domain.

CHAPTER SIX

PSD-95 Multimerization *In Vivo*

Summary

The observation that PSD-95 contains concatamerized repeats that bind to synaptic channels and receptors led to the hypothesis that it plays a role in the formation or maintenance of receptor clustering. Subsequently, experiments in heterologous cells demonstrated that the N-terminus of PSD-95 is crucial for clustering of cotransfected $K_V1.4$ and NMDA receptor subunits. This was proposed to be due to the formation of intramolecular disulfide bonds between pairs of cysteines located at the N-terminus (Hsueh et al., 1997). However, work form the Bredt lab showed that these cysteines are highly palmitoylated, offering an alternative explanation for their importance in PSD-95 function (Topinka and Bredt, 1998). Since palmitoylation can affect specific intracellular localization, it is not surprising that is also affects the ability of PSD-95 to bind $K_V1.4$ in heterologous cells [Topinka, 1998 #35. However, we also find that PSD-95 requires these palmitoylated cysteines in order to self-associate. Furthermore, only the first 13 amino acids of PSD-95 are required for coimmunoprecipitation from heterologous cells, arguing that the palmitoyl groups are sufficient for multimerization. This suggests that these groups either directly interact in the membrane or that they are coimmunoprecipitated together by associate with membrane lipids or other components that are not solubilized during extraction.

Results

Disulfide bond-formation does not account for PSD-95 association in heterologous cells

We were interested in the question of whether *in vivo* PSD-95 might be associated by a mechanism that was not relevant in an *in vitro* system. The only current hypothesis at the time was a study suggesting that intramolecular disulfide bonds led to the formation of tetramers that were detectable in brain extracts (Hsueh et al., 1997). We thought this was an unusual finding, and considered whether the disulfide bond-formation observed in this study might be due to oxidation of the extract after cell lysis. To test this, we made a crude brain extract and either immediately ran an aliquot on a non-denaturing gel or allowed it to incubate overnight in lysis buffer before the same analysis. We were unable to detect any multimerized PSD-95 in the freshly prepared sample. However, after incubation we observed a species of PSD-95 that appeared to the size of a tetramer (Figure 6-1), suggesting that post-lysis oxidation was indeed occurring, allowing intramolecular disulfide-bond formation between PSD-95 molecules *in vitro*.

Although the above result does not dismiss the possibility that a very small percentage of PSD-95 in brain may be oxidized at a given time, it indicated that it could not be the explanation for the robust coimmunoprecipitation reported between molecules of PSD-95 expressed in heterologous cells (Hsueh et al., 1997). We therefore considered an alternative mechanism, and we first addressed this by confirming that we were able to see association of PSD-95 monomers in this system. We cotransfected COS cells with

two full-length PSD-95 constructs that were differentially tagged on the C-terminus with either GFP or a FLAG epitope tag. We found that these two proteins are indeed efficiently coimmunoprecipitated (Figure 6-2*A*). Although we did not find any evidence for *in vivo* disulfide bond-formation, we knew from the experiment in Figure 6-1 that this can happen post-lysis, for instance while the extract is incubated in lysis buffer during the immunoprecipitation procedure. To confirm our hypothesis that this coimmuno-precipitation is not due to disulfide bond-formation, we performed the same immunoprecipitation experiment after first boiling the lysate for two minutes (Figure 6-2*B*). Although the disulfide bond is not heat labile, we found that this treatment completely abolished coimmunoprecipitation of these two differentially tagged PSD-95 molecules, suggesting that some other mechanism was responsible for their association.

Palmitoylation is required for PSD-95 association in heterologous cells

Considering these results, were wanted to verify that the N-terminal cysteine residues were required for PSD-95 multimerization, as had been reported previously (Hsueh et al., 1997). Indeed, when full-length PSD-95-GFP was mutated to serines at the two N-terminal cysteine residues, it was no longer able to associate with full-length wild-type PSD-95 (Figure 6-3). Since PSD-95 is palmitoylated at these sites (Topinka and Bredt, 1998), it was of interest to us to determine whether these cysteine residues are important in this assay because they are palmitoylated. The lab has recently identified point mutants at residues other that these cysteines in the PSD-95 N-terminus that abolish palmitoylation (A. E. El-Husseini and D. S. Bredt, manuscript submitted), and we tested

one of these (IVTT to TEIN) in the coimmunoprecipitation assay (see Figure 6-4*C*). Although the PSD-95-IVTT-GFP mutant protein retains the cysteines residues, it also is unable to coimmunoprecipitate with a wild-type PSD-95 partner (Figure 6-4*B*), suggesting it is palmitoylation that is the key requirement for *in vivo* association.

One reason that palmitoylation may be required for association is that PSD-95 palmitoylation mutants are differentially targeted in heterologous cells. Since many of these constructs are GFP-tagged, the distribution of the bulk of the protein can be directly visualized. Wild-type PSD-95 has very specific distribution, appearing to be targeted to a specific perinulcear structure within the cell (data not shown). The double-cysteine mutant, however, has a drastically different distribution, in that it is diffusely expressed throughout the cell. This difference in intracellular distribution is reflected in a crude fractionation experiment. Transfected cell lysates were lysed by homogenization in the absence of detergent and the cytosolic fraction separated by ultracentrifugation at 100,000 x *g*. The pellet was then sequentially extracted with 1% Triton X-100 followed by RIPA buffer containing 0.5% deoxycholate and 0.1% SDS. When samples of these fractions are analyzed by western blotting, almost all of the cysteine mutant-form (C35S) is present in the cytosol (Figure 6-5*A*). Conversely, almost all of the wild type PSD-95 protein is present in the Triton-extracted fraction, with almost none in the cytosolic fraction. In this experiment, these cells were also transfected with a shorter PSD-95 construct, 1-13 GFP, which contains only the N-terminal residues required for palmitoylation fused to GFP (A. E. El-Husseini and D. S. Bredt, manuscript submitted). The distribution of this fusion protein is the same as full-length wild-type (Figure 6-5*B*),

demonstrating that palmitoylation is sufficient to convey this fractionation behavior on a heterologous protein.

Palmitoylation is sufficient for PSD-95 association in heterologous cells

Although we had been unable to detect multimerization between PSD-95 molecules expressed in bacteria, heterologous mammalian cells, or from brain extracts, it was formally possible that coimmunoprecipitation was occurring indirectly through an "adaptor"-like protein in these experiments. We therefore sought to determine the minimal region of PSD-95 required for multimerization. It had been published that a fusion protein containing only the first 64 residues of PSD-95 was sufficient for this association (Hsueh et al., 1997), which still leaves room for a protein-protein interaction motif that could lie somewhere in that region. We therefore tested a fusion protein that contains only the first thirteen residues of PSD-95, which is the minimum motif required for palmitoylation (see above). This construct efficiently associated with full length PSD-95 (Figure 6-6*A*), demonstrating that it is unlikely that there are any other protein-protein interaction sites that participate in this self-association. In addition, a truncated PSD-95 protein that contains only residues from the very N-terminus through PDZ 2 also coimmunoprecipitated with PSD-95 1-13-GFP (Figure 6-6*B*).

Discussion

In these experiments, we have addressed the mechanism for multimerization of PSD-95 *in vivo*, observed by coimmunoprecipitation of differentially-tagged PSD-95 molecules expressed in heterologous cells. As shown in Chapter 5, this association can not be due to direct binding between protein domains of PSD-95. Although the two N-terminal cysteines at positions 3 and 5 are required for this association, we find that this is not due to intramolecular disulfide bond-formation between these residues. Instead, it seems that these cysteines are required for coimmunoprecipitation because they are palmitoylated in heterologous cells, as well as in brain. In fact, we find that only the palmitoylation motif (residues 1-13) of PSD-95 is required for coimmunoprecipitation, suggesting that the mechanism of association does not involve a protein-protein interaction interface but only the palmitoyl group.

Our finding that palmitoylation is required for this multimerization is not surprising considering the effect of this lipid modification on the intracellular distribution of PSD-95. We analyzed the distribution of PSD-95, both biochemically and by visualization of GFP-tagged constructs, and both the cysteine mutant presented in this chapter and a palmitoylation mutant (A. E. El-Husseini and D. S. Bredt, manuscript submitted), are diffusely expressed in COS cells. This is in marked contrast to the wild type protein, which is found in a distinct perinuclear region and is not present in the cytosolic fraction of cell lysates. These observations also explain previous data from the lab (Topinka and Bredt, 1998), indicating that the cysteine mutant form of PSD-95 does

not coimmunoprecipitate with the potassium channel $K_V1.4$, although it binds the channel just as well as wild type PSD-95 *in vitro*. It was suggested that these two proteins needed to be targeted to the same intracellular location in order to interact, and that the cysteine mutations disrupt proper targeting due to lack of palmitoylation.

Therefore, in addition to affecting the *in vivo* association of PSD-95 and $K_V1.4$, palmitoylation also affects multimerization of PSD-95 in heterologous cells. In fact, at this point in time the *only* function that has been identified for PSD-95 with respect to channel localization or clustering has been its ability to cluster $K_V1.4$ and the NMDA receptor into large plasma membrane aggregates in COS cells (Kim et al., 1996; Kim et al., 1995). Our data is consistent with the idea that palmitoylation is required for clustering because it is required for *both* colocalization of PSD-95 and $K_V1.4$ to the same intracellular compartment, and also for PSD-95 multimerization, which is necessary for the formation of large clusters containing these proteins.

We have provided evidence that the palmitoyl groups themselves are the only requirement for two molecules of PSD-95 to coimmunoprecipitate. This leads to the question of the mechanism of association. Many palmitoylated proteins have been intensely studied because they are targeted to specific plasma membrane microdomains that are enriched in signaling proteins [reviewed in (Brown and London, 1998; Casey, 1995; Simons and Ikonen, 1997)]. These microdomains are also enriched in certain lipids, namely sphingolipids (glycolipids and sphingomyelin) and cholesterol, and have been purified from cells based on their detergent insolubility in Triton X-100. These detergent-resistant membranes (DRMs) have been postulated to be in a liquid-ordered state (l_o) based on biophysical experiments with model lipid bilayers (Brown and London,

1998). It has been suggested that the higher T_m of these lipids causes a phase separation in the membrane, such that these lipids are brought together into microdomains where they exist in this l_o state, in between the fluid liquid-disordered state (l_d) of the bulk of the membranes and the liquid-crystalline (l_c) state that lipid bilayers adopt at low temperatures.

It is thought that saturated acyl chains, such as palmitoyl groups, prefer this ordered environment and are thus preferentially associated with these microdomains. This association with an ordered domain would also be more favored as the temperature is lowered, and in fact the standard protocol for purification of DRMs requires extraction at 4^0C. These DRMs have been proposed to represent purified caveolae, plasma membrane invaginations that are visible by EM. This is based on the fact that the protein marker for caveolae, caveolin, is enriched in DRMs, as are other proteins that have been localized to caveolae by immunomicroscopy. In fact, DRMs probably contain certain other intracellular membranes, in addition to plasma membrane caveolae, that have similar biochemical characteristics.

PSD-95 has also been shown to be enriched in the "caveolar" fraction (more precisely DRMs) (Perez and Bredt, 1998). This fraction is purified biochemically by centrifugation of a Triton X-100 extract through a sucrose gradient, after which caveolin is found in the low density or "floating" membrane fraction. This suggests that PSD-95 is also targeted to specific membrane microdomains. The fact that the bulk of GFP-tagged PSD-95 appears localized to a perinuclear compartment is not inconsistent with this idea, as a significant amount of caveolin, as well as sphingolipids and cholesterol, are also found in the Golgi and other membrane components of the endosomal pathway

(Brown and London, 1998). This suggests that when PSD-95 is expressed in heterologous cells, it is targeted to microdomains in the perinuclear regions by the presence of the N-terminal palmitoyl groups, and that multimerization is occurring by virtue of association with these microdomains.

Our current hypothesis is that PSD-95 is being coimmunoprecipitated by virtue of its presence in these lipid microdomains, or "rafts, in which multiple molecules are associated with DRMs that have not been fully solubilized. The fact that these proteins can be extracted with buffer containing Triton X-100 is consistent, since the protocol for DRM preparation involves this same extraction as a first step, although DRM proteins are clearly not fully solubilized by this extraction. There are other examples of proteins that exist in DRMs that are not pelleted by detergent extraction and ultracentrifugation, but are still associated with DRM lipid (Brown and London, 1998; Naslavsky et al., 1997). Furthermore, in a study of the N-terminus of G_i, which is also dually acylated, the first 32 residues were sufficient for coimmunoprecipitation with the acylated caveolin protein from detergent extracts (Galbiati et al., 1999), suggesting that other acylated proteins can be coimmunoprecipitated by virtue of their association with DRMs. Further experiments need to be done to address the role of lipid membrane microdomain association in the proper synaptic targeting and function of PSD-95 in neurons.

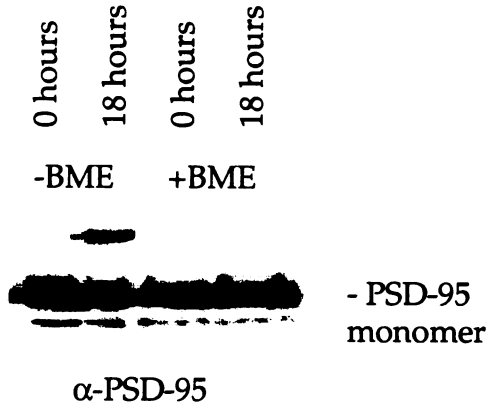

0 hours
18 hours
0 hours
18 hours
-BME
+BME
- PSD-95
monomer
α-PSD-95

Figure 6-1

Figure 6-1 PSD-95 from rat brain is not significantly disulfide-bonded.

Rat brain was homogenized in TEE buffer and either frozen immediately in liquid N_2 or incubated for 18 hours at 4°C before separation by SDS-PAGE in the presence or absence of BME. Samples were analyzed by western blotting using an antibody to PSD-95. A disulfide-bonded PSD-95 multimer appears over time when sample is exposed to oxidizing conditions but is not present immediately after homogenization.

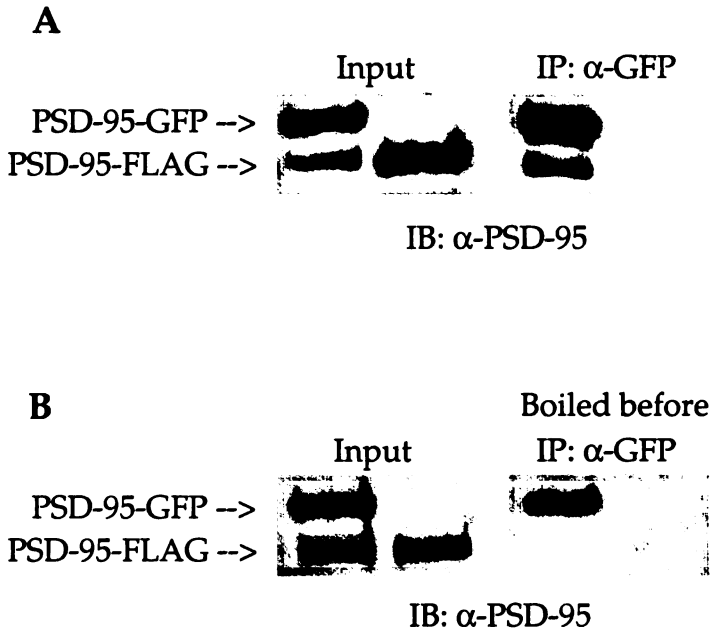

A
Input
IP: α-GFP
PSD-95-GFP -->
PSD-95-FLAG -->
IB: α-PSD-95
B
Boiled before
Input
IP: α-GFP
PSD-95-GFP -->
PSD-95-FLAG -->
IB: α-PSD-95

Figure 6-2

Figure 6-2 PSD-95 coimmunoprecipitation with itself in heterologous cells is not due to disulfide bonding.

A. COS cells were cotransfected with expression vectors encoding PSD-95-FLAG and PSD-95-GFP or PSD-95-FLAG alone. Cell lysates were immunoprecipitated with an antibody to GFP and analyzed by western blotting for the presence of PSD-95.

B. Same as in (A) except the COS cell lysate was subjected to boiling for two minutes prior to immunoprecipitation. Boiling destroys self-association of the PSD-95 molecules.

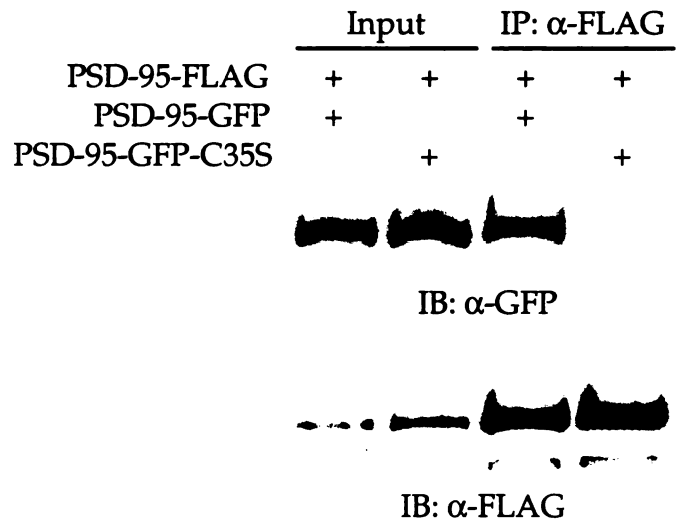

Input
IP: α-FLAG
PSD-95-FLAG + + + +
PSD-95-GFP + +
PSD-95-GFP-C35S + +
IB: α-GFP
IB: α-FLAG

Figure 6-3

Figure 6-3 N-terminal cysteine residues are required for multimerization.

COS cells were cotransfected with expression vectors encoding PSD-95-FLAG and PSD-95-GFP or PSD-95-FLAG and PSD-95-GFP-C35S. Cell lysates were immuno-precipitated with an antibody to FLAG and analyzed by western blotting for the presence of PSD-95-FLAG or PSD-95-GFP.

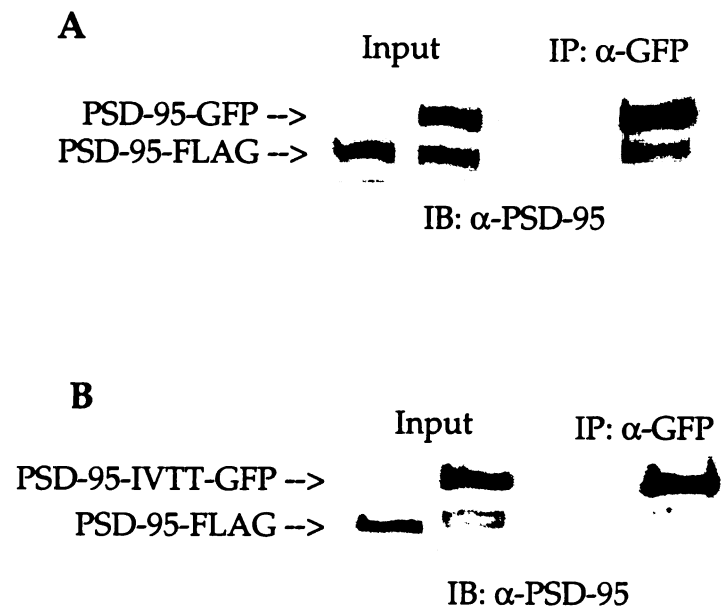

A
Input
IP: α-GFP
PSD-95-GFP -->
PSD-95-FLAG -->
IB: α-PSD-95
B
Input
IP: α-GFP
PSD-95-IVTT-GFP -->
PSD-95-FLAG -->
IB: α-PSD-95
C
PSD-95 N-terminus
NH2-Met-Asp-Cys-Leu-Cys-Ile-Val-Thr-Thr-Lys-Lys-Tyr-Arg

Figure 6-4

Figure 6-4 Palmitoylation mutant is defective in multimerization.

A. and B. COS cells were cotransfected with expression vectors encoding PSD-95-FLAG and PSD-95-GFP (A) or PSD-95-FLAG and PSD-95-GFP-IVTT (B). Cell lysates were immuno-precipitated with an antibody to GFP and analyzed by western blotting for the presence of PSD-95-FLAG or PSD-95-GFP.

C. The N-terminus of PSD-95. The palmitoylated cysteine residues are in bold.

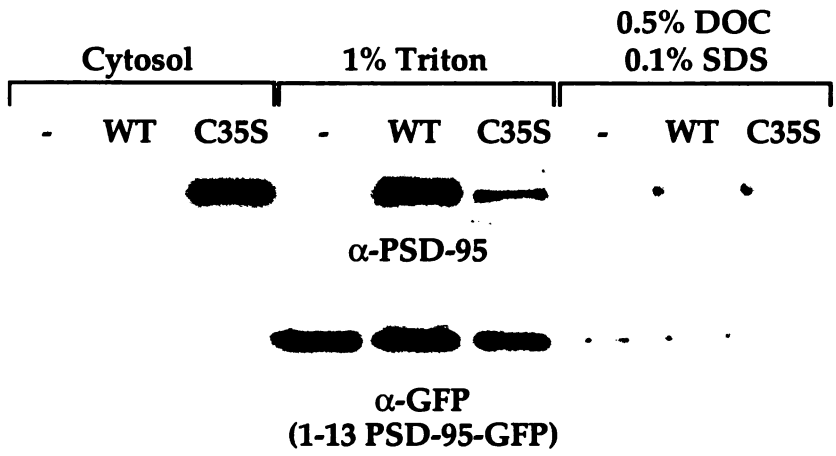

Cytosol
1% Triton
0.5% DOC
0.1% SDS
- WT C35S - WT C35S - WT C35S
α-PSD-95
α-GFP
(1-13 PSD-95-GFP)

Figure 6-5

Figure 6-5 Palmitoylation affects the intracellular distribution of PSD-95.

COS cells were cotransfected with either full-length wild-type PSD-95 or full-length PSD-95 in which the two N-terminal cysteines are mutated to serines (C35S), and a fusion protein containing the first thirteen residues of PSD-95 fused to GFP (1-13 GFP). Cells were lysed by homogenization in detergent-free buffer, and the cytosolic fraction was separated by ultracentrifugation. The pellets were sequentially extracted with 1% Triton X-100, and then RIPA buffer containing 0.5% deoxycholate and 0.1% SDS. Fractions were analyzed by western blotting for the presence of full-length PSD-95 protein (A) or 1-13 GFP (B).

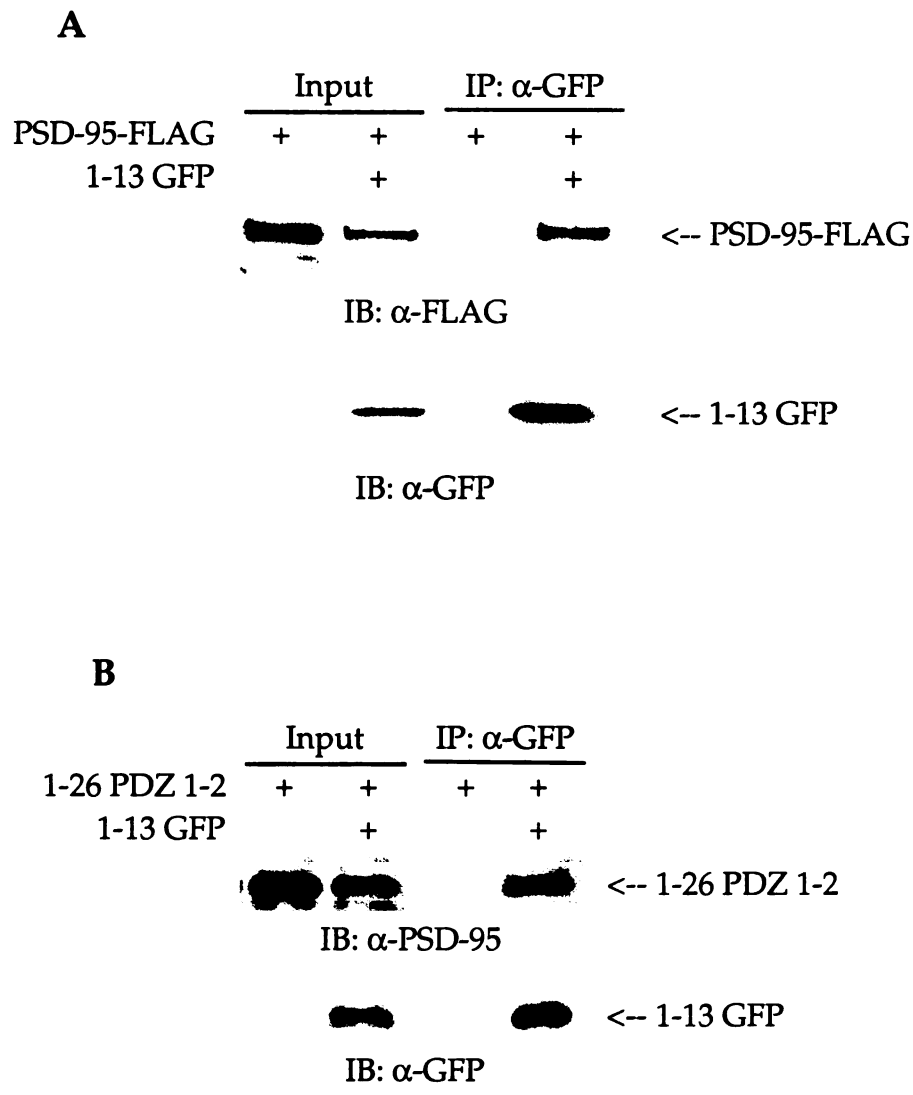

A
Input
IP: α-GFP
PSD-95-FLAG + + + +
1-13 GFP + +
<-- PSD-95-FLAG
IB: α-FLAG
<-- 1-13 GFP
IB: α-GFP
B
Input
IP: α-GFP
1-26 PDZ 1-2 + + + +
1-13 GFP + +
<-- 1-26 PDZ 1-2
IB: α-PSD-95
<-- 1-13 GFP
IB: α-GFP

Figure 6-6

Figure 6-6 Amino acids 1-13 in PSD-95 are sufficient for multimerization

COS cells were cotransfected with expression vectors encoding PSD-95-FLAG and 1-13-GFP (A) or 1-26 PDZ 1-2 and 1-13-GFP (B). Cell lysates were immunoprecipitated with an antibody to GFP and analyzed by western blotting for the presence of the indicated proteins.

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