UCSF
UC San Francisco Electronic Theses and Dissertations

Title

Biochemical and structural analysis of the SH3GK module from the synaptic scaffolding protein PSD-95

Permalink

https://escholarship.org/uc/item/4b17n499

Author

McGee, Aaron W.

Publication Date

2001

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

Biochemical and Structural Analysis of the SH3GK Module from the Synaptic Scaffolding Protein PSD-95

by

Aaron W. McGee

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Copyright (2001)
by
Aaron W. McGee

Acknowledgements

This dissertation is the product of numerous discussions with my advisor, thesis committee members, and classmates, and hours of labor contributed by my collaborators, especially Srikanth Dakoji and Ken Prehoda.

Foremost, I am indebted to my advisor David Bredt. David is a scientist of remarkable enthusiasm and scholarship. He has guided my scientific development by both suggestion and example. During our meetings he has been a whirlwind of focus, determination and insight, but at the bench he is nothing less than a hurricane. I'm grateful for the time and energy he has expended to improve both my scientific capacity and philosophy. Accordingly, I would also like to thank Roger Nicoll, Lily Jan and Henry Bourne.

The members of the Bredt lab, past and present, deserve thanks and consolation. Specifically, I would like to recognize to Jay Brenman, who was unfortunate enough to sit across the bench from me in the 'early days', and Srikanth Dakoji, who endured the difficult task of working with me on protein purification strategies. The lab environment has been beyond reproach.

I owe much to my classmates: Chris Patil, Steve McCarroll, Oren Beske, and Sharif Taha. These gentlemen made the experience. I would like to extend a special thanks to Lynn Almli for her friendship. Last, I'm grateful to my parents for their support and encouragement and to my brother Bryan for excursions to snow and sand as well as valuable, anytime, road-trip philosophical discourse.

Preface

The material presented as Chapters 1 and 2 in this thesis is published. Chapter 1, 'Identification of an Intramolecular Interaction between the SH3 and Guanylate Kinase Domains of PSD-95', was published in *The Journal of Biological Chemistry* (1999) 274, 17431-17436. Chapter 2, 'Structure of the SH3-Guanylate Kinase-like Module from PSD-95 Suggests a Mechanism for Regulated Assembly of MAGUK Scaffolding Proteins', was published in *Molecular Cell* (2001) 8, 1291-1301.

BERKELEY • DAVIS • IRVINE • LOS ANGELES • RIVERSIDE • SAN DIEGO • SAN FRANCISCO

SANTA BARBARA • SANTA CRUZ

DEPARTMENT OF PHYSIOLOGY
SCHOOL OF MEDICINE
(415) 476-6310
(415) 476-4929 (FAX)
EMAIL BREDT@PHY.UCSF.EDU

ROOM S-859
513 PARNASSUS AVENUE
SAN FRANCISCO, CALIFORNIA 94143-0444

December 19, 2001

Editor, *The Journal of Biological Chemistry*
Permissions
9650 Rockville Pike
Bethesda, MD 20814

To Whom It May Concern:

I would like to request permission to include in my thesis dissertation the paper cited below:

McGee, A. W., and Bredt, D. S. (1999). Identification of an intramolecular interaction between the SH3 and guanylate kinase domains of PSD-95. Journal of Biological Chemistry 274, 17431-6.

The dissertation will be microfilmed by University Microfilms Incorporated, and they request permission to supply single copies upon demand. Please respond to FAX to (415) 476-4929.

Sincerely,

Aaron W. McGee

PERMISSION GRANTED
contingent upon obtaining that of the author

for the copyright owner
THE AMERICAN SOCIETY FOR BIOCHEMISTRY
& MOLECULAR BIOLOGY

DEC 29 2001

ELSEVIER
SCIENCE

Aaron McGee
UCSF
Department of Physiology
University of California – San Francisco
513 Parnassus Box # 0444
San Francisco, CA 94143-0444
USA

Elsevier Science

Global Rights
PO Box 800
Oxford OX5 1DX
England

Tel: +44 (0) 1865 843830
Fax: +44 (0) 1865 853333
E-mail: permissions@elsevier.co.uk
Home Page: www.elsevier.com

Dear Mr McGee

MOLECULAR CELL, Vol 8, No 6, 2001, pp 1291-1301, "Structure of the SH3..."

As per your letter dated 10 January 2002, we hereby grant you permission to reprint the aforementioned material at no charge **in your thesis** subject to the following conditions:

1. If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies.

2. Suitable acknowledgment to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:

"Reprinted from Publication title, Vol number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier Science".

3. Reproduction of this material is confined to the purpose for which permission is hereby given.

4. This permission is granted for non-exclusive world **English** rights only. For other languages please reapply separately for each one required. Permission excludes use in an electronic form. Should you have a specific electronic project in mind please reapply for permission.

5. This includes permission for UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission.

Yours sincerely

Helen Wilson

Frances Rothwell (Mrs)
Global Rights Manager

Your future requests will be handled more quickly if you complete the online form at www.elsevier.com

Imprints:
Elsevier
Pergamon
North-Holland
Excerpta Medica

TOTAL P.01

Biochemical and Structural Analysis of the SH3GK Module from the Synaptic Scaffolding Protein PSD-95

by

Aaron W. McGee

Lily Y. Jan
Chair, Thesis Committee

Abstract

Synaptic transmission requires the proper assembly of complex signal transduction networks at specialized plasma membrane domains. The proteins and mechanisms that contribute to the establishment, maintenance, and plasticity of the synapse are subjects of intense study. Identification and characterization of proteins enriched in the postsynaptic density has provided insight into synaptic development and function. The membrane-associated guanylate kinase PSD-95, which comprises three PDZ domains, an SH3 domain and a guanylate kinase-like (GK) domain, binds neurotransmitter receptors, cytosolic signaling proteins and cytoskeletal elements within the postsynaptic density. Recent studies have revealed that an amino-terminal lipid moiety and the PDZ domains are required to target PSD-95 to synapses, but the functions

of this proposed scaffolding molecule are not clear. Evidence from invertebrate genetic systems suggests a critical role for the SH3 and GK domains.

This work presents a biochemical and structural characterization of the SH3GK region of PSD-95. The SH3 and GK domains appear to bind intramolecularly; but the interaction is also observed between protein fragments intermolecularly *in vitro*. The SH3GK structure reveals that this interaction does not involve binding between intact, modular, domains but rather represents the assembly of a 'split' SH3 fold. The PSD-95 SH3 domain is unique; the primary amino acid sequences that contribute to the SH3 fold are not contiguous but form two subdomains separated by an insertion: a 'core' subdomain and a pair of β-strands flanking the GK domain. The two subdomains can interact intramolecularly or intermolecularly to complete the SH3 fold. Rather than binding canonical SH3 ligands, one function of the SH3 fold in PSD-95, and other MAGUKs, may be to reassemble intermolecularly. We propose a model whereby regulatory proteins bind the SH3GK module to promote intermolecular assembly of the SH3 fold to generate interlocked MAGUK networks. The structure of the SH3GK region of PSD-95 provides one of the first examples of how normally discrete, modular, protein domains have evolved into an integrated module.

Table of Contents

Acknowledgements iii

Preface iv

Abstract vii

Table of Contents ix

List of Tables and Figures x

Introduction: **Synapses and the postsynaptic density** 1

Chapter One: **Identification of an intramolecular interaction between the SH3 and Guanylate Kinase Domains of PSD-95** **12**

Summary 13

Results 14

Discussion 19

Table and Figures 22

Chapter Two: **Structure of the SH3-Guanylate Kinase-like Module from PSD-95 Suggests a Mechanism for Regulated Assembly of MAGUK Scaffolding Proteins** **34**

Summary 35

Results and Discussion 36

Conclusion 45

Table and Figures 47

Experimental Procedures 62

References 72

List of Tables and Figures

Chapter One

Table 1-1: Structural requirements for the PSD-95 SH3/GK domain interaction 22

Figure 1-1: Interaction of the SH3 and GK domains of PSD-95 24

Figure 1-2: The SH3 / GK domain interactions is conserved between PSD-95 and DLG, but is not detectable with LIN-2 26

Figure 1-3: An intramolecular SH3 / GK domain interaction within PSD-95 prevents similar intermolecular interactions 28

Figure 1-4: Immunoprecipitations from whole brain homogenate and transfected HEK 293 cells 30

Figure 1-5: Co-immunoprecipitation of GKAP is unaffected by the SH3 mutation L460P 32

Chapter Two

Table 1: Data Collection and Refinement Statistics 47

Figure 2-1: Structure of the SH3GK module of PSD-95 48

Figure 2-2: Sequence alignment of SH3GK modules from MAGUKs 50

Figure 2-3: The PSD-95 GK domain structurally resembles apo-yeast guanylate kinase but does not bind GMP 52

Figure 2-4: Comparison of the PSD-95 split SH3 structure with a canonical SH3 domain structure 54

Figure 2-5: The SH3-GK interaction involves reassembly of the split SH3 fold 56

Figure 2-6: The Hinge region is variable and can modulate inter- vs. intramolecular SH3 assembly 58

Figure 2-7: Model for role of SH3GK module in regulated assembly - ligand binding to the hinge region may promote oligomerization by 3D-domain swapping 60

Introduction

Synapses and the Postsynaptic Density

UCSF LIBRARY

Efficient and specific intercellular signaling requires assembly of complex signal transduction networks at specialized plasma membrane domains. This organization is exemplified in the central nervous system (CNS), where neurotransmitter receptors and associated downstream signaling elements are concentrated at synapse. Morphologically, synapses are characterized by a presynaptic accumulation of synaptic vesicles, the close apposition of presynaptic and postsynaptic membranes, and the presence of an electron-dense submembraneous structure (Peters et al., 1976). Excitatory synapses generally have an asymmetric thickening at the synaptic junction that is larger beneath the postsynaptic membrane (Carlin et al., 1980; Peters et al., 1976). This structure was initially termed the 'subsynaptic web' but later referred to as the 'postsynaptic thickening' or 'postsynaptic density' (PSD) (Ziff, 1997). The PSD, adjacent to the postsynaptic membrane, is uniquely positioned to couple chemical signals received from the presynaptic cell by postsynaptic transmembrane receptors to cytosolic signal transduction cascades and second messenger systems. The PSD also may contribute to the structural integrity of the synapse, perhaps helping to hold presynaptic and postsynaptic elements in register. Thus, in an effort to determine the synaptic constituents and mechanisms that contribute to the establishment, maintenance and plasticity of synaptic transmission, many proteins in the PSD have been identified and characterized (Ziff, 1997).

Postsynaptic densities can be isolated by biochemical fractionation (Carlin et al., 1980; Cohen et al., 1977). Characterization of the proteins in this fraction by electrophoresis, immunoblotting and peptide sequencing has revealed that a number of enzymes, cytoskeletal proteins, receptors and channels are components of the PSD

(Walsh and Kuruc, 1992). Subsequent work has determined that many proteins that are enriched in the PSD preparation also have a punctate distribution indicative of synaptic localization (Walikonis et al., 2000; Ziff, 1997). The major components of the PSD fraction include the enzyme Ca^{2+}-calmodulin dependent protein kinase II (CamKII) (Kennedy et al., 1983), the 2A/B subunits of the N-methyl-D-aspartate (NMDA) responsive subclass of ionotropic glutamate receptors (NMDARs) (Moon et al., 1994; Nusser et al., 1994; Walikonis et al., 2000), and the cytoskeleton-associated proteins spectrin and α-actinin (Walsh and Kuruc, 1992; Wyszynski et al., 1998; Wyszynski et al., 1997). Similar studies have reported that a number of kinases, phosphatases, and G-proteins are also in the PSD (Walikonis et al., 2000; Ziff, 1997). Detergent extraction of the PSD fraction with the mild anionic detergent Triton X-100 followed by the ionic detergent Sarkosyl (N-lauroyl sarcosinate) strips away the majority of the membrane-associated proteins, leaving a 'core' of more detergent insoluble proteins (Cho et al., 1992). The remaining proteins include the cytoskeletal proteins tubulin, actin and fodrin, as well as the α and β subunits of CAMK II. Recently, two groups utilized mass spectroscopy to identify other proteins present in the PSD (Walikonis et al., 2000) and proteins associated with NMDARs (Husi et al., 2000). These experiments yielded over thirty proteins, many of which have unknown synaptic functions. This analysis confirmed that members of a growing family of proposed scaffolding proteins are found both at the PSD and associated with NMDARs. These proteins, alternatively termed postsynaptic density proteins (PSD) and synapse-associated proteins (SAP), are some the first identified membrane associated guanylate kinase-like molecules (MAGUKs) (Gomperts, 1996). Postsynaptic density 95kD (PSD-95) (Cho et al., 1992), also known as synapse-

associated protein 90kD (SAP90) (Kistner et al., 1993), was the first MAGUK identified in the PSD. Other MAGUKs present in the PSD include SAP102 (Muller et al., 1996) and PSD-93 (Brenman et al., 1996), also known as Chapsyn-110 (Kim et al., 1996).

MAGUKS comprise multiple protein-protein interaction domains. They contain one or three PDZ domains, an SH3 domain and a region of homology to yeast guanylate kinase (GK) (Woods and Bryant, 1993). PDZ domains were identified as homology regions between three MAGUKs, PSD-95, the *Drosophila* protein Discs Large and the tight junction component Zona Occludens-1 (ZO-1). Also referred to as discs large homology regions (DHR) and GLGF repeats, these small (80 to 100 amino acid) domains bind the cytosolic carboxylate termini (C-termini) of proteins that contain one of several short (3 amino acid) consensus sequences (Kornau et al., 1995; Sheng and Sala, 2001) but also bind to PDZ domains that contain a unique structural extension termed a 'beta finger' (Brenman et al., 1996; Christopherson et al., 1999; Hillier et al., 1999). PDZ domains are a characteristic feature of MAGUKs but also are present in a variety of signaling and other cytoskeletal proteins (Sheng and Sala, 2001). SH3 domains are modular protein interaction domains that typically bind to proteins containing a poly-proline motif that forms a type II left-handed helix (Feng et al., 1994; Kohda et al., 1994; Lim et al., 1994; Musacchio et al., 1994; Wittekind et al., 1994; Yu et al., 1994). In the crystal structures of some cytosolic tyrosine kinases, SH3 domains have been observed to bind intramolecularly to regions that form this same helical structure (Sicheri et al., 1997; Xu et al., 1997). SH3 domains are present in a diverse array of signaling proteins (Birge et al., 1996). Guanylate kinase domains are unique to MAGUKs and a related protein, S-SCAM/MAGI-1 (Dobrosotskaya et al., 1997; Hirao et al., 1998). These domains bear

homology to the enzyme guanylate kinase, which phosphorylates GMP to GDP, but is not catalytically active in PSD-95 and most other MAGUKs (Hoskins et al., 1996; Kistner et al., 1995; Kuhlendahl et al., 1998). Instead, this domain appears to be yet another protein binding domain of MAGUK proteins.

PSD-95 interacts with many different synaptic proteins through its multiple protein binding domains. Neuronal nitric oxide synthase (nNOS) was one of the first proteins shown to interact with the second PDZ domain of PSD-95 (Brenman et al., 1996). The carboxylate terminus of the NMDAR 2A receptor also binds to this domain (Kornau et al., 1995), as do a number of other transmembrane proteins that end with the type I PDZ consensus sequence S/T-X-V (Sheng and Sala, 2001; Songyang et al., 1997), where the last amino acid residue is a valine, the preceding residue can be any amino acid, and the 'minus two' position is serine or threonine. These last three residues are critical for binding but the ligand specificity for PDZ domains is, in part, dependent upon the more amino-terminal residues (Songyang et al., 1997). This specificity is exemplified by the observation that phosphorylation of the K^+ channel Kir 2.3 by protein kinase A (PKA) on the serine at this 'minus three' position in the consensus sequence disrupts channel binding to PSD-95 (Cohen et al., 1996). Many proteins that bind the PDZ domains of PSD-95 interact with both the first and second PDZ domains *in vitro*. The third PDZ domain of PSD-95 also interacts with identified components of the PSD, such as SynGAP (Kim et al., 1998), the cell adhesion protein neuroligin (Irie et al., 1997) and the neuronal Rho-GDP/GTP exchange factor Kalirin-7 (Penzes et al., 2001). The SH3 domain has been reported to bind to the KA2 subunit of the kainate responsive subclass of glutamate receptors (Garcia et al., 1998). A pair of proline rich regions within the C-

DUKE LIBRARY

terminal tail of this receptor subunit can interact *in vitro* with the SH3 domains. However, surprisingly few other ligands have been identified which bind to the SH3 domain of MAGUKs (Bellaïche et al., 2001; Maximov et al., 1999). The KA2 subunit also interacts with the GK domain of PSD-95, but this interaction has not been characterized. Similarly, the PKA anchoring protein AKAP79/150 interacts with both the SH3 and GK domains of PSD-95 (Colledge et al., 2000). The GK domain interacts with MAP1a (Brenman et al., 1998), the RapGAP SPAR (Pak et al., 2001), the kinesin-like protein GAKIN (Hanada et al., 2000) and a family of proteins termed guanylate kinase associated proteins (GKAPs, or SAPAPs or DAPs) (Kim et al., 1997; Satoh et al., 1997; Takeuchi et al., 1997). GKAPs are present in the 'core' PSD but have not been described functionally other than they have been reported to recruit PSD-95 into a detergent resistant fraction in co-transfected heterologous cells (Deguchi et al., 1998).

To determine where PSD-95 may function, several approaches have been used to identify its expression profile and subcellular localization. PSD-95 is expressed almost exclusively in the brain and spinal cord (Brenman et al., 1996; Cho et al., 1992). Immunohistochemical analysis of primary neuronal cultures (Rao et al., 1998) and brain sections in conjunction with *in situ* hybridization (Brenman et al., 1996) reveal that PSD-95 localizes to synapses. Higher resolution immunoelectron microscopic analysis of cortical sections suggests that PSD-95 is predominantly beneath the postsynaptic membrane (Hunt et al., 1996) but can be presynaptic, such as at the pinceaus of basket cells in the cerebellum (Kim et al., 1995; Kistner et al., 1993). Transfection of primary hippocampal neurons and hippocampal neurons in slice cultures with expression constructs containing PSD-95 fused to green fluorescent protein (GFP) has confirmed

UCSF LIBRARY

these findings, as the PSD-95-GFP fusion proteins have a somatodendritic, punctate distribution that colocalizes with synaptic markers (Arnold and Clapham, 1999; Craven et al., 1999). The MAGUKs PSD-93, SAP97 and SAP102 are also enriched in the CNS but are expressed in the periphery in various polarized cell types, including lung and intestinal epithelia (Brenman et al., 1996; Makino et al., 1997; Muller et al., 1995). Parallel localization studies of MAGUKS suggest that these proteins are often localized at sites of cell-cell contact or are asymmetrically distributed in polarized cells (Fanning and Anderson, 1999).

Most of the evidence for the functions of MAGUKs comes from genetic studies in invertebrates. The *Drosophila discs large (dlg)* gene encodes the homologue of PSD-95 (Woods and Bryant, 1989), and *lin-2* encodes a related MAGUK in *C.elegans* (Hata et al., 1996; Hoskins et al., 1996). *Dlg* was identified in a 'tumor suppressor' screen for fly mutants that affect viability (Murphy, 1974; Stewart et al., 1972). In *dlg* mutant flies, the epithelial layer that ensheaths the imaginal discs loses polarity and proliferative control. In addition to defects in the development of imaginal discs, *dlg* mutant flies have abnormal neuromuscular synapse development (Budnik et al., 1996; Guan et al., 1996; Lahey et al., 1994). *Dlg* is expressed in both the motor neuron and developing muscle, where it is concentrated at the developing postsynaptic membrane, the subsynaptic reticulum (SSR). Loss of *dlg* decreases the elaboration of the SSR during development and increases synaptic currents (Budnik et al., 1996). In addition, transmembrane proteins that interact with the PDZ domains of DLG, such as the Shaker K^+ channel and the cell adhesion protein Fasciclin II, are mislocalized in the more severe *dlg* alleles (Tejedor et al., 1997). Transgenic studies have confirmed that the localization of these

proteins is dependent upon *dlg*, as chimeric transmembrane proteins that express only the C-terminal tail of the Shaker protein that contains the PDZ binding consensus sequence localize to the SSR in wild type but not *dlg* mutant flies (Zito et al., 1997). Mutant flies homozygous also lack or have a reduced number of septate junctions, the invertebrate analog of tight junctions, between epithelial cells of the imaginal discs and salivary gland (Woods et al., 1996). In *C.elegans*, *lin-2* promotes the activity of a receptor tyrosine kinase, *let-23*, that directs the development precursor cells to form the nematode vulva (Kaech et al., 1998). In *lin-2* mutants, the *let-23* receptor no longer localizes to the intercellular junctions between the vulval precursor cells, and vulva development is aberrant.

Experiments *in vitro* have demonstrated that the co-expression of Kv1.4, a potassium channel similar to the *Drosophila Shaker*, with PSD-95 in heterologous cells clusters the channel into large aggregates on the plasma membrane (Kim et al., 1995). This clustering activity of MAGUK proteins is dependent upon PDZ binding. In similar experiments, PSD-95 and PSD-93, have been observed to cluster NMDARs (Kim et al., 1996) and the AMPA receptor trafficking molecule stargazin (Chen et al., 2000). In mice genetically altered to express an NMDAR 2A subunit that lacks the C-terminus containing the PDZ binding motif, these truncated receptors were less concentrated at synaptic sites (Mori et al., 1998; Steigerwald et al., 2000) but see (Sprengel et al., 1998). However, 'knock-out' mice which fail to express PSD-93 (McGee et al., 2001), or express low levels of a truncated fragment of PSD-95 (Migaud et al., 1998), appear to have normal synaptic morphology and excitatory synaptic transmission. Interestingly, the PSD-95 'knock-out' mice have a deficiency in synaptic plasticity; they exhibit non-

USF LIBRARY

saturating hippocampal long term potentiation (LTP), lack long term depression (LTD) and fail certain spatial learning tasks (Migaud et al., 1998). Together, these findings suggest that either more than one neuronal MAGUK can localize these receptors to synaptic sites or that MAGUKs, while competent to bind NMDARs and other channels, may be involved in some aspect of scaffolding receptors to downstream signaling components but are not required for synaptic targeting of interacting proteins. This appears to be the case for nNOS, which is specifically responsive to Ca^{2+} influx through NMDA receptors (Bredt and Snyder, 1990; Garthwaite et al., 1988; Kiedrowski et al., 1992). Anti-sense oligonucleotide inhibition of PSD-95 protein expression in primary neuronal cultures diminishes the production of nitric oxide in response to NMDA treatment, although both nNOS and NMDA receptors remain expressed at wild-type levels (Sattler et al., 1999).

The scaffolding function of PSD-95 may contribute to the formation and maintenance of excitatory synapses. Immunohistochemical analysis of primary cultures indicates NMDA receptors and PSD-95 are some of the first proteins detected at sites, which later form synapses, as determined by colocalization with presynaptic markers (Rao et al., 1998). PSD-95 clusters are resistant to disruption by agents which take apart both microtubule and actin-based cytoskeletal networks (Allison et al., 2000; Allison et al., 1998). However, in time-lapse imaging studies PSD-95-GFP fusions appear to be dynamic (Friedman et al., 2000; Marrs et al., 2001; Okabe et al., 1999; Okabe et al., 2001); they can rapidly appear or disappear, grow, shrink and move within shafts and spines. Overexpression of PSD-95-GFP fusions drives increased localization of some synaptic proteins, such as GKAP and GluR1, and can promote the maturation of

MUSC LIBRARY

excitatory synapses (El-Husseini et al., 2000). During development *in vitro*, primary hippocampal neurons increase the ratio of AMPA-subtype glutamate receptors to NMDA-subtype glutamate receptors. Neurons transfected with PSD-95 produce currents similar to more mature cultures. These cells have larger synapses and a corresponding increase in synaptic vesicle content (El-Husseini et al., 2000). These changes in synaptic morphology and composition appear to depend only on the PDZ domains although recruitment of GKAP requires the interacting GK domain.

The mechanism and functional importance of the PDZ domains in MAGUKs are better established than the role for their SH3 and GK domains. Crystal structures have been solved for the third PDZ domain of PSD-95 binding a peptide ligand (Doyle et al., 1996; Morais Cabral et al., 1996) and for a PDZ domain from hCASK (Daniels et al., 1998). The crystal structure of the PDZ domain of nNOS complexed with the PDZ domain from syntrophin has also been determined (Hillier et al., 1999). These structures reveal that the PDZ domain contains an elongated surface groove which binds the C-terminus of proteins that end with certain hydrophobic amino acids, such as valine, and contain either a serine or a threonine at the 'minus two' position (Songyang et al., 1997; Stricker et al., 1997). The PDZ domains are required for both the clustering of interacting transmembrane proteins *in vitro* and at the neuromuscular junction in *Drosophila* (Thomas et al., 2000). They are essential but not sufficient for proper targeting of MAGUKs to synapses, septate junctions and the neuromuscular junction in *Drosophila* (Arnold and Clapham, 1999; Craven et al., 1999; Hough et al., 1997; Thomas et al., 2000). There is some evidence that the GK domain contributes to the targeting of MAGUKs (Craven and Bredt, 2000; Thomas et al., 2000). Despite genetic studies that

USSF LIBRARY

demonstrate an essential role for the SH3 and GK domains (Hoskins et al., 1996; Woods et al., 1996), their function has not been elucidated biochemically or by studies in hippocampal primary and slice cultures (Arnold and Clapham, 1999; Craven et al., 1999; El-Husseini et al., 2000)

This study presents a biochemical characterization of the SH3-GK module from PSD-95 and the corresponding crystal structure. First, Chapter One describes an intramolecular interaction between the SH3 and GK domains that can be reconstituted intermolecularly *in vitro*. In Chapter Two, the SH3GK structure reveals that the interaction between protein fragments containing the SH3 and GK homology regions, respectively, does not involve binding between intact, modular, domains but rather represents the assembly of a 'split' SH3 fold. The PSD-95 SH3 domain is unique; it is comprised of secondary structure elements that are distributed throughout the C-terminal half of the protein. These elements can associate *in trans* to complete the SH3 fold. Rather than binding canonical SH3 ligands, one function of the SH3 fold in PSD-95, and other MAGUKs, may be to reassemble intermolecularly. We propose a model whereby regulatory proteins bind the SH3GK module to promote intermolecular assembly of the SH3 fold to generate interlocked MAGUK networks. The structure of the SH3GK region of PSD-95 provides one of the first examples of how normally discrete, modular, protein domains have evolved into an integrated module.

UCSF LIBRARY

Chapter One

Identification of an Intramolecular Interaction between the SH3 and Guanylate Kinase Domains of PSD-95

UCSF LIBRARY

Summary

Postsynaptic density-95 (PSD-95 / SAP-90) is a member of the membrane-associated guanylate kinase (MAGUK) family of proteins that assemble protein complexes at synapses and other cell junctions. MAGUKs comprise multiple protein-protein interaction motifs including PDZ, SH3 and guanylate kinase (GK) domains, and these binding sites mediate the scaffolding function of MAGUK proteins. Synaptic binding partners for the PDZ and GK domains of PSD-95 have been identified, but the role of the SH3 domain remains elusive. We now report that the SH3 domain of PSD-95 mediates a specific interaction with the GK domain. The GK domain lacks a poly-proline motif that typically binds to SH3 domains; instead, SH3 / GK binding is a bi-domain interaction that requires both intact motifs. Although isolated SH3 and GK domains can bind *in trans*, experiments with intact PSD-95 molecules indicate that intramolecular SH3 / GK binding dominates and prevents intermolecular associations. SH3 / GK binding is conserved in the related *Drosophila* MAGUK protein DLG but is not detectable for *C. elegans* LIN-2. Many previously identified genetic mutations of MAGUKs in invertebrates occur in the SH3 or GK domains, and all of these mutations disrupt intramolecular SH3 / GK binding.

MSSF LIBRARY

Results

Because SH3 domains can regulate protein function through intramolecular interactions (Andreotti et al., 1997; Moarefi et al., 1997), we determined whether the SH3 domain of PSD-95 might associate with other domains from the protein. We first evaluated this possibility using the yeast two-hybrid system. Yeast strains HF7c and SFY526 were transformed with appropriate GAL4-fusion expression vectors encoding the SH3 domain of PSD-95 together with either the PDZ, SH3 or GK domains. Using both an assay for β-galactosidase activity and an assay for growth on His(-) plates, we found that the SH3 domain specifically interacts with the GK domain, and that the SH3 domain does not interact with the PDZ or SH3 domains (Fig. 1-1A). A quantitative liquid culture assay indicated that this association is robust, and appears similar in magnitude to the PSD-95 PDZ domain interaction with the PDZ domain of neuronal nitric oxide synthase (Brenman et al., 1996) and to the PSD-95 GK domain interaction with GKAP (Kim et al., 1997; Takeuchi et al., 1997). To verify that the SH3 / GK interaction is authentic we also evaluated binding of protein fragments *in vitro*. We found that the SH3 domain of PSD-95 fused to green fluorescent protein (GFP), expressed in HEK 293 cells, binds specifically to a bacterial GST fusion protein of the GK domain. Similarly, the GK domain of PSD-95, expressed in HEK cells, interacts with a GST-SH3 fusion protein (Fig. 1-1B).

The SH3 / GK interaction identified here is atypical because the GK domain lacks a P-X-X-P motif that typically binds to SH3 domains (Mayer and Eck, 1995; Musacchio et al., 1994; Ren et al., 1993). We therefore characterized the structural requirements within the GK domain that are essential for interaction. Using the yeast two-hybrid

USF LIBRARY

system, we found that an intact GK domain is required to bind the SH3 domain. The GK domain in PSD-95 comprises amino acids 534-712 (Cho et al., 1992). Yeast transformed with constructs encoding GK domains that were missing 36 amino acids from the N-terminus or 13 amino acids from the C-terminus do not turn blue in β-galactosidase assays nor do they form colonies on synthetic media plates lacking histidine (Table 1-1).

We also characterized features of the SH3 domain that mediate GK domain binding. Three-dimensional structures of SH3 domains are well characterized and are conserved (Musacchio et al., 1994). W470 of PSD-95 corresponds to an amino acid in other SH3 domains that mediates interaction with proline-containing peptides (Lim and Richards, 1994). Mutation of this tryptophan to phenylalanine typically disrupts SH3 / proline-containing peptide interactions without compromising the SH3 domain structure (Lim and Richards, 1994), but we found it does not abolish SH3 / GK binding (Table 1-1). One allele of *dlg*, a *Drosophila* MAGUK, contains a point mutation that changes a conserved leucine in the SH3 domain to proline (Woods et al., 1996). We introduced the corresponding mutation, L460P, into the PSD-95 SH3 domain constructs and found it disrupts the SH3 / GK domain interaction (Table 1-1).

We next asked whether the SH3 / GK domain interaction found in PSD-95 is unique or is conserved in other MAGUK proteins. We tested for SH3 / GK binding in both *Drosophila* DLG (Woods and Bryant, 1991), which is highly homologous to PSD-95, and in *C. elegans* LIN-2 (Hoskins et al., 1996), a MAGUK that is more distantly related. We found that SH3 domain from DLG interacts with its GK domain, but that the SH3 and GK domains from LIN-2 do not (Fig. 1-2). Furthermore, SH3 / GK domain binding was detected in SH3 and GK combinations between PSD-95 and DLG (Fig. 1-2).

MIT LIBRARIES

Our inability to detect SH3 / GK interactions with the protein domains from LIN-2 either reflects the lower affinity for this interaction within LIN-2 or suggests that this interaction is not a general feature of more distantly related MAGUK proteins.

Intermolecular SH3 / GK domain binding could mediate multimerization of MAGUKs whereas intramolecular SH3 / GK binding could mediate a regulatory interaction. To determine which mode of binding predominates, we evaluated binding of isolated SH3 or GK domains to intact PSD-95 *in vitro*. We found that GST-fusions of either the SH3 or GK domain alone did not bind to a full length PSD-95-GFP fusion expressed in HEK 293 cells (Fig. 1-3B). Further, in yeast two-hybrid experiments, no binding was detected between isolated SH3 or GK domains and SH3GK (Fig. 1-3A). This absence of binding may indicate that intramolecular SH3 / GK binding in intact PSD-95 molecules is highly favored and prevents intermolecular associations. This interpretation predicts that introducing mutations that disrupt the intramolecular binding would facilitate intermolecular binding of isolated SH3 or GK domains to intact PSD-95 or SH3GK. Indeed, we found that an isolated GK domain interacts only with an SH3GK construct that contains a C-terminal GK domain truncation (Fig. 1-3A). We also determined, by yeast two-hybrid analysis and with GST-fusion protein binding experiments, that an isolated SH3 domain can bind full length PSD-95 or SH3GK constructs that contain a disruptive L460P point mutation in the SH3 domain (Fig. 1-3B). This latter interaction is specific as an SH3GK construct with both the L460P SH3 mutation and a truncation of the GK domain does not bind to SH3 (Fig. 1-3B)

As an additional tool to evaluate the SH3 / GK domain interaction in PSD-95 protein extracted from brain, we generated an antibody to the last 14 amino acids of the

MIT LIBRARY

GK domain (amino acids 698 – 711). We find that this antibody immunoprecipitates PSD-95 from whole brain homogenates solubilized with 0.2% SDS (SDS extracts), but does not immunoprecipitate PSD-95 from brain homogenates solubilized with 1% Triton X-100 alone (Fig. 1-4A). In contrast, another antibody directed to the PDZ domains immunoprecipitates PSD-95 equally from both the SDS and Triton extracts (Fig. 1-4A).

The selective immunoprecipitation of PSD-95 by the GK domain antibody in SDS extracts may indicate that the antibody only interacts with a denatured epitope. Alternatively, the antigenic epitope of the GK domain may not be accessible to the antibody under native conditions due to intermolecular protein interactions, such as with the GKAPs or MAP1A, or due to intramolecular interactions, such as with the SH3 domain. To help distinguish between these possibilities, we evaluated immunoprecipitation of PSD-95, SH3GK-GFP or GK-GFP expressed in HEK 293 cells, which lack GKAPs and MAP1A. The GK antibody avidly immunoprecipitates GK-GFP in both SDS and Triton extracts (Fig. 1-4B), indicating that the GK antibody indeed can react with the GK domain in Triton extracts. However, we find that the transfected PSD-95 proteins containing both the SH3 and GK domains immunoprecipitate with the GK antibody only from SDS extracts and not from extracts solubilized with Triton X-100 alone (Fig. 1-4C). These results would seem to indicate that, under native conditions, the SH3 domain from PSD-95 masks the GK domain epitope. We next repeated these transfection / immunoprecipitation experiments with constructs that contained the SH3 L460P point mutation, which disrupts SH3 / GK binding. Remarkably, introduction of this SH3 domain mutation into constructs containing the SH3 and GK domains restores antigenic accessibility of the GK domain in Triton extracts (Fig. 1-4C). Taken together

USSF LIBRARY

with other data in this study, the simplest model consistent with these results is that intramolecular binding between the SH3 and GK domains of PSD-95 is sensitive to SDS denaturation and that the SH3 / GK interaction blocks antigenic access of the GK domain antibody. This parsimonious interpretation would indicate that the intramolecular SH3 / GK domain interaction occurs to a significant extent within PSD-95 from both brain and heterologous cells.

To determine if the SH3 domain interaction might regulate GK binding to GKAP or MAP1A, we evaluated the association of wild type and mutant PSD-95 with GKAP. We co-transfected HEK cells with expression constructs encoding GKAP (Kim et al., 1997; Takeuchi et al., 1997) together with either full length PSD-95, GFP-SH3GK, or with PSD-95 or GFP-SH3GK constructs containing the SH3 L460P mutation, which disrupts SH3/GK binding. In extracts from these cells, we immunoprecipitated PSD-95 and evaluated association of GKAP (Fig. 1-5). We found that GKAP co-immunoprecipitates equally with all these PSD-95 constructs indicating that SH3 / GK binding does not influence GK binding activity.

USC LIBRARY

Discussion

This work identifies a specific intramolecular association between the SH3 and GK domains within PSD-95 and shows that this interaction is conserved in other MAGUK proteins. Although isolated SH3 and GK domains of PSD-95 can bind one another *in trans*, this intermolecular interaction is not detected between intact PSD-95 molecules or fusion proteins that contain both the SH3 and GK domain. Instead, an intramolecular SH3 / GK association appears to predominate within full-length PSD-95 molecules, suggesting a regulatory rather than scaffolding role for the interaction.

Several lines of evidence suggest that the intramolecular interaction between the SH3 and GK domains identified here is likely to play a physiological role in MAGUK function. First, SH3 / GK binding is conserved even between the distantly related MAGUK proteins PSD-95 and DLG. Second, genetically identified mutations of the SH3 and GK domains in *dlg* disrupt the intramolecular SH3 / GK association. A point mutation in the SH3 domain that disrupts SH3 / GK binding was identified by its strong phenotype (Woods et al., 1996). Conversely, C-terminal GK domain truncations that resemble genetic mutations of *dlg* disrupt the SH3 domain interaction. These truncations of DLG in *Drosophila* are lethal in the absence of maternal contribution (Woods et al., 1996). It is also intriguing that certain genetic mutants of the SH3 and GK domains of *dlg* complement one another in mixed heterozygous flies, negating the lethality of the recessive mutations (Woods et al., 1996). Our data suggest that a MAGUK protein with a mutant SH3 domain will bind *in trans* to a MAGUK with a GK domain mutation. If such a mixed bimolecular complex restores *dlg* function, this could explain the genetic complementation that has been observed.

DUKE LIBRARY

The SH3 domain interactions described here are atypical. SH3 domains have classically been shown to interact with proline-containing motifs that contain the consensus sequence P-X-X-P (Mayer and Eck, 1995). This binding model cannot explain the SH3 / GK interactions, as GK domains lack a P-X-X-P motif. Moreover, mutation of the SH3 domain of PSD-95 at the conserved W470 that normally mediates interaction with proline-containing peptides (Lim and Richards, 1994) does not disrupt SH3 / GK binding. Rather than the SH3 domain recognizing a short peptide sequence within the GK domain, our data suggest that SH3 / GK binding represents a bi-domain interaction and requires proper folding of both intact motifs.

The intramolecular interactions described here are reminiscent of recent studies showing that intramolecular SH3 domain associations mediate autoinhibition of Src and Tec family tyrosine kinases (Andreotti et al., 1997; Moarefi et al., 1997). In the Src family kinase, Hck, the SH3 motif binds to and blocks the catalytic activity of the adjacent tyrosine kinase domain. This intramolecular SH3 domain interaction within Hck is displaced and tyrosine kinase activity is restored when the Hck SH3 domain binds to an appropriate protein ligand *in trans*. By analogy, the intramolecular SH3 domain interaction within PSD-95 may regulate the GK domain. While we find that this interaction does not alter GK domain binding to either GKAP (Fig. 1-5) or MAP1A (data not shown), the SH3 domain may regulate an as yet unidentified catalytic activity of the GK domain. It is also possible that the intramolecular SH3 / GK interaction mediates functional interactions between the SH3 domain and other unknown proteins, as studies with DLG in *Drosophila* have suggested that the GK domain may act a negative regulator of DLG in controlling cell proliferation (Hough et al., 1997). Recent studies indicate that

UCSF LIBRARY

PDZ domains within PSD-95 can negatively regulate GK binding activity (Brenman et al., 1998), though the PDZ domains themselves do not bind to GK (Brenman et al., 1998). As the SH3 domains is interposed between the PDZ and GK domains, the SH3 / GK interaction described here could play a role in autoinhibition of GK binding by PDZ domains. Alternatively, while our data best support a model in which intramolecular associations between the SH3 and GK domains predominate, it does not eliminate the possibility that other factors present *in vivo* may facilitate intermolecular interactions that could contribute to the scaffolding functions of PSD-95.

UCSF LIBRARY

Gal4 DNA Binding hybrid	Gal4 activation hybrid	β-galactosidase activity	Growth
Interactions between PSD-95 SH3 and guanylate kinase-like domain deletions			
GK-(503-724)	SH3-(408-509)	++	+
GKΔN57-(560-724)	SH3-(408-509)	-	-
GKΔN109-(611-724)	SH3-(408-509)	-	-
GKΔC25-(503-699)	SH3-(408-509)	-	-
Interactions between PSD-95 SH3 point mutants and the guanylate kinase-like domain			
GK-(503-724)	SH3 L460P-(408-509)	-	-
SH3 L460P-(408-560)	GK-(503-724)	-	-
SH3 W470F-(408-560)	GK-(503-724)	+	+
SH3-(408-560)	GK-(503-724)	++	+

UCSF LIBRARY

Table 1-1. Yeast SFY526 and HF7c cells were cotransformed with expression vectors encoding various GAL4-binding domain and GAL4 activation domain fusion proteins. Each transformation mixture was plated on two synthetic dextrose plates, one lacking tryptophan and leucine and the other lacking tryptophan, leucine and histidine. β-Gal activity was scored as the time required for colonies of co-transformed yeast to turn blue at 30°C: ++ <60 min; +, 60 - 240 min; -, no significant activity. Growth on His(-) plates was positive if yeast streaked to -LWH plates produced isolated colonies within 3 days.

UCSF LIBRARY

A

Gal4 DNA Binding Hybrid	Gal4 Activation Hybrid	β-Galactosidase Units
PSD-95 GK (503-724)	pGAD424 vector	0.5
PSD-95 GK (503-724)	SV40 large T antigen (84-708)	0.6
PSD-95 GK (503-724)	GKAP (1-344)	5.5
PSD-95 GK (503-724)	PSD-95 SH3 (408-509)	8.9
PSD-95 GK (503-724)	PSD-95 PDZ I-III (40-384)	0.5
pGBT9 vector	PSD-95 SH3 (408-509)	0.2
PSD-95 SH3 (408-509)	PSD-95 GK (503-724)	11.7
PSD-95 SH3 (408-509)	PSD-95 SH3 (408-509)	0.6
PSD-95 SH3 (408-509)	PSD-95 PDZ I-III (40-384)	0.4
nNOS (1-195)	PSD-95 PDZ I-III (40-384)	7.6

UCSF LIBRARY

B

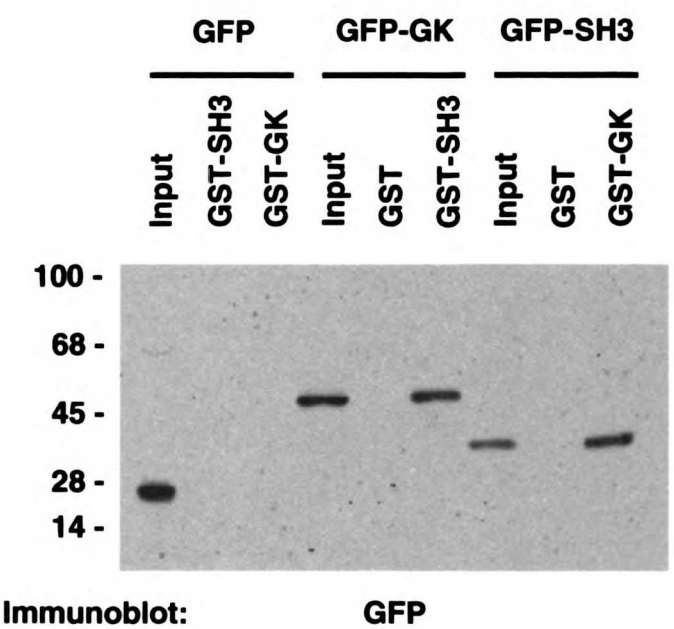


Figure 1-1. Interaction of the SH3 and GK domains of PSD-95. (A) Yeast strain SFY526 were transformed with expression vectors encoding various GAL4 DNA binding domain and GAL4 activation domain fusion proteins. The amino acid residues from PSD-95 encoded in each construct are shown in parentheses. Each transformation was plated on synthetic dextrose plates lacking tryptophan and leucine. Interactions were quantified using an ONPG liquid culture assay. Values are representative of an experiment that was repeated twice with similar results. (B) Glutathione-Sepharose beads (25 µl) were charged with 3 µg of GST, GST-SH3 or GST-GK protein and were incubated with extracts from HEK cells expressing either GFP alone, GFP fused to the GK domain of PSD-95 (GFP-GK) or GFP fused to the SH3 domain of PSD-95. After washing the beads, bound proteins were eluted and analyzed by immunoblotting. Note that GFP-GK binds specifically to GST-SH3 and that GFP-SH3 binds to GST-GK. The input lanes represent 5% of the cell extract using for the binding assays. Positions of molecular weight marker proteins (in kDa) are on the left.

USC LIBRARY

GAL4 DNA Binding Hybrid	GAL4 Activation Hybrid: PSD-95 SH3 (408-509)	LIN2 SH3 (614-736)	DLG SH3 (581-691)	pGAD424
PSD-95 GK (503-724)	++	–	++	–
DLG GK (684-960)	++	–	++	–
LIN2 GK (731-960)	–	–	–	–
pGBT9	–	–	–	–

β-Galactosidase Activity

UCSF LIBRARY

Figure 1-2. The SH3 / GK domain interactions is conserved between PSD-95 and DLG, but is not detectable with LIN-2. Yeast strain SFY526 were transformed with plasmids encoding combinations of SH3 and GK domains from PSD-95, DLG, and LIN-2. Constructs encoding SH3 domains were fusions with the GAL4 activation domain, and those encoding GK domains were fusions with the GAL4 DNA binding domain. Interactions were assayed with the β-gal colony filter-lift assay. β-Gal activity was scored as the time required for colonies of transformed yeast to turn blue at 30°C: ++ <60 min; + 60 - 240 min; - no significant activity.

UCSF LIBRARY

A

Gal4 DNA Binding Hybrid	Gal4 Activation Hybrid	β-Gal / Growth
PSD-95 GK interactions with SH3 and SH3GK constructs:		
GK (503-724)	SH3 (408-509)	++/+
GK (503-724)	SH3GK (408-724)	–/–
GK (503-724)	SH3GKΔC (408-699)	+/+
GK (503-724)	SH3(L460P)GK (408-724)	–/–
GK (503-724)	SH3(L460P)GKΔC (408-699)	–/–
PSD-95 SH3 interactions with GK and SH3GK constructs:		
SH3GK (408-724)	SH3 (408-509)	–/–
SH3GKΔC (408-699)	SH3 (408-509)	–/–
SH3(L460P)GK (408-724)	SH3 (408-509)	+/+
SH3(L460P)GKΔC (408-699)	SH3 (408-509)	–/–
PSD-95 L460P SH3GK interactions with SH3GK constructs:		
SH3(L460P)GK (408-724)	SH3GK (408-724)	–/–
SH3(L460P)GK (408-724)	SH3GKΔC (408-699)	+/+
SH3(L460P)GK (408-724)	SH3(L460P)GKΔC (408-699)	–/–
SH3(L460P)GK (408-724)	SH3(L460P)GK (408-724)	–/–

UCSF LIBRARY

B

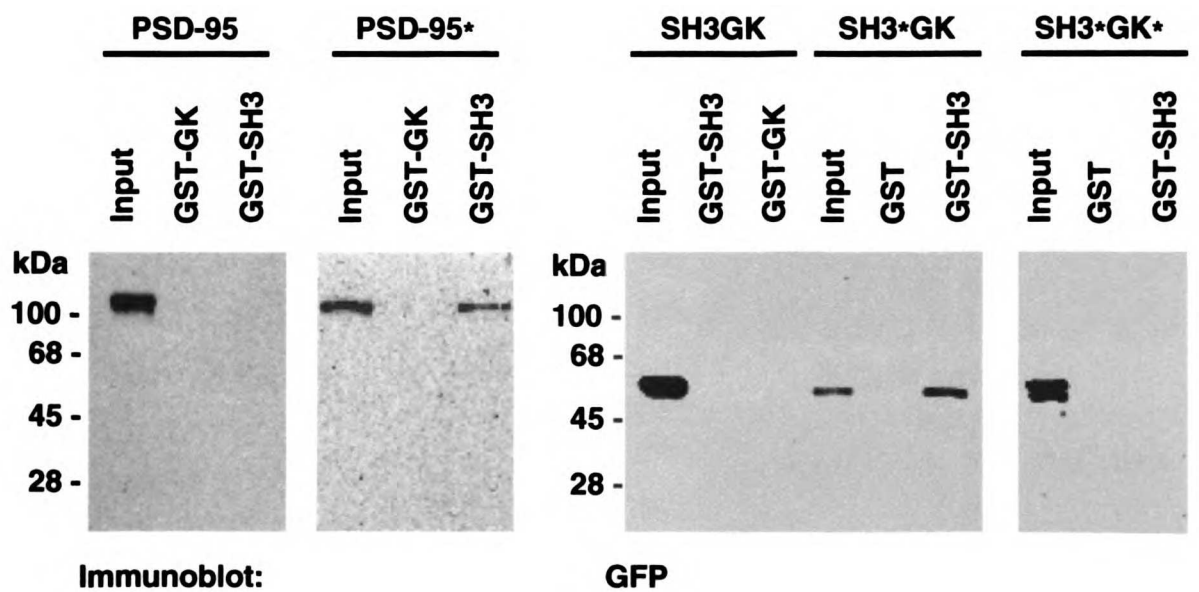
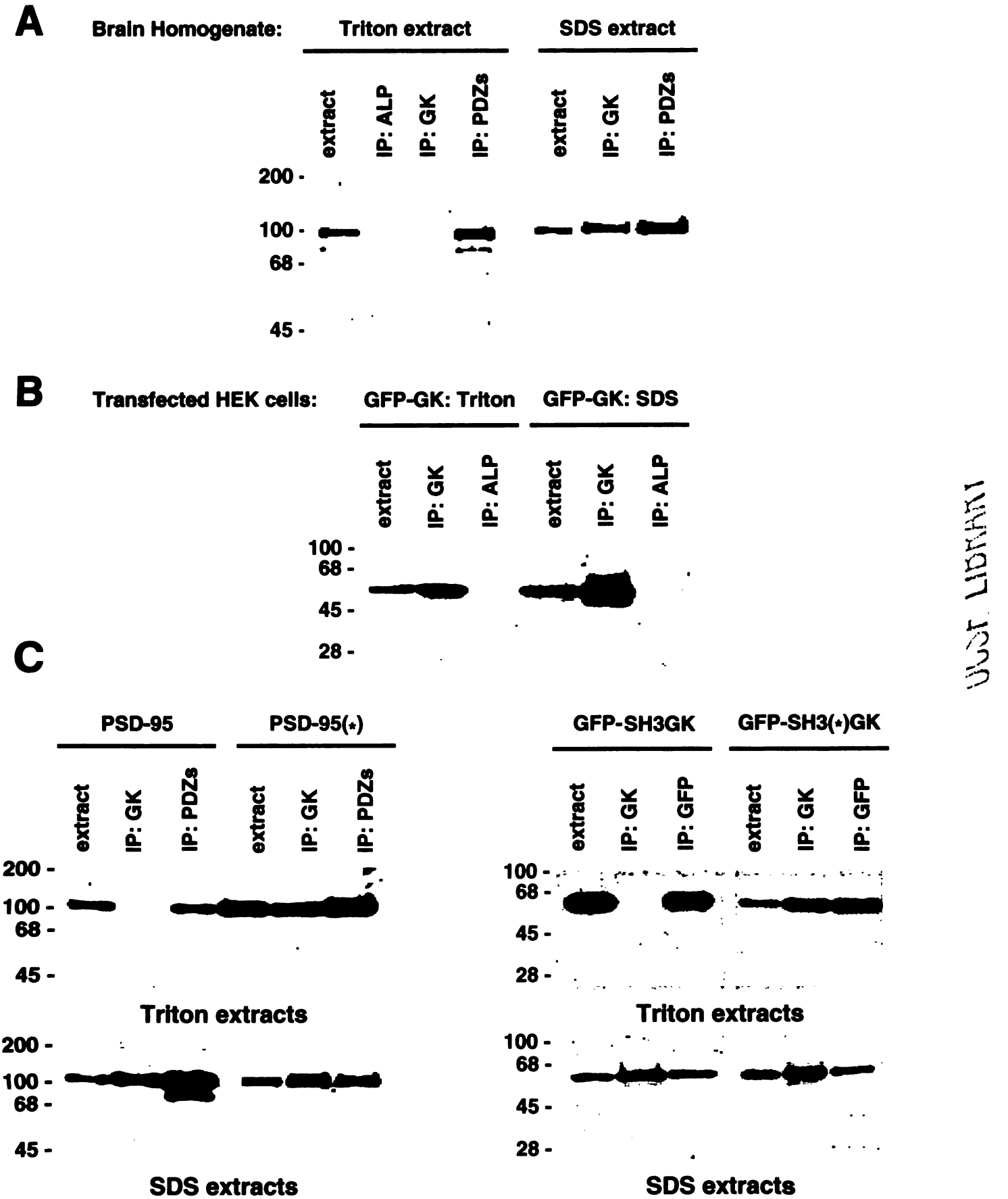


Figure 1-3. An intramolecular SH3 / GK domain interaction within PSD-95 prevents similar intermolecular interactions. (A) Yeast strain HF7c were transformed with two-hybrid constructs encoding various combinations of the SH3, GK and SH3GK domains of PSD-95. The amino acid residues from PSD-95 encoded in each construct are shown in parentheses. Interactions between hybrid proteins were assayed with the β-gal colony filter lift assay and growth on His(-) plates. β-Gal activity was scored as the time required for colonies of transformed yeast to turn blue at 30°C: ++ <60 min; + 60 - 240 min; - no significant activity. Growth on His(-) plates was scored as positive if yeast streaked to -LWH plates produced isolated colonies within 3 days. Note that the SH3GK constructs do not bind SH3 or GK domains unless the corresponding domain is mutated in SH3GK. (B) Glutathione-Sepharose beads (25 μl) were charged with 3 μg of GST, GST-SH3 or GST-GK protein and were incubated with extracts from HEK cells expressing *(1)* GFP-PSD-95, *(2)* GFP-PSD95(L460P) containing the SH3 domain mutation *(3)* GFP-SH3GK, *(4)* GFP-SH3(L460P)GK containing the SH3 domain mutation or *(5)* GFP-SH3(L460P)GKΔC25 containing both the SH3 domain mutation and the GK C-terminal truncation. After washing the beads, bound proteins were detected by western blotting. The input lanes represent 5% of the cell extract using for the binding assays. Positions of molecular weight marker proteins (in kDa) are on the left.

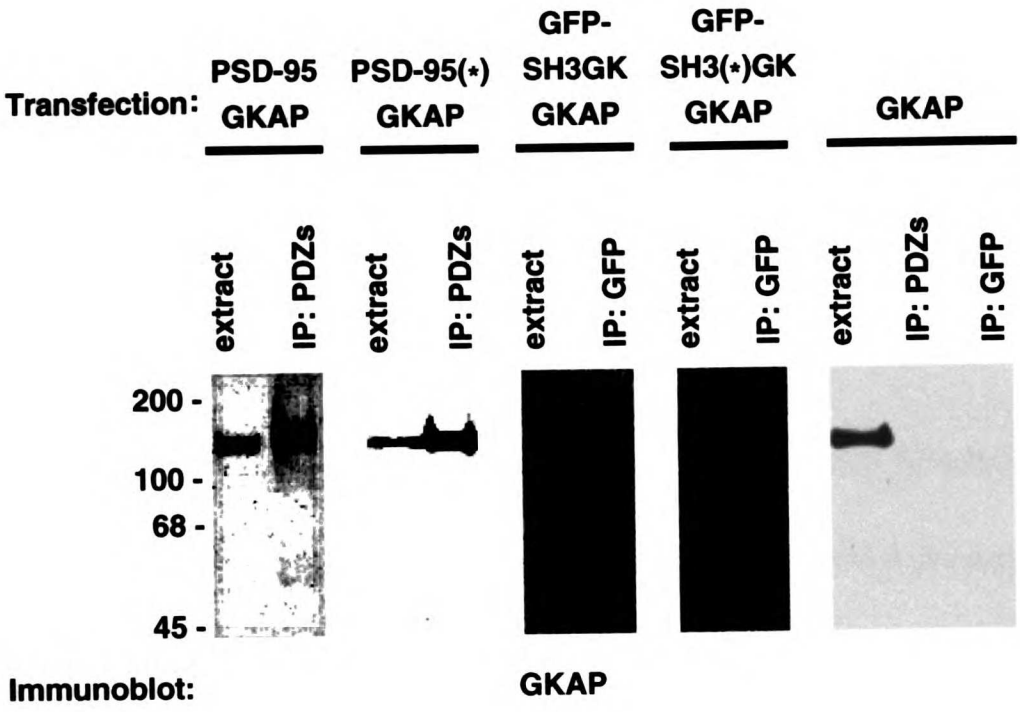
USC LIBRARY


A
Brain Homogenate:
Triton extract
SDS extract
extract
IP: ALP
IP: GK
IP: PDZs
extract
IP: GK
IP: PDZs
200 -
100 -
68 -
45 -
B
Transfected HEK cells:
GFP-GK: Triton
GFP-GK: SDS
extract
IP: GK
IP: ALP
extract
IP: GK
IP: ALP
100 -
68 -
45 -
28 -
C
PSD-95
PSD-95(*)
extract
IP: GK
IP: PDZs
extract
IP: GK
IP: PDZs
200 -
100 -
68 -
45 -
Triton extracts
200 -
100 -
68 -
45 -
SDS extracts
GFP-SH3GK
GFP-SH3(*)GK
extract
IP: GK
IP: GFP
extract
IP: GK
IP: GFP
100 -
68 -
45 -
28 -
Triton extracts
100 -
68 -
45 -
28 -
SDS extracts

USC LIBRARY

Figure 1-4. Immunoprecipitations from whole brain homogenate and transfected HEK 293 cells. PSD-95 was detected with a monoclonal PSD-95 antibody (ABR). GFP-fusion constructs were detected with a monoclonal antibody (Clontech). Positions of marker proteins are given on the left (in kDa). (A) Rat brain extracts were solubilized with 1% Triton (Triton extracts) or with 0.2% SDS and were immunoprecipitated with antibodies against either ALP (negative control), the GK domain of PSD-95, or the PDZ domains of PSD-95. Note that the PDZs antibody immunoprecipitates PSD-95 from both Triton and SDS extracts whereas the GK antibody only immunoprecipitates from SDS extracts. Extract lanes represent 10% and 5% of the volume of Triton and SDS extracts used for immunoprecipitations, respectively. (B) Triton and SDS extracts were prepared from HEK 293 cells expressing the GK domain of PSD-95 fused to GFP. Immunoprecipitations and analyses were done as in A. The extract lanes contain 5% of the extract used for each condition. (C) Immunoprecipitations were performed with Triton and SDS extracts prepared from transfected HEK 293 cells expressing PSD-95, PSD-95 containing the SH3 mutation L460P (PSD-95(*)), GFP-SH3GK, or GFP-SH3(*)GK with the L460P point mutation. The extract lanes contain 5% of the extract used for each condition.

UCSF LIBRARY



USSF LIBRARY

Figure 1-5. Co-immunoprecipitation of GKAP is unaffected by the SH3 mutation L460P. HEK 293 cells were co-transfected with expression constructs for GKAP and either PSD-95, PSD-95 containing the SH3 mutation L460P (PSD-95(*)), GFP-SH3GK, or GFP-SH3(*)GK with the L460P point mutation. Cell extracts were solubilized with 1.0% Triton. Immunoprecipitations of these extracts were analyzed by immunoblotting for GKAP. The extract lanes represent 5% of the cell extract used for immunoprecipitations. Positions of molecular weight marker proteins (in kDa) are on the left.

UCSF LIBRARY

Chapter Two

Structure of the SH3-Guanylate Kinase-like Module from PSD-95 Suggests a Mechanism for Regulated Assembly of MAGUK Scaffolding Proteins

UCSF LIBRARY

Summary

Membrane associated guanylate kinases (MAGUKs), such as PSD-95, are modular scaffolds that organize signaling complexes at synapses and other cell junctions. MAGUKs contain PDZ domains, which recruit signaling proteins, as well as a Src homology 3 (SH3) and a guanylate kinase-like (GK) domain, which are implicated in scaffold oligomerization. The crystal structure of the SH3-GK module from PSD-95 reveals that these domains form an integrated structural unit: the SH3 fold comprises non-contiguous sequence elements that are divided by a large hinge region and the GK fold. These sequence elements compose two subdomains that can assemble in either an intramolecular or intermolecular fashion to complete the SH3 fold. These findings suggest a model for MAGUK oligomerization in which complementary subdomains of the SH3 fold associate by 3D domain swapping. This model provides a mechanism by which ligand binding to the hinge region could regulate assembly of a MAGUK network.

UCSF LIBRARY

Results and Discussion

Crystal Structure of the SH3-GK Region of PSD-95

The C-terminal region of PSD-95 (residues 417-724) spanning the SH3 and GK domains was crystallized by vapor diffusion against 2 M ammonium sulfate, 2% PEG400, and the structure determined by multiple wavelength anomalous dispersion (MAD) with selenomethionyl-substituted protein (Table 2-1). The solvent flattened electron density map was readily interpretable and allowed a model of most of the SH3 and the entire GK domain of PSD-95 to be built. The final refined model contains all residues except for 12 NH_2-terminal residues and residues 504-509 for which only weak electron density was observed (Fig. 2-1A). The refined SH3-GK structure has an R value of 23% and a free R value of 25% at 1.8 Å resolution against a native synchrotron data set. (Table 2-1).

A pair of anti-parallel β-strands (E and F) links the SH3 and GK domains. A Cα contact map indicates that these linker strands make contact with both globular folds, but that they are primarily part of the SH3 fold (Fig. 2-1B). Thus the SH3 fold comprises several non-contiguous sequence elements. Aside from the contacts mediated by the E and F β-strands, there are remarkably few contacts between the two domains. Below we present the structural properties of each domain, then discuss how this unanticipated domain arrangement explains the previously reported SH3-GK interactions.

The GK Domain Resembles Catalytic Homologues

The GK domain of PSD-95 shares 40% sequence identity with yeast guanylate kinase (Fig. 2-2), the enzyme that catalyzes the phosphorylation of GMP to GDP. Yeast

UCSF LIBRARY

guanylate kinase, like many kinases, has a clamshell-like structure surrounding the active site (Blaszczyk et al., 2001; Stehle and Schulz, 1992). The yeast enzyme can adopt at least two states, an open conformation observed in the absence of ligand, and a closed conformation, observed in the presence of guanosine mono-phosphate (GMP), in which the clamshell fold closes down on the substrate (Blaszczyk et al., 2001). The structure of the PSD-95 GK domain superimposes better (RMSD= 1.49 Å) with the open (apo) form of the yeast guanylate kinase structure than the closed conformation (RMSD = 1.94 Å) (Fig. 2-3A). Presumably, the PSD-95 GK fold may also form a similar closed state upon binding of the appropriate ligand. It is also noteworthy that the homology between the yeast guanylate kinase structure and the PSD-95 GK structure does not extend to the flanking E and F β-strands, as these strands are not present in the yeast enzyme.

. Although PSD-95 lacks guanylate kinase catalytic activity (Kistner et al., 1995; Kuhlendahl et al., 1998), we wondered whether it binds GMP. The reported binding affinity of yeast guanylate kinase for GMP is 29 μM (Li et al., 1996). Whereas we observed yeast GK bound GMP with an affinity of 20-50 μM, we were unable to detect binding of GMP to the SH3GK module using identical equilibrium dialysis conditions (data not shown). These data do not preclude the possibility that SH3GK binds with a significantly reduced affinity compared to yeast GK. Comparison of the structures suggests why the GK domain from PSD95 may not bind GMP with high affinity. Only two of the four residues that coordinate the guanine base in yeast guanylate kinase are present in PSD-95. By comparison, the five residues that coordinate the GMP phosphate group are all conserved in sequence and structural arrangement. This observation

UCSF LIBRARY

suggests that the GK domain from PSD-95 may bind a phosphorylated molecule other than GMP.

The SH3 Domain Has a Split Fold Incompatible with Canonical Peptide Binding

The SH3 fold in the SH3GK module differs radically from typical SH3 domains (Fig. 2-4). First, this domain has an extra strand in its fold. Canonical SH3 domains consist of an anti-parallel β-barrel with orthogonal packing of the β-sheets (Kuriyan and Cowburn, 1997; Musacchio et al., 1994). While the canonical SH3 fold has five β-strands (A-E), the PSD-95 SH3 fold has six β-strands (A-F). Second, the PSD-95 SH3 domain is composed of discontinuous segments of sequence; a large insert, which we refer to as the hinge region, separates β-strands D and E. Additionally, the GK domain is inserted between β-strands E and F. Structure-based sequence alignments suggest this unusual split configuration is conserved within the extended MAGUK family (Fig. 2-2). Third, in canonical SH3 domains a segment of 3_{10} helix separates β-strands D and E (Larson and Davidson, 2000). This conformation is conserved in known SH3 structures and forms part a conserved hydrophobic binding surface that interacts with proline-containing peptides presented as polyproline type II helices (Fig. 2-4B and D) [Yu, 1994 #1683; Feng, 1994 #1684; Lim, 1994 #1685; Musacchio, 1994 #1686; Kohda, 1994 #1688; Wittekind, 1994 #1689]. A conserved tyrosine in the 3_{10} helix is a crucial element in the peptide recognition interface (Lim and Richards, 1994; Nguyen et al., 1998). In MAGUK SH3 domains, the large hinge insert replaces this short segment. Not only does the PSD-95 SH3 fold lack the conserved tyrosine residue, but also the hinge region sterically occludes the peptide binding site (Fig. 2-4A).

As a consequence of this unusual structure, it seems unlikely that MAGUK SH3 domains bind proline-rich peptide ligands in a canonical manner. Nevertheless, some studies have reported proline-containing ligands for MAGUK SH3 domains (Garcia et al., 1998; Maximov et al., 1999). However, because of the unexpected insertion of the hinge region in the SH3GK structure, the constructs used in these previous binding studies lack β-strands E and F. Thus, ligands identified in previous studies most likely interact in an atypical fashion. The unique features of the PSD-95 SH3 fold suggest that regulated association with the GK domain, and not peptide binding, may be its primary role.

The SH3-GK Interaction Reflects Reassembly of the Split SH3 Domain

While previous models have presumed that the interaction between the SH3 and GK fragments involves docking between two prefolded domains, the structure of the SH3GK module reveals that these domains are intertwined in sequence (Fig. 2-2). We used the structure to define the boundaries for the interacting fragments. Specifically, we wanted to determine the role of β-strands E and F in the SH3-GK interaction as these β-strands appear to be structurally part of the SH3 fold, but are adjacent to the GK domain. We therefore tested a series of deletion constructs for their ability to bind one another by GST-pulldown assays (Fig. 2-5A). In this series, the β-strands E and F were fused to the core SH3 fragment (β-strands A-D), or the GK fragment. This interaction matrix reveals that a GK fragment lacking either the E or F β-strands cannot interact with any of the SH3 fragments tested. In contrast, adding the E and F β-strands to the core SH3 fragment does not influence binding; even an SH3 fragment only consisting of β-strands A through

D can bind the GK fragment including β-strands E and F. Thus for binding to occur, the E and F β-strands must be part of the GK fragment.

However, the E and F β-strands appear to be part of the SH3 fold. To determine if β-strands E and F contribute structurally to the SH3 fold we used guanidine hydrochloride denaturation to measure the stability of four SH3 constructs: β-strands A-D, β-strands A-E, β-strands A-F* (where F is appended to β-strand E by a $(Gly\text{-}Ser)_3$ linker), and the full SH3GK module (Fig. 2-5B). The fragment containing only β-strands A-D is unstable. Most likely this fragment is only partially structured at the experimental temperature of 25 °C. In contrast, a fragment including β-strand E displays a cooperative unfolding transition but the protein is still quite unstable (ΔG_{fold} = ~4 kcal/mol). Adding β-strand F, by artificially appending it to β-strand E, increases domain stability (ΔG_{fold} = ~5 kcal/mol). Finally, in the SH3GK module, SH3 domain is stability is further increased (ΔG_{fold} = ~10 kcal/mol). These findings suggest that β-strands E and F are part of the SH3 fold. In addition, they show that the GK domain plays a role in stabilizing the SH3 fold. Because the GK domain makes only limited contacts with the core SH3 fold (Fig. 2-1B) its role in stabilizing the SH3 domain appears indirect, most likely by properly orienting β-strands E and F.

Thus, the previously described SH3-GK interaction is unusual because it does not involve docking of two prefolded domains, but rather reassembly of an SH3 fold from two separable subdomains, β-strands A-D of the SH3 fold and β-strands E and F flanking the GK fold. At least one of the subdomains, the β-strand A-D fragment, is unstable in the absence of the complementary subdomain (β-strands E and F). This interaction is

distinct from canonical domain-domain interactions as the boundaries of the interacting fragments are different from the boundaries of folded domains (Fig. 2-5C).

Intermolecular Interaction Between SH3GK Modules May Involve Three-Dimensional Domain Swapping

The PSD-95 SH3 fold can assemble intramolecularly or intermolecularly with individual subdomains (Fig. 2-5A). The structure solved here represents the intramolecular configuration. Intramolecular assembly is favored, as the intact SH3GK module does not interact with either isolated subdomain *in vitro* (Fig. 2-5A). Intermolecular assembly *in vitro* is only observed between SH3GK modules if the complementary subdomains are disrupted within separate protein molecules (McGee and Bredt, 1999; Shin et al., 2000). Nonetheless, prior studies indicate that intermolecular SH3 assembly does occur *in vivo*. First, the MAGUK proteins hCASK and hDLG associate in intestinal epithelia, and this interaction requires the SH3GK modules (Nix et al., 2000). Second, genetic studies of *discs large*, a MAGUK that clusters receptors and ion channels at the fly neuromuscular junction (Zito et al., 1997) and is also present at septate junctions in epithelia (Woods et al., 1996) demonstrate interallelic complementation between mutations that disrupt separate subdomains in the SH3GK module. Specifically, two different recessive lethal alleles, one containing a missense mutation in the β-strand A-D subdomain, dlg^{m30}, the other truncating the protein before the E-F subdomain, dlg^{X1-2}, are viable as transheterozygotes (Woods et al., 1996). Intermolecular assembly of a 'functional' SH3 by the two proteins with mutations in separate SH3 subdomains most simply explains this finding.

The SH3GK structure, along with *in vitro* binding studies, suggests that the intermolecular SH3 assembly may occur via three-dimensional (3D) domain swapping, a mechanism in which proteins exchange complementary substructures to generate dimers or higher order oligomers (Bennett et al., 1995; Newcomer, 2001; Schlunegger et al., 1997). In SH3GK 3D domain swapping, the β-strand A-D subdomain from one polypeptide would interact with the β-strand E and F subdomain from a separate polypeptide. This type of interaction could occur either as a closed dimer or a higher order oligomeric chain (Fig. 2-6A).

The Hinge Element May Regulate Domain Swap Assembly

In a 3D domain swap model of SH3GK oligomerization, how could assembly be regulated? Studies of protein systems that undergo domain swapping indicate that the hinge region linking the two interacting subdomains plays an important role in determining the preference for inter- versus intramolecular assembly (Newcomer, 2001; Schlunegger et al., 1997). Experimental manipulation of the hinge loop region in p13suc1 has revealed that imposing a conformational constraint on this region of the protein can alter the kinetics as well as the equilibrium distribution of monomer vs. domain swapped dimer (Rousseau et al., 2001; Schymkowitz et al., 2000). Interestingly, in MAGUK SH3 domains, the hinge region replaces a structurally conserved 3_{10} helix (Fig. 2-6B). Moreover, in several MAGUKs the hinge region has been identified as the binding site for several potential regulatory proteins such as protein 4.1 and calmodulin (Fig. 2-6C)(Cohen et al., 1998; Lue et al., 1994; Marfatia et al., 1995; Masuko et al., 1999). We therefore hypothesize that ligand binding to the hinge region could alter its

conformational properties, either by destabilizing the monomeric form or reducing the kinetic barrier, to promote oligomerization. However, no proteins have yet been identified that bind to the hinge region of PSD-95.

To test our hypothesis that the hinge region is a potential point for regulation of inter- vs. intramolecular SH3 assembly, we perturbed the conformational properties of this region by mutagenesis. To mimic rigidity that might be imposed by protein binding we replaced residues 490 - 523 with a linker containing five prolines (Pro_5Δhinge) (Fig. 2-6D). We predicted that this substitution would stiffen the peptide backbone and extend the distance between the ends of the hinge that separate the SH3 subdomains (MacArthur and Thornton, 1991). In similar constructs, we replaced the hinge with a flexible linker consisting of five glycines (Gly_5Δhinge), or inserted no linker (Δhinge). We analyzed each of these proteins by gel filtration. All of these mutant proteins, like the wild-type SH3GK module, eluted as monomers except for Pro_5Δhinge, for which a significant fraction eluted as a dimer (Fig. 2-6E). Rechromatography of the Pro_5Δhinge dimer species revealed that exchange between the monomer and dimer states is relatively slow ($t_{1/2}$ = ~ 2 hrs). Therefore, we conclude that replacement of the hinge with a conformationally constrained segment promotes oligomerization.

To confirm that the dimer observed for this mutant SH3GK module is formed by 3D domain swapping and not by novel interactions introduced by the inserted Pro_5 element, we performed control experiments to eliminate the possibility that the Pro_5 element served as a docking site for the SH3 domain. First, we mutated the putative peptide binding surface of the SH3 domain with the Pro_5Δhinge construct. Specifically, we changed tryptophan 470 to phenylalanine as this amino acid is critical for binding of

proline-rich peptides to canonical SH3 domains (Lim and Richards, 1994), but does not affect intermolecular assembly of the SH3 fold (McGee and Bredt, 1999). By gel filtration, this mutant protein yielded a dimer species with the same elution profile as the Pro_5Δhinge SH3GK protein (data not shown). Second, we made an analogous Pro_5 insertion mutant, without completely deleting the hinge region (Pro_5+hinge). This variant failed to dimerize, presumably because the remaining hinge sequence retains sufficient flexibility. We conclude that the overall rigidity of the hinge region, and not the specific Pro_5 sequence element, is critical for inducing oligomerization.

Conclusion

A Model of Regulated Assembly by MAGUKs

Assembly of ordered structures such as the PSD requires interaction between many proteins. A fundamental question is how these interactions are coordinated and regulated to achieve spatial and temporal specificity. Why do these scaffolds only assemble at the correct membrane sites? Previous studies have suggested that the intermolecular SH3GK interactions may contribute to MAGUK scaffolding (Nix et al., 2000), and that this process may be regulated by extrinsic factors (Masuko et al., 1999). Using the structure of the PSD-95 SH3GK module, we refine this model by proposing that the intermolecular interactions observed for MAGUKs are mediated by 3D domain swapping of structural components of the split SH3 fold. We hypothesize that ligand binding may constrain the flexibility of the hinge region, thereby promoting the switch from intra- to intermolecular assembly (Fig. 2-7).

This model offers potential advantages as a scaffolding mechanism. First, because 3D domain swapping regenerates complete folds, this mechanism facilitates oligomerization without occluding sites on the SH3 and GK folds that may bind associated signaling proteins. Second, regulatory proteins with the appropriate subcellular localization could direct the correct temporal and spatial assembly of interlocked MAGUK networks. Third, heteromeric 3D domain swapping of MAGUKs, perhaps directed by sets of regulatory proteins, could provide combinatorial scaffold diversity, which could specify differential protein recruitment. This model of regulated assembly is consistent with the function of MAGUK proteins and is one of several

mechanisms that may participate in the proper assembly of supramolecular signaling complexes at cell junctions.

The structure of the SH3GK module reveals how a simple, modular protein fold, such as the SH3 fold, can be used in diverse modes to mediate protein complex assembly. This module provides one of the first structural examples in which normally discrete protein domains have evolved into an integrated functional unit.

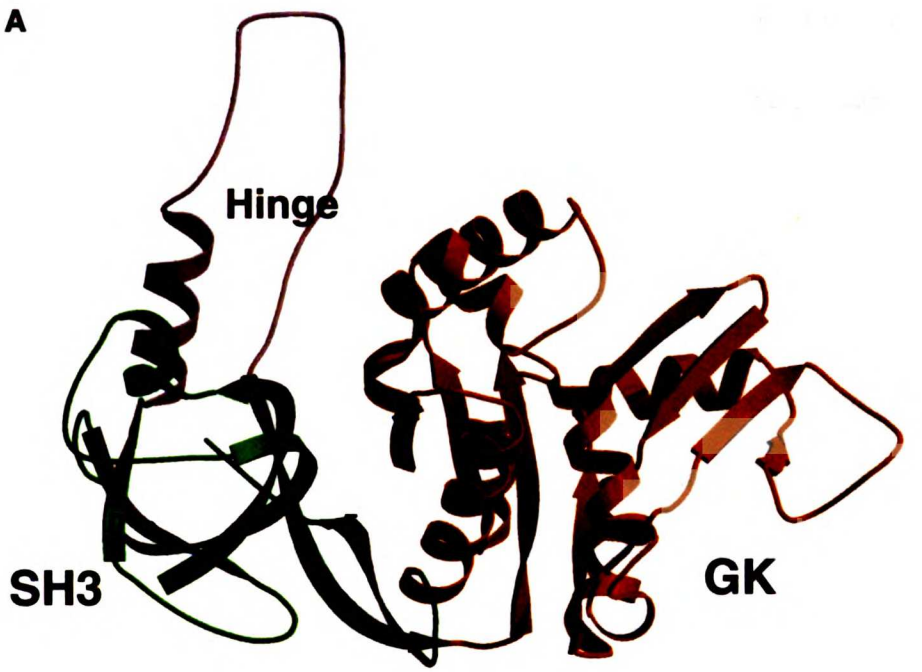
Table 1. Data Collection and Refinement Statistics

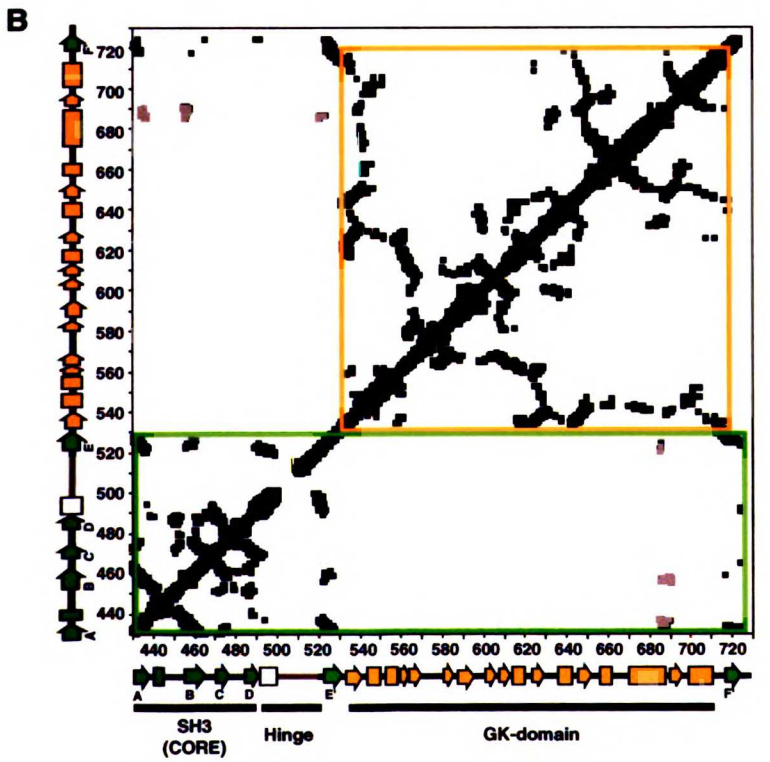
MAD Phasing				
Energies	No. of Reflections (Total/Unique)	Completeness (%)[a]	Overall I/σ	Rsym[a,b]
λ_1 12657	164,138/17,911	98.5 (88.8)	10.5	10.2 (45.0)
λ_2 12655	165,419/18,051	98.8 (90.2)	11.1	8.4 (34.6)
λ_3 12800	168,966/18,187	99.0 (92.2)	9.3	15.7 (42.0)
Mean overall figure of merit (30.0-2.3 Å) (centric/acentric) = 0.52/0.65.				
Native (30.0 – 1.8 Å)				
	294,814/37,032	99.4 (94.4)	11.1	7.7 (35.1)

Refinement and Stereochemical Statistics (All Data 30.0 – 1.8 Å)	
R value	23%
Free R value	25%
Solvent Molecules	108
Average B Factors (Å^2)	
PSD95 430-724	34
RMS Deviations	
Bonds (Å)	0.005
Angles (°)	1.08

[a]Statistics for highest resolution shell (1.9 - 1.8 Å) in parentheses.

[b]$R_{sym} = \Sigma |I-\langle I\rangle|/\Sigma I$, where I is the integrated intensity of a given reflection.


A
Hinge
SH3
GK


B
720
700
680
660
640
620
600
580
560
540
520
500
480
460
440
440 460 480 500 520 540 560 580 600 620 640 660 680 700 720
A B C D E F
SH3
(CORE)
Hinge
GK-domain

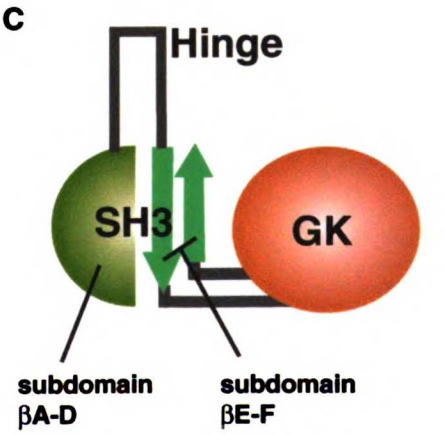
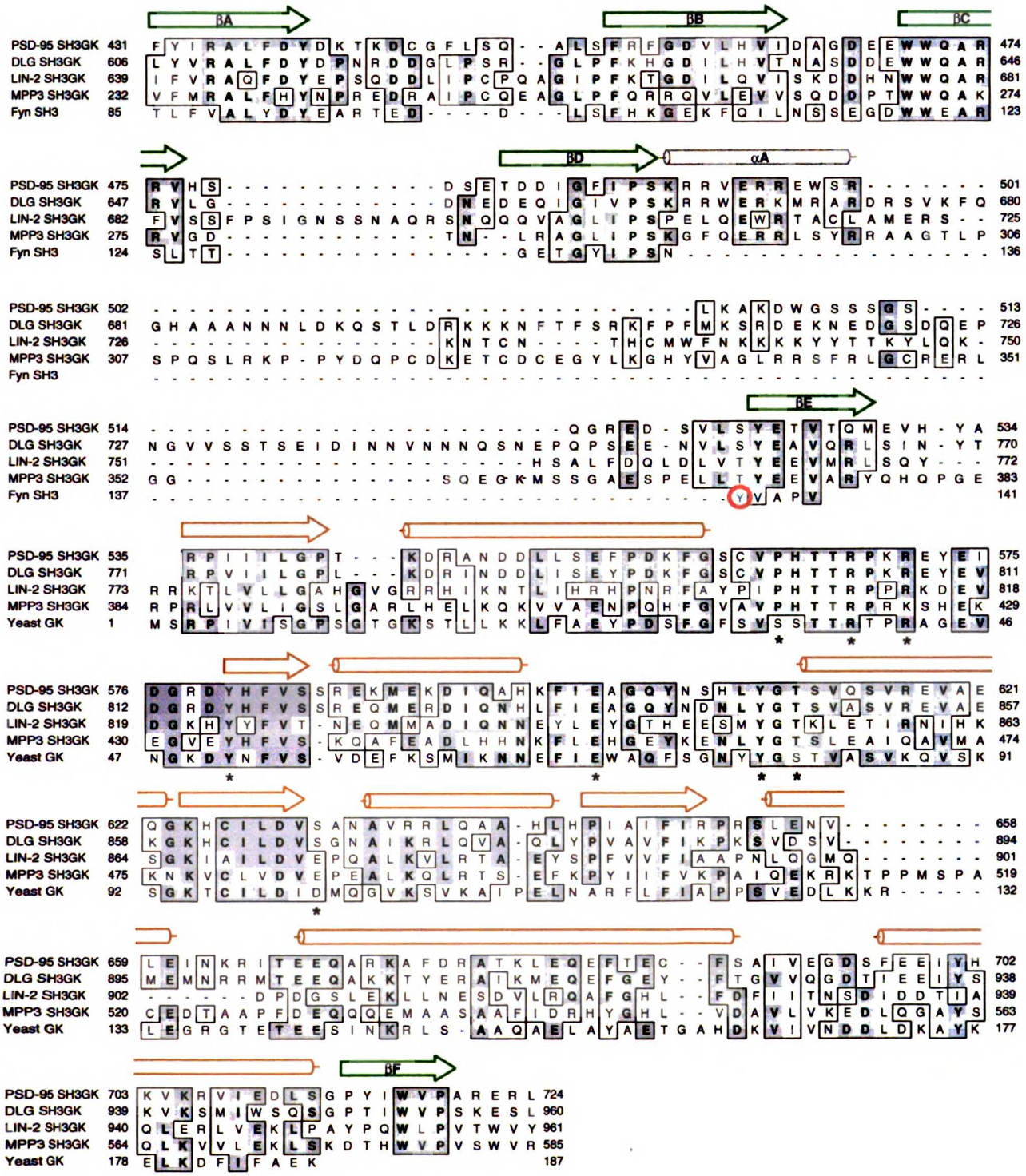

C
Hinge
SH3
GK
subdomain
βA-D
subdomain
βE-F

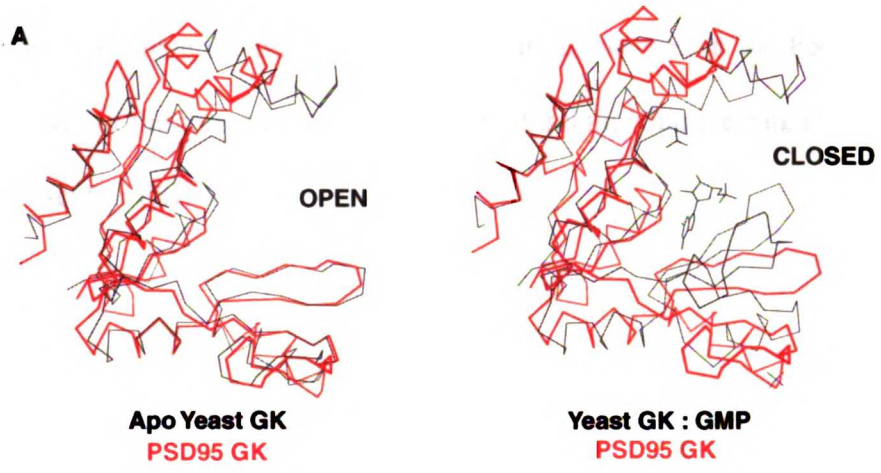
Figure 2-1. Structure of the SH3GK module of PSD-95. (A) Ribbon diagram of the SH3 (green) and guanylate kinase-like (orange) domains of PSD-95. The HINGE (grey) is a large insert containing an α-helix that separates structural components of the SH3 fold. (B) A Cα contact map of the SH3GK module of PSD-95 reveals that β-strand E and β-strand F make more numerous and closer contacts with structural components of the SH3. Map indicates pairs of Cα atoms that are <10 Å apart. Map was generated with CNS (Brünger et al., 1998). The rectangles outlined in orange and green outline the GK and SH3 folds, respectively. The structure shows remarkably few interdomain contacts, which are colored in grey. (C) A schematic representation of the SH3GK module. The hinge divides the SH3 domain into two subdomains: a core of four β-strands A-D (green half-circle), and a pair of β-strands (E and F, light green arrows) flanking the GK domain (orange circle). Panel A was generated using MOLSCRIPT and RASTER3D.


βA
βB
βC
βD
αA
βE
βF
PSD-95 SH3GK
DLG SH3GK
LIN-2 SH3GK
MPP3 SH3GK
Fyn SH3
Yeast GK

Library
Withdrawn

Figure 2-2. Sequence alignment of SH3GK modules from MAGUKs. Sequences corresponding to the SH3GK regions of Rat PSD-95, *Drosophila* Discs Large (DLG), *C.elegans* LIN-2 and human palmitolylated membrane protein 3 (MPP3), a p55 subfamily member, were aligned using the program CLUSTALW. For comparison, the sequences of the canonical SH3 domain from Fyn and yeast guanylate kinase were also aligned, using the structure as a guide. Amino acid identities are shaded, and similarities are boxed. Secondary structure elements for the SH3 and GK domains of PSD-95 are shown above the sequences, with β-strands as arrows and α-helices as cylinders. The βE strand of Fyn SH3 has been displaced to align with the structurally analogous sequence in PSD-95. The tyrosine in this β-strand that is critical for binding poly-proline ligands is circled in red. Although this residue is conserved in canonical SH3 domains, it is absent in the MAGUK sequences. The residues of yeast guanylate kinase (yeast GK) that bind GMP are marked with asterisks.

Library
Withdrawn


A
OPEN
CLOSED
Apo Yeast GK
PSD95 GK
Yeast GK : GMP
PSD95 GK

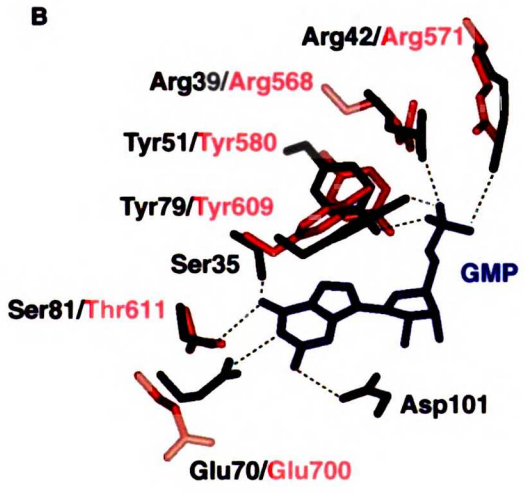

B
Arg42/Arg571
Arg39/Arg568
Tyr51/Tyr580
Tyr79/Tyr609
Ser35
GMP
Ser81/Thr611
Asp101
Glu70/Glu700

Figure 2-3. The PSD-95 GK domain structurally resembles apo-yeast guanylate kinase but does not bind GMP. (A) Comparison of the PSD-95 GK domain structure (red) with the apo-yeast guanylate kinase structure (black) (top; RMSD = 1.49 Å) and with the yeast guanylate kinase structure in complex with guanosine monophosphate (GMP) (black) (bottom; RMSD = 1.94 Å). (B) The five residues of yeast guanylate kinase that coordinate the phosphate in GMP are conserved in the PSD-95 GK domain. However, two of the four residues of YGK that bind the guanine ring (Ser35 and Asp101) are not present in PSD-95.

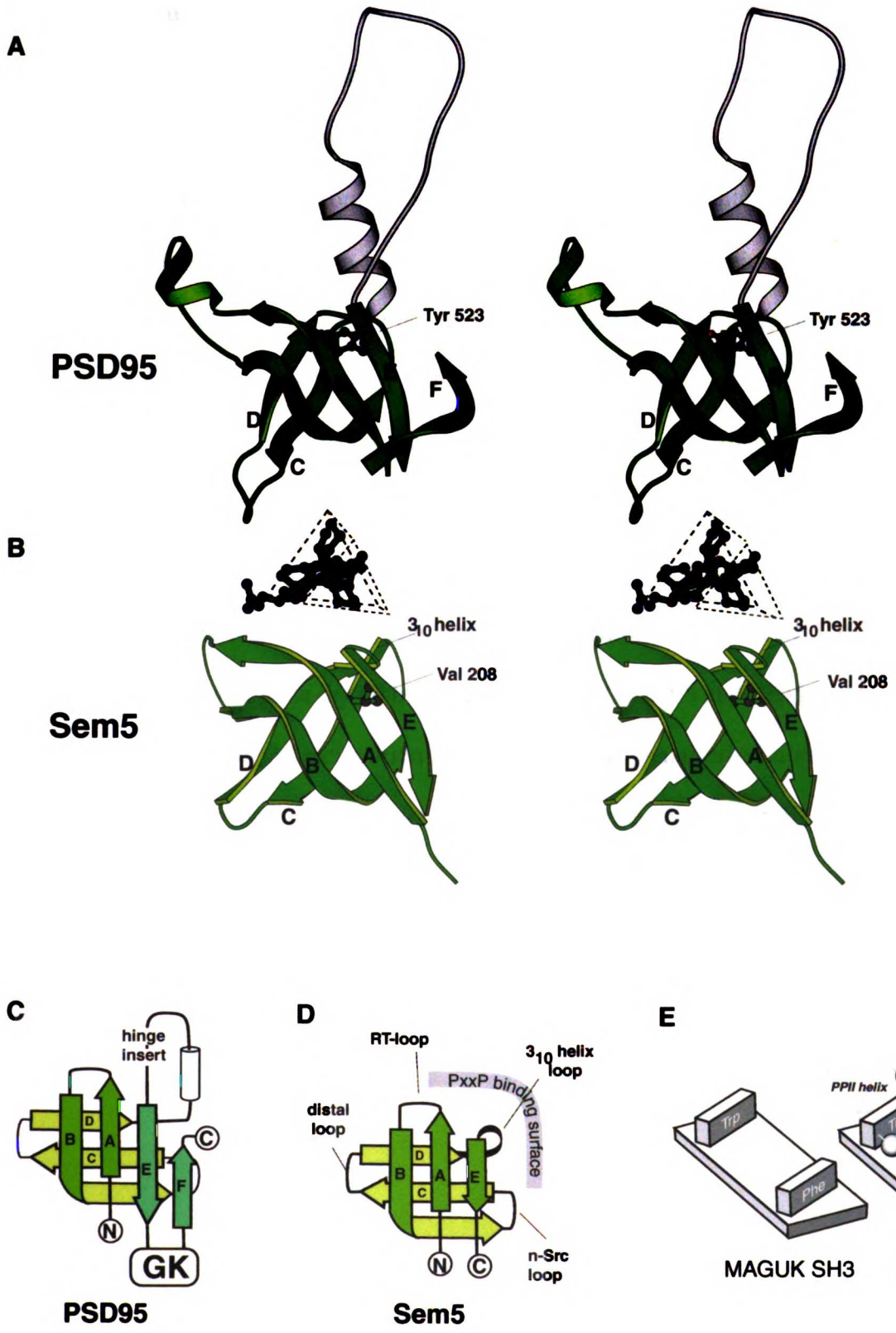
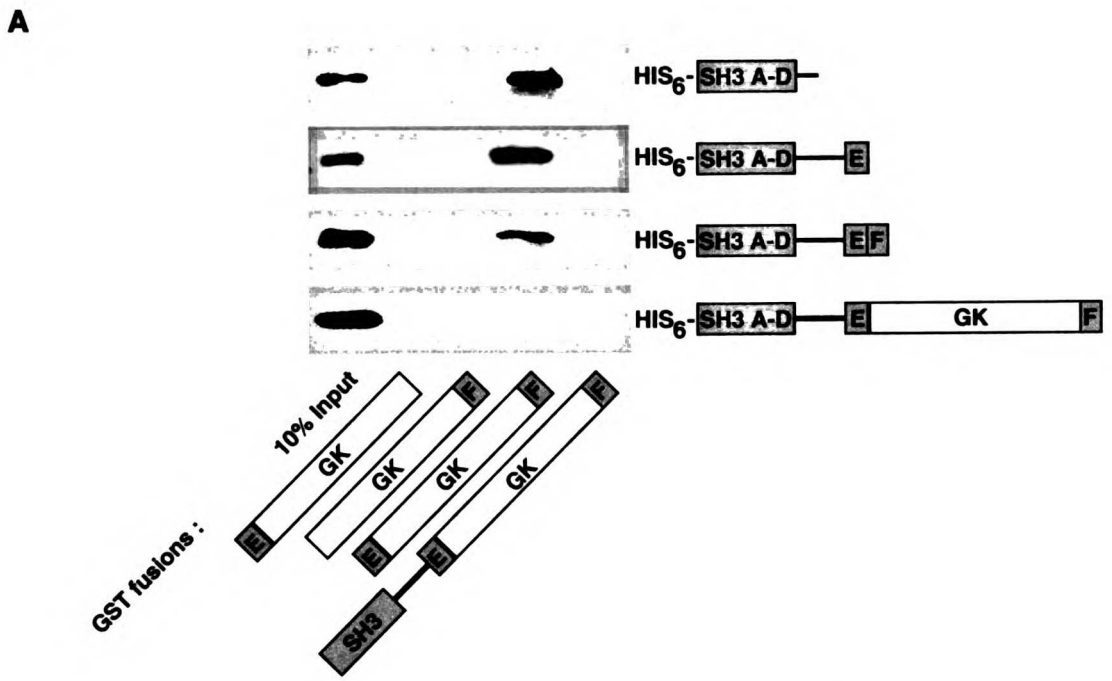
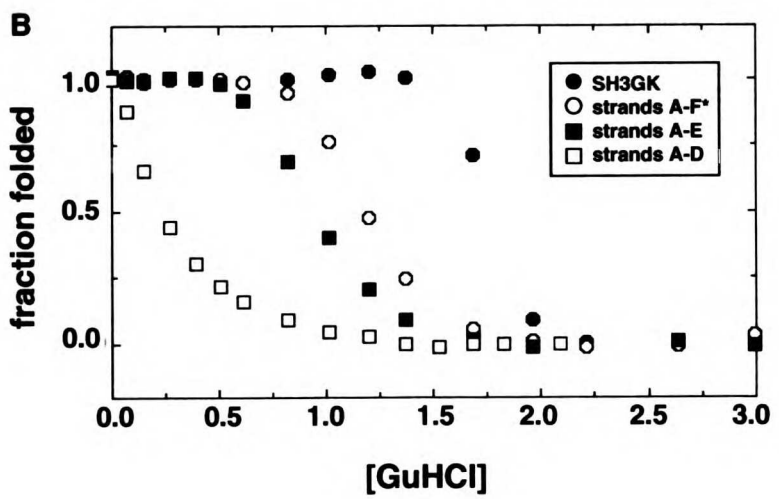

A
PSD95
Tyr 523
F
D
C
Tyr 523
F
D
C
B
Sem5
3 10 helix
Val 208
E
D
B
A
C
3 10 helix
Val 208
E
D
B
A
C
C
hinge insert
D
B
A
C
E
F
C
N
GK
PSD95
D
RT-loop
3 10 helix loop
PxxP binding surface
distal loop
D
B
A
C
E
N
C
n-Src loop
Sem5
E
PPII helix
required prolines
Trp
Phe
Trp
Tyr
Phe
MAGUK SH3
canonical SH3

Figure 2-4. Comparison of the PSD-95 split SH3 structure with a canonical SH3 domain structure. (A) Stereo ribbon diagrams of the PSD-95 SH3 fold (upper), including the intervening hinge region and the additional β-strand F, and the C-terminal SH3 domain of SEM-5 (lower) complexed with a peptide ligand (Lim et al., 1994). In PSD-95, a tyrosine (523) from the separated E β-strand packs into the hydrophobic core of the SH3 fold, in place of a valine normally observed in canonical SH3 domains (Val 208 in SEM-5).. The position of the 3_{10} helix in SEM-5 is indicated. The beta strands for PSD-95 and SEM-5 are labeled A-F and A-E, respectively. (B) Secondary structure diagrams of PSD-95 and SEM-5 reveal the conserved overall topology. The hinge region in PSD-95, which separates β-strands D and E, occludes the canonical peptide binding surface. The GK domain is inserted between β-strands E and F. The positions of the various loops and the conserved segment of 3_{10} helix are shown in SEM-5 (C) Schematic comparison of the peptide ligand binding surfaces for PSD-95 and SEM-5. Aromatic side chains critical for ligand recognition are represented as planar projections. PSD-95 lacks the central conserved tyrosine residue. A docked polyproline II (PPII) helical ligand is shown for the canonical SH3 domain.


A
HIS6-SH3 A-D
HIS6-SH3 A-D E
HIS6-SH3 A-D E F
HIS6-SH3 A-D E GK F
10% Input
GST fusions :
GK
SH3


B
fraction folded
1.0
0.5
0.0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
[GuHCl]
SH3GK
strands A-F*
strands A-E
strands A-D

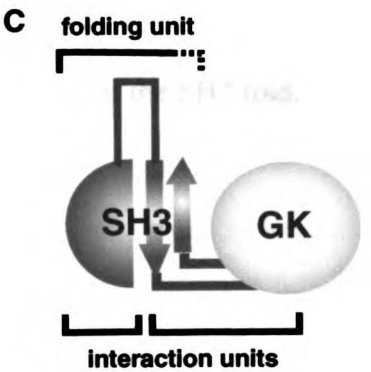

C
folding unit
SH3
GK
interaction units

Figure 2-5. The SH3-GK interaction involves reassembly of the split SH3 fold.

(A) GST pulldowns demonstrate that the SH3-GK interaction requires that β-strands E and F are contiguous with the GK fold. GST-fusions of the GK domain containing one or both of the flanking β-strands E and F or GST-SH3GK were incubated with His-tagged SH3 proteins that contained β-strands A-D, A-E, A-F* (with β-strand F attached by a $(Gly\text{-}Ser)_3$ linker) or the complete SH3GK module. Only GST-GK fusion proteins containing β-strands E and F bound the His_6-SH3 proteins that did not contain GK. (B) Stability of corresponding SH3 fragments to guanidine hydrochloride denaturation was monitored by tryptophan fluorescence. Fragments lacking the E and F β-strands are not stably folded. Addition of β-strands E and F sequentially stabilizes the fold. Stability is most significantly increased by the addition of β-strands E, F, and the GK fold. (C) A schematic comparison of the differences between the folding and interacting units within the SH3GK module. The SH3-GK interaction requires that β-strands E and F be attached to the GK fold. However, these two strands are structural components of the SH3 fold.

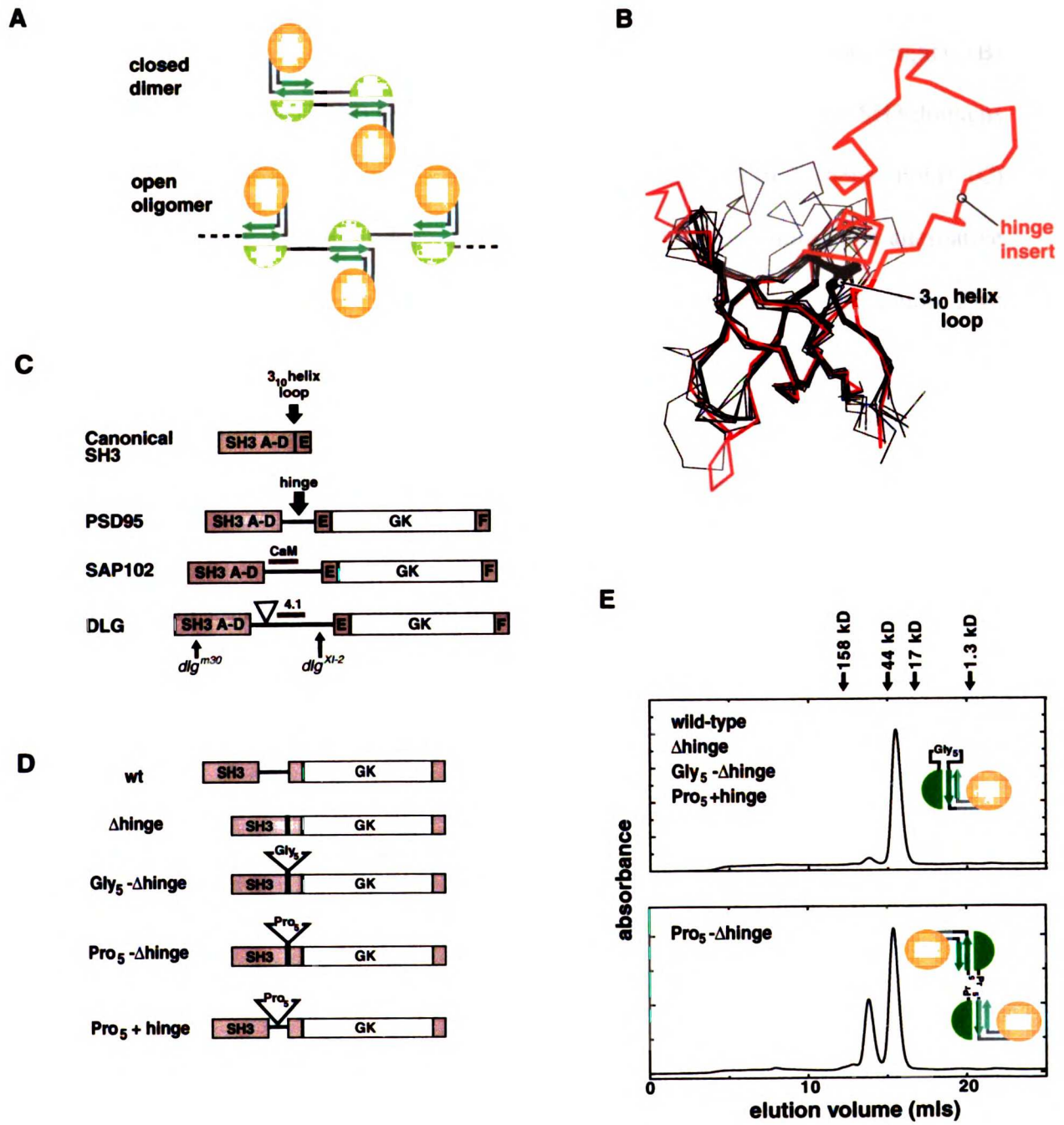

A
closed dimer
open oligomer
B
hinge insert
3₁₀ helix loop
C
3₁₀ helix loop
Canonical SH3
SH3 A-D E
hinge
PSD95
SH3 A-D E GK F
CaM
SAP102
SH3 A-D E GK F
4.1
DLG
SH3 A-D E GK F
dlg m30
dlg X1-2
D
wt
SH3 GK
Δhinge
SH3 GK
Gly5 -Δhinge
SH3 GK
Pro5 -Δhinge
SH3 GK
Pro5 + hinge
SH3 GK
E
158 kD
44 kD
17 kD
1.3 kD
wild-type
Δhinge
Gly5 -Δhinge
Pro5 +hinge
Pro5 -Δhinge
absorbance
0
10
20
elution volume (mls)

Figure 2-6. The Hinge region is variable and can modulate inter- vs. intramolecular SH3 assembly. (A) Schematic representations of intramolecular SH3 assembly (upper), a 3D domain swapped open oligomeric chain (left) and a closed dimer (right). (B) Alignment of the PSD-95 SH3 fold (red) with the structures of 8 canonical SH3 domains (black) shows that the hinge insert replaces a conserved segment of 3_{10} helix (bold). (C) The hinge region is variable in length and is the site of protein binding and alternative splicing. Calmodulin and protein 4.1 bind some MAGUKs within the hinge region. Two mutations in *Drosophila* DLG that are capable of interallelic complementation are indicated with arrows. (D) Schematic representations of the SH3GK mutant proteins used to probe the role of the hinge region in SH3 3D-domain swapped assembly. (E) Elution profiles for the proteins diagrammed in (D). Unlike the wt and other mutants, the Pro_5-Δhinge protein elutes with a significant peak corresponding to the dimer species.

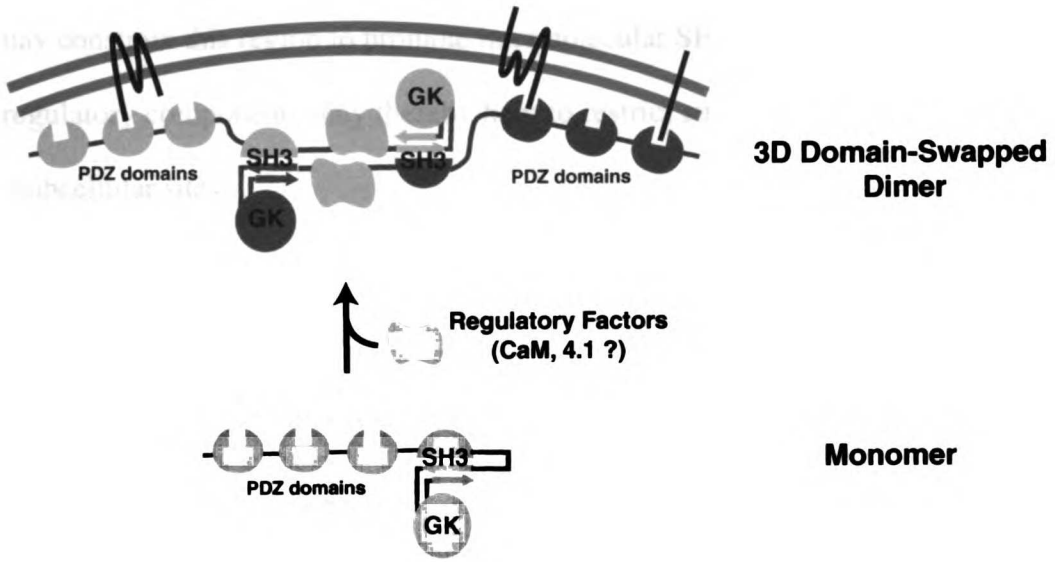

GK
PDZ domains
SH3
SH3
PDZ domains
GK
3D Domain-Swapped Dimer
Regulatory Factors (CaM, 4.1 ?)
SH3
PDZ domains
GK
Monomer

Figure 2-7. Model for role of SH3GK module in regulated assembly - ligand binding to the hinge region may promote oligomerization by 3D-domain swapping.

Proteins binding to the hinge region (red), such as calmodulin, protein 4.1 or unknown factors, may constrain this region to promote intermolecular SH3 assembly. Localization of these regulatory components may thereby help to restrict supramolecular assembly to specified subcellular sites.

UNIVERSITY OF CALIFORNIA
SOUTHERN REGIONAL LIBRARY FACILITY

Experimental Procedures

Antibodies

Monoclonal antibodies to PSD-95 (clone 7E3-18; Affinity Bioreagents), GFP (Clontech), the His_6-tag (Omni-probe(M21)) (Santa Cruz Biotechnology, Inc.) and polyclonal antibodies to GKAP (Kim et al., 1997), actinin associated LIM protein (ALP) (Xia et al., 1997), and the GAL4 DNA binding domain (Santa Cruz Biotech) have been previously characterized. A polyclonal antiserum to the PDZ domains of PSD-95 was raised by immunizing a sheep with a GST fusion protein of amino acids 1-386 of rat PSD-95. Polyclonal antisera to the GK domain of PSD-95 were raised by injecting rabbits with a 14-mer peptide (amino acids 698-711 of PSD-95) coupled to KLH. Polyclonal antisera to GFP were raised by injecting guinea pigs with a GST fusion of GFP. All antisera were affinity purified on Affigel-10 columns charged with the immunizing antigen.

Construction of cDNA plasmids

For yeast two-hybrid experiments domains of PSD-95 were amplified by PCR with primers containing endonuclease restriction sites, and PCR products were restricted with appropriate enzymes and ligated into pGBT9 or pGAD424 (Clontech). All constructs were sequenced to confirm that inserted nucleotide sequences were correct. The primers for PSD-95 sequences are as follows: PSD-95 SH3 sense 5'-CGG-GAA-CAG-CTC-ATG-AAT-3'; PSD-95 SH3 antisense 5'-GGA-GCC-CCA-GTC-CTT-GGC-3'; PSD-95 GK sense 5'-AAG-GCC-AAG-GAC-TGG-GGC-TCC-3'; PSD-95 GK antisense 5'-CTA-GAG-TCT-CTC-TCG-GGC-TGG-3'; PSD-95 GK611 sense 5'-ACC-AGC-GTC-

CAG-TCT-GTG-3'. The constructs containing the W470F mutation were assembled by sequential PCR with a codon changed in both the sense and antisense primer from W = TGG to F = TTT: sense 5'-GAA-GAG-TTT-TGG-CAA-GCA-CGG-CGG-GTG-CAC-TCC-3'; antisense 5'-GGA-GTG-CAC-CCG-CCG-TGC-TTG-CCA-AAA-CTC-TTC-3'. The SH3 (408-560) constructs (with or without point mutations) were subcloned by excising the SH3 domain at the EcoRI site in the polylinker and the BamHI site in the GK domain from the SH3GK construct and ligating into pGBT9 at EcoRI / BamHI. The constructs containing the SH3 L460P mutation were made by inverse PCR using SAP90:pGEX4T-1 as a template with the following primers: sense 5'-CAT-GTC-ATC-GAT-GCT-GGT-GAC-GAA-GAG-TGG-TGG-CAA-GCA-3'; antisense 5'-ACC-AGC-ATC-GAT-GAC-ATG-AGG-CAC-ATC-CCC-GAA-GCG-GAA-3'. Yeast two-hybrid constructs were then made by using this plasmid as a template for the 95SH3 sense and anti-sense primers described above. GKΔN109 was constructed by subcloning the DNA fragment between the BamHI site in the PSD-95 GK domain and the BamHI site in the polylinker into the BamHI site in pGBT9.

Sequences from DLG, LIN-2, GKAP and nNOS were also amplified by PCR with primers containing endonuclease restriction sites, restricted with the appropriate enzymes and ligated into pGBT9 or pGAD424. The primers for DLG sequences are as follows: DLG GK sense 5'-GCA-GCT-AAT-AAT-AAT-CGT-GAT-AAG-3'; DLG GK antisense 5'-TCA-TAG-AGA-TTC-CTT-GGA-AGG-3'; DLG SH3 sense 5'-AAA-CAA-CAG-GCT-GCC-CTC-3'; DLG SH3 antisense 5'-CTA-CTT-ATC-CAG-ATT-ATT-ATT-AGC-TGC-3'. The primers for LIN-2 sequences are as follows: LIN-2 SH3 sense 5'-GAT-GCT-CGA-GGT-CAA-GTC-3'; LIN-2 SH3 antisense 5'-CTA-GAA-CCA-CAT-

GCA-GTG-AGT-3'; LIN-2 GK sense 5'-ACT-CAC-TGC-ATG-TGG-TTC-3'; LIN-2 GK antisense 5'-TCA-GTA-GAC-CCA-AGT-GAC-TGG-3'. The primers for GKAP sequences are as follows: GKAP sense 5'-ATG-ATC-GAC-CTT-TTT-AAG-GCT-3'; GKAP antisense 5'-CTG-TAT-CCC-AAT-AGA-TAG-GCA-3'. The nNOS and PSD-95 PDZ1-3 constructs were previously described (Brenman et al., 1996). The C-terminal GFP fusion constructs were subcloned by ligating a PCR amplified sequence of PSD-95 SH3 or GK (with the primers described above) into EGFP-C2 (Clontech).

Yeast two-hybrid analysis

Yeast co-transformation, colony lift β-galactosidase filter assays, and ONPG liquid culture assays were done as described in the Matchmaker Library protocol (Clontech) with the yeast strain HF7c or SFY526. Growth on His(-) was tested by streaking several individual colonies to -LWH plates. Positives were scored by the presence of individual colonies after 3 days at 30°C.

Cell transfection

HEK 293 cells were transiently transfected with EGFP-C2 constructs using Lipofectamine Plus (Life Technologies). Transfections were done in six-well culture plates with cells at 50%-80% confluence. Two μg of plasmid DNA were used for each transfection. Six hours after transformation, the media was changed to DMEM-21 plus 10% heat inactivated fetal bovine serum, and the cells were harvested 48 hours later.

***In vitro* binding assays with Affinity-tagged Fusion Proteins**

In Chapter One, to test for interactions between SH3 and GK domains *in vitro*, GST fusion proteins were incubated with HEK cell extracts expressing GFP fusion proteins. GST fusion proteins were expressed and purified as previously described (Brenman et al., 1995). Extracts from transfected HEK 293 cells were prepared from a six-well plate. Cells were washed with 1 ml of PBS and then scraped from the substrate into resuspension buffer: 25 mM TrisHCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 5 mM EGTA. Cells were sheared with 10 passes through a 25-gauge needle. PMSF was added to 1 mM and Triton X-100 added to 1%. The solution was gently agitated at 4°C for 30 minutes and then centrifuged 20 min at 14,000 rpm in a microcentrifuge. The soluble fraction of the extracts was removed to new tubes. Three μg of GST protein coupled to Sepharose beads was added and the samples were incubated at 4°C for 60 minutes. The protein-coupled GST beads were pelleted by centrifugation and then washed 5 times with 1 ml of resuspension buffer. The beads were resuspended in 5X protein loading buffer and samples separated by SDS-PAGE with 12% acrylamide gels, transferred to PVDF membranes (Millipore) and analyzed by western blotting as previously described (Brenman et al., 1996).

In Chapter Two, sequences encoding various regions of PSD-95 were amplified by PCR and cloned into either pGEX 4T-1 (Pharmacia) or a His-tagged expression vector (Hillier et al., 1999). The amino acid sequences corresponding to following constructs are in parentheses: His_6-SH3 A—D (417—490), His_6-SH3 A—E (417—532), His_6-SH3 A—F* (417—532-$(GS)_3$-712—724, His_6-SH3-GK (417—724), GST-E-GK (523—711),

GST-GK-F (532—724), GST-E-GK-F (523—724), GST-SH3-GK (417—724), GST-Δhinge (417—490-(Δ)-521—724), GST-Gly_5Δhinge (417—490-$(G)_5$-521—724), GST-Pro_5Δhinge (417—490-$(P)_5$-521—724), GST-Pro_5+hinge (417—502-$(P)_5$-514—724). GST fusion proteins and His-tagged proteins were expressed and purified as described (Brenman et al., 1996). The His-tagged proteins were diluted to 0.1 mg/ml with elution buffer (200 mM imidazole, 50 mM Na-PO_4 (pH 8.0), 300 mM NaCl, 10% glycerol) containing 1 mM DTT. Approximately 10 μg of GST-fusion protein was coupled to glutathione-Sepharose (Pharmacia) beads and incubated with 250 μl (25μg) His-tagged proteins at 4° C for 30 minutes. The beads were washed extensively with a phosphate buffered saline containing 300 mM NaCl, 2 mM EDTA, 2 mM EGTA and 1 mM DTT. Retained proteins were eluted with SDS protein loading buffer, separated by PAGE, and analyzed by immunoblotting.

Immunoprecipitations

Adult rat brain was homogenized in 20 volumes resuspension buffer. After centrifugation at 15,000g to remove the soluble fraction, membranes were solubilized for 30 minutes in resuspension buffer + 0.5% Triton (Triton Extracts) or + 0.2% SDS (SDS extracts). Triton X-100 was then added to 1% to the SDS extracts to sequester the ionic detergent. Solubilized proteins were recovered by centrifuging the extracts at 100,000g for 30 minutes. Immunoprecipitations were performed by first adding the appropriate antibody, and then, after a 60-minute incubation at 4°C, adding 20 μl of protein G Sepharose to precipitate the antibodies. The protein G beads were then washed five times with resuspension buffer and immunoprecipitated proteins were recovered in 5X protein

UNCLASSIFIED

loading buffer. Immunoprecipitates were resolved by 10% SDS-PAGE and analyzed by immunoblotting.

Protein Expression and Purification

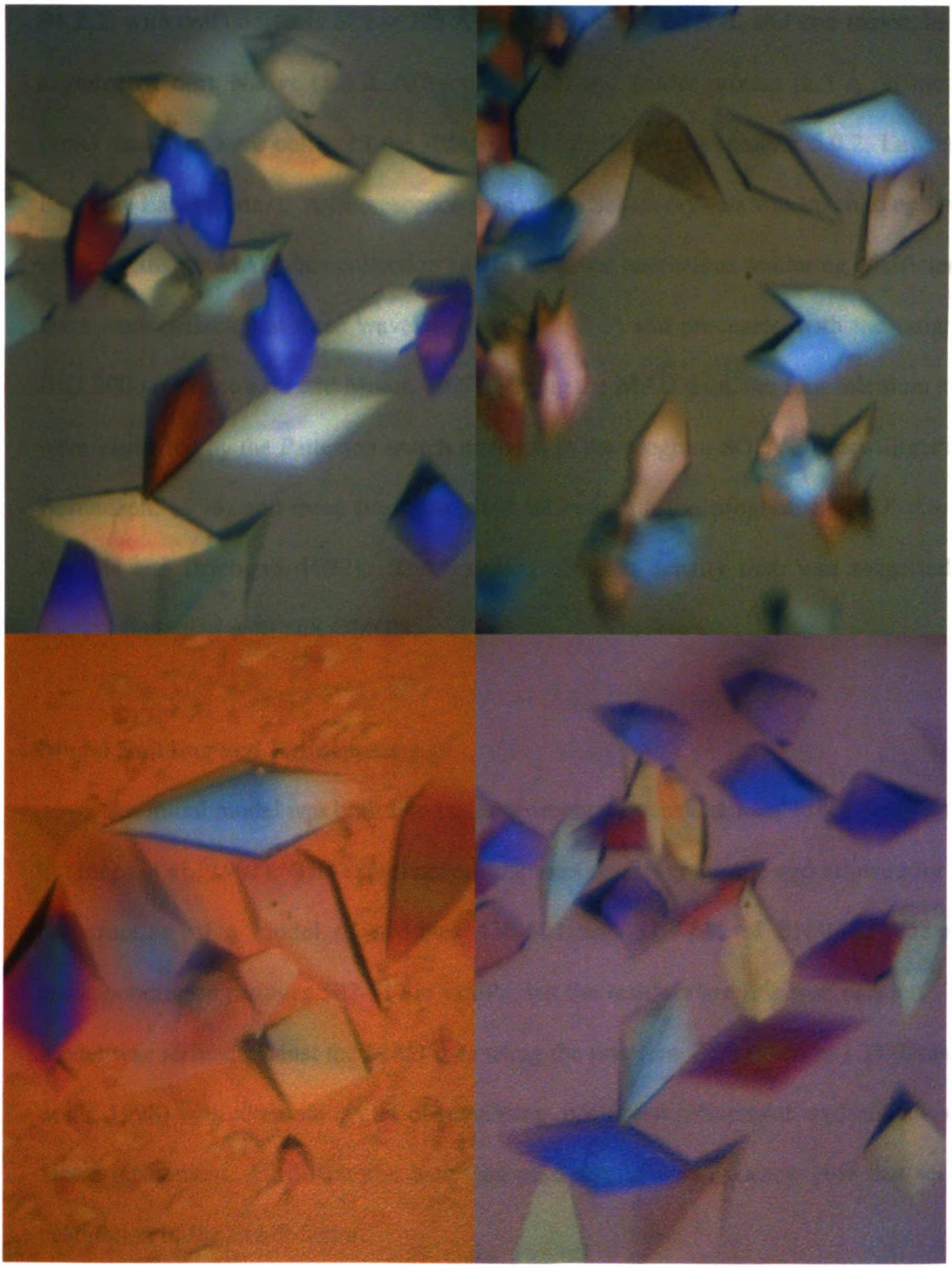
The DNA sequence encoding residues 417 to 724 of rat PSD-95 (SH3-GK) was amplified by PCR and cloned into a His-tagged expression vector containing a TEV site that permits proteolytic removal of the tag (Hillier et al., 1999). Twelve liters of *E. coli* strain BL21 (DE3; pLysS) expressing SH3GK were grown to OD_{600} = 0.8, induced with 1 mM IPTG for 3 hr, harvested by centrifugation, freeze-thawed with liquid nitrogen, and lysed by sonication in 240 ml of 50 mM Na-PO_4 (pH 8.0), 300 mM NaCl, 10% glycerol. Protein was purified on 12 ml Ni-NTA resin (Qiagen) by elution with 200 mM imidazole following extensive washing with 50 mM Na-PO_4 (pH 6.5), 300 mM NaCl, 10% glycerol, and 20 mM imidazole, 50 mM Na-PO_4 (pH 8.0), 300 mM NaCl, 10% glycerol. The protein was dialyzed against 100 mM Tris-HCl, 400 mM NaCl, 10% glycerol, 2mM DTT and the His-tag was removed by cleavage with TEV protease. Following dialysis into 25 mM Tris-HCl (pH 8.0), final purification was achieved by chromatography on a 10ml Resource Q column (Pharmacia) that was eluted with a gradient of 0-150 mM NaCl in 25 mM Tris-HCl (pH 8.0), 1 mM DTT. Protein was dialyzed against 10 mM HEPES (pH 8.0), 100 mM NaCl, 2 mM DTT and concentrated to 10 mg ml^{-1} by ultrafiltration (Amicon). Purity of >99% was established by SDS-PAGE, mass spectroscopy and isoelectric focusing gel electrophoresis. For the MAD data set, two additional methionines were inserted into the SH3GK expression construct used for protein production. Residues T543 and A547 were both mutated to methionine. Both these

LIBRARY

mutations were conservative as T543 is a methionine and A547 is a valine or isoleucine in closely related MAGUKs. Selenomethionine (SeMet)-labeled SH3GK was produced by growth in minimal media supplemented with selenomethionine (Van Duyne et al., 1993) and purified in buffers that were purged with N_2 to prevent selenium oxidation. Labeling was confirmed by mass spectroscopy.

Crystallographic Methods

Small bi-pyramidal crystals of SH3GK were grown by vapor diffusion in hanging drops (6 mg/ ml) in 2 M ammonium sulfate, 2% PEG4000, 10 mM HEPES pH 7.0 at 15°C (buffer 39; Hampton Research); larger crystals were grown by microseeding in sitting drops. For microseeding, 6 µl of SH3GK (10 mg ml^{-1}) was mixed with 4 µl of buffer 39 and were seeded in serial dilution with microcrystals obtained by crushing a previously obtained crystal. Following 2 weeks of growth, average crystal dimensions were 150 x 150 x 450 µm. A representative cluster of crystals is shown on the next page. Prior to data collection, crystals were transferred to a cryoprotectant solution containing mother liquor and 20% glycerol, then flash frozen in liquid nitrogen.



Crystals of both the native and SeMet protein grew in space group space group $P4_12_12$, with cell constants of a = 205 Å, b = 60.5 Å, c = 60.5 Å, and one molecule per asymmetric unit. Native (1.8 Å diffraction limit) and SeMet protein (2.3 Å diffraction limit) data sets were collected the Advanced Light Source (beamline 5.0.2, Lawrence Berkeley Laboratories). A fluorescence scan of the SeMet crystals was taken to optimize wavelengths for MAD data collection and to calculate anomalous scattering coefficients. Data were collected at three wavelengths (Table 2-1) and processed with the program HKL2000 (Otwinowski and Minor, 1997). Using the MAD data, the four selenium sites were identified by the Patterson search methods in the program SOLVE (Terwilliger and Berendzen, 1996) and these positions were refined with the program SHARP (De La Fortelle and Bricogne, 1997). The resulting electron density map was subjected to solvent flattening with SOLOMON.

Model Building and Refinement

An initial model was built into the experimental electron density using the program O (Jones et al., 1991). The experimental map was readily traceable and allowed for the construction of a model of residues 430-501 and 518-724 of SH3GK. Density corresponding to residues 502-517 is visible, but the residues are less well ordered. The model was refined against the MAD data using the program CNS (version 1.1) (Brünger et al., 1998) with alternate cycles of rebuilding, positional refinement, and restrained B factor refinement. All diffraction data was used throughout, except a 10% test set for calculation of the free R factor.

Guanidine hydrochloride denaturation

Stability of SH3 constructs to guanidine hydrochloride (GuHCl) denaturation was measured as previously described (Lim et al., 1994). The folded SH3 structure has a buried tryptophan residue (W471) within its hydrophobic core but the GK domain has no tryptophan residues. Unfolding was monitored by tryptophan fluorescence (excitation 295 nM; emission 340 nM) at 25 °C. Assays were performed using a PTI fluorimeter with His_6-tagged protein at a concentration 1 μM in 50 mM Tris HCl pH 7.5, 50 mM NaCl. ΔG of unfolding was calculated as described assuming a two-state model for unfolding. The construct His_6-SH3 A-D showed no folded state baseline, indicating that at 25 °C, the protein was already partially unfolded. The fluorescence at 0 M GuHCL was therefore used as an estimate for the upper limit corresponding to folded protein.

Gel filtration

GST fusion proteins were purified on glutathione-sepharose, cleaved from the beads with thrombin (Sigma), concentrated to 5 mg/ml by ultrafiltration in Centricon units, and ultracentrifuged to remove aggregates. Samples (100μl) were chromatographed on a Superdex 200 column by isocratic elution with 50 mM Tris pH 7.5, 150 mM NaCl and 1 mM DTT at a flow rate of 0.5 ml/min.

References

Allison, D. W., Chervin, A. S., Gelfand, V. I., and Craig, A. M. (2000). Postsynaptic scaffolds of excitatory and inhibitory synapses in hippocampal neurons: maintenance of core components independent of actin filaments and microtubules. Journal of Neuroscience *20*, 4545-54.

Allison, D. W., Gelfand, V. I., Spector, I., and Craig, A. M. (1998). Role of actin in anchoring postsynaptic receptors in cultured hippocampal neurons: differential attachment of NMDA versus AMPA receptors. Journal of Neuroscience *18*, 2423-36.

Andreotti, A. H., Bunnell, S. C., Feng, S., Berg, L. J., and Schreiber, S. L. (1997). Regulatory intramolecular association in a tyrosine kinase of the Tec family. Nature *385*, 93-7.

Arnold, D. B., and Clapham, D. E. (1999). Molecular determinants for subcellular localization of PSD-95 with an interacting K+ channel. Neuron *23*, 149-157.

Bellaïche, Y., Radovic, A., Woods, D. F., Hough, C. D., Parmentier, M. L., O'Kane, C. J., Bryant, P. J., and Schweisguth, F. (2001). The Partner of Inscuteable/Discs-large complex is required to establish planar polarity during asymmetric cell division in Drosophila. Cell *106*, 355-66.

Bennett, M. J., Schlunegger, M. P., and Eisenberg, D. (1995). 3D domain swapping: a mechanism for oligomer assembly. Protein Science *4*, 2455-68.

Birge, R. B., Knudsen, B. S., Besser, D., and Hanafusa, H. (1996). SH2 and SH3-containing adaptor proteins: redundant or independent mediators of intracellular signal transduction. Genes To Cells *1*, 595-613.

Blaszczyk, J., Li, Y., Yan, H. G., and Ji, X. H. (2001). Crystal structure of unligated guanylate kinase from yeast reveals GMP-induced conformational changes. Journal of Molecular Biology *307*, 247-257.

Bredt, D. S., and Snyder, S. H. (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proc Natl Acad Sci U S A *87*, 682-5.

Brenman, J. E., Chao, D., S., Xia, H., Aldape, K., and Bredt, D., S. (1995). Nitric oxide synthase complexed with dystrophin and absent from skeletal muscle sarcolemma in Duchenne muscular dystrophy. Cell *82*, 743-752.

Brenman, J. E., Chao, D. S., Gee, S. H., McGee, A. W., Craven, S. E., Santillano, D. R., Huang, F., Xia, H., Peters, M. F., Froehner, S. C., and Bredt, D. S. (1996). Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and α-1 syntrophin mediated by PDZ motifs. Cell *84*, 757-767.

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

Brenman, J. E., Topinka, J. R., Cooper, E. C., McGee, A. W., Rosen, J., Milroy, T., Ralston, H. J., and Bredt, D. S. (1998). Localization of postsynaptic density-93 to dendritic microtubules and interaction with microtubule-associated protein 1A. Journal of Neuroscience *18*, 8805-13.

Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J. S., Kuszewski, J., Nilges, M., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998). Crystallography & NMR system: A new software suite for macromolecular structure determination. Acta Crystallographica. Section D: Biological Crystallography *54*, 905-21.

Budnik, V., Koh, Y. H., Guan, B., Hartmann, B., Hough, C., Woods, D., and Gorczyca, M. (1996). Regulation of synapse structure and function by the Drosophila tumor suppressor gene dlg. Neuron *17*, 627-40.

Carlin, R. K., Grab, D. J., Cohen, R. S., and Siekevitz, P. (1980). Isolation and characterization of postsynaptic densities from various brain regions: enrichment of different types of postsynaptic densities. Journal of Cell Biology *86*, 831-45.

Chen, L., Chetkovich, D. M., Petrailia, R., Sweeney, N., Kawaski, Y., Wenthold, R., Bredt, D. S., and Nicoll, R. A. (2000). Stargazin mediates synaptic targeting of AMPA receptors by two distinct mechanisms. Nature *408*, 936-943.

Cho, K. O., Hunt, C. A., and Kennedy, M. B. (1992). The rat brain postsynaptic density fraction contains a homolog of the Drosophila discs-large tumor suppressor protein. Neuron *9*, 929-42.

Christopherson, K. S., Hillier, B. J., Lim, W. A., and Bredt, D. S. (1999). PSD-95 assembles a ternary complex with the N-methyl-D-aspartic acid receptor and a bivalent neuronal NO synthase PDZ domain. Journal of Biological Chemistry *274*, 27467-73.

Cohen, A. R., Woods, D. F., Marfatia, S. M., Walther, Z., Chishti, A. H., Anderson, J. M., and Wood, D. F. (1998). Human CASK/LIN-2 binds syndecan-2 and protein 4.1 and localizes to the basolateral membrane of epithelial cells. Journal of Cell Biology *142*, 129-38.

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

Cohen, R. S., Blomberg, F., Berzins, K., and Siekevitz, P. (1977). The structure of postsynaptic densities isolated from dog cerebral cortex. I. Overall morphology and protein composition. Journal of Cell Biology *74*, 181-203.

Colledge, M., Dean, R. A., Scott, G. K., Langeberg, L. K., Huganir, R. L., and Scott, J. D. (2000). Targeting of PKA to glutamate receptors through a MAGUK-AKAP complex. Neuron *27*, 107-19.

Craven, S. E., and Bredt, D. S. (2000). Synaptic targeting of the postsynaptic density protein PSD-95 mediated by a tyrosine-based trafficking signal. Journal of Biological Chemistry *275*, 20045-51.

Craven, S. E., Husseini, A. E., and Bredt, D. S. (1999). Synaptic targeting of the postsynaptic density protein PSD-95 mediated by lipid and protein motifs. Neuron *22*, 497-509.

Daniels, D. L., Cohen, A. R., Anderson, J. M., and Brünger, A. T. (1998). Crystal structure of the hCASK PDZ domain reveals the structural basis of class II PDZ domain target recognition. Nature Structural Biology *5*, 317-25.

De La Fortelle, E., and Bricogne, G. (1997). Maximum-likelihood heavy-atom parameter refinement for muliple isomorphous replacement and multiwavelength anomalous diffraction methods. Methods Enzymol. *276*, 472-494.

Deguchi, M., Hata, Y., Takeuchi, M., Ide, N., Hirao, K., Yao, I., Irie, M., Toyoda, A., and Takai, Y. (1998). BEGAIN (brain-enriched guanylate kinase-associated protein), a novel neuronal PSD-95/SAP90-binding protein. Journal of Biological Chemistry *273*, 26269-72.

Dobrosotskaya, I., Guy, R. K., and James, G. L. (1997). MAGI-1, a membrane-associated guanylate kinase with a unique arrangement of protein-protein interaction domains. Journal of Biological Chemistry *272*, 31589-97.

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

El-Husseini, A. E., Schnell, E., Chetkovich, D. M., Nicoll, R. A., and Bredt, D. S. (2000). PSD-95 involvement in maturation of excitatory synapses. Science *290*, 1364-8.

Fanning, A. S., and Anderson, J. M. (1999). Protein modules as organizers of membrane structure. Current Opinion in Cell Biology *11*, 432-9.

Feng, S., Chen, J. K., Yu, H., Simon, J. A., and Schreiber, S. L. (1994). Two binding orientations for peptides to the Src SH3 domain: development of a general model for SH3-ligand interactions. Science *266*, 1241-7.

Friedman, H. V., Bresler, T., Garner, C. C., and Ziv, N. E. (2000). Assembly of new individual excitatory synapses: time course and temporal order of synaptic molecule recruitment [see comments]. Neuron *27*, 57-69.

Garcia, E. P., Mehta, S., Blair, L. A., Wells, D. G., Shang, J., Fukushima, T., Fallon, J. R., Garner, C. C., and Marshall, J. (1998). SAP90 binds and clusters kainate receptors causing incomplete desensitization. Neuron *21*, 727-39.

Garthwaite, J., Charles, S. L., and Chess-Williams, R. (1988). Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. Nature *336*, 385-8.

Gomperts, S. N. (1996). Clustering membrane proteins: It's all coming together with the PSD-95/SAP90 protein family. Cell *84*, 659-62.

Guan, B., Hartmann, B., Kho, Y. H., Gorczyca, M., and Budnik, V. (1996). The Drosophila tumor suppressor gene, dlg, is involved in structural plasticity at a glutamatergic synapse. Curr Biol *6*, 695-706.

Hanada, T., Lin, L., Tibaldi, E. V., Reinherz, E. L., and Chishti, A. H. (2000). GAKIN, a novel kinesin-like protein associates with the human homologue of the Drosophila discs large tumor suppressor in T lymphocytes. J Biol Chem *275*, 28774-84.

Hata, Y., Butz, S., and Sudhof, T. C. (1996). CASK: a novel dlg/PSD95 homolog with an N-terminal calmodulin-dependent protein kinase domain identified by interaction with neurexins. J Neurosci *16*, 2488-94.

Hillier, B. J., Christopherson, K. S., Prehoda, K. E., Bredt, D. S., and Lim, W. A. (1999). Unexpected modes of PDZ domain scaffolding revealed by structure of nNOS-syntrophin complex. Science *284*, 812-5.

Hirao, K., Hata, Y., Ide, N., Takeuchi, M., Irie, M., Yao, I., Deguchi, M., Toyoda, A., Sudhof, T. C., and Takai, Y. (1998). A novel multiple PDZ domain-containing molecule interacting with N-methyl-D-aspartate receptors and neuronal cell adhesion proteins. Journal of Biological Chemistry *273*, 21105-10.

Hoskins, R., Hajnal, A. F., Harp, S. A., and Kim, S. K. (1996). The C. elegans vulval induction gene lin-2 encodes a member of the MAGUK family of cell junction proteins. Development *122*, 97-111.

Hough, C. D., Woods, D. F., Park, S., and Bryant, P. J. (1997). Organizing a functional junctional complex requires specific domains of the Drosophila MAGUK Discs large. Genes and Development *11*, 3242-53.

Hunt, A. C., Schenker, L. J., and Kennedy, M. B. (1996). PSD-95 is associated with the postsynaptic density and not with the presynaptic membrane at forebrain synapses. Journal of Neuroscience *16*, 1380-1388.

Husi, H., Ward, M. A., Choudhary, J. S., Blackstock, W. P., and Grant, S. G. (2000). Proteomic analysis of NMDA receptor-adhesion protein signaling complexes [see comments]. Nature Neuroscience *3*, 661-9.

Irie, M., Hata, Y., Takeuchi, M., Ichtchenko, K., Toyoda, A., Hirao, K., Takai, Y., Rosahl, T. W., and Sudhof, T. C. (1997). Binding of neuroligins to PSD-95. Science *277*, 1511-5.

Jones, T. A., Zou, J.-Y., and Cowan, S. W. (1991). Improved methods for building models in electron density maps and the location of errors in these models. Acta Crystallogr. *A 47*, 110–119.

Kaech, S. M., Whitfield, C. W., and Kim, S. K. (1998). The LIN-2/LIN-7/LIN-10 complex mediates basolateral membrane localization of the C. elegans EGF receptor LET-23 in vulval epithelial cells. Cell *94*, 761-71.

Kennedy, M. B., Bennett, M. K., and Erondu, N. E. (1983). Biochemical and immunochemical evidence that the "major postsynaptic density protein" is a subunit of a calmodulin-dependent protein kinase. Proceedings of the National Academy of Sciences of the United States of America *80*, 7357-61.

Kiedrowski, L., Costa, E., and Wroblewski, J. T. (1992). Glutamate receptor agonists stimulate nitric oxide synthase in primary cultures of cerebellar granule cells. J Neurochem *58*, 335-41.

Kim, E., Cho, K.-O., Rothschild, A., and Sheng, M. (1996). Heteromultimerization and NMDA receptor clustering activity of chapsyn-110, a novel member of the PSD-95 family of synaptic proteins. Neuron *17*, 103-113.

Kim, E., Naisbitt, S., Hsueh, Y. P., Rao, A., Rothschild, A., Craig, A. M., and Sheng, M. (1997). GKAP, a novel synaptic protein that interacts with the guanylate kinase-like domain of the PSD-95/SAP90 family of channel clustering molecules. J Cell Biol *136*, 669-78.

Kim, E., Niethammer, M., Rothschild, A., N., J. Y., and Sheng, M. (1995). Clustering of Shaker-type K+ channels by direct interaction with the PSD-95/SAP90 family of membrane-associated guanylate kinases. Nature *378*, 85-88.

Kim, J. H., Liao, D., Lau, L.-F., and Huganir, R. L. (1998). SynGAP: a synaptic RasGAP that associates with the PSD-95 / SAP90 protein family. Neuron *20*, 683-691.

Kistner, U., Garner, C. C., and Linial, M. (1995). Nucleotide binding by the synapse associated protein SAP90. FEBS Lett *359*, 159-63.

Kistner, U., Wenzel, B. M., Veh, R. W., Cases-Langhoff, C., Garner, A. M., Appeltauer, U., Voss, B., Gundelfinger, E. D., and Garner, C. C. (1993). SAP90, a rat presynaptic protein related to the product of the Drosophila tumor suppressor gene dlg-A. J Biol Chem *268*, 4580-3.

Kohda, D., Terasawa, H., Ichikawa, S., Ogura, K., Hatanaka, H., Mandiyan, V., Ullrich, A., Schlessinger, J., and Inagaki, F. (1994). Solution structure and ligand-binding site of the carboxy-terminal SH3 domain of GRB2. Structure *2*, 1029-40.

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

Kuhlendahl, S., Spangenberg, O., Konrad, M., Kim, E., and Garner, C. C. (1998). Functional analysis of the guanylate kinase-like domain in the synapse-associated protein SAP97. European Journal of Biochemistry *252*, 305-13.

Kuriyan, J., and Cowburn, D. (1997). Modular peptide recognition domains in eukaryotic signaling. Annual Review of Biophysics and Biomolecular Structure *26*, 259-88.

Lahey, T., Gorczyca, M., Jia, X. X., and Budnik, V. (1994). The Drosophila tumor suppressor gene dlg is required for normal synaptic bouton structure. Neuron *13*, 823-35.

Larson, S. M., and Davidson, A. R. (2000). The identification of conserved interactions within the SH3 domain by alignment of sequences and structures. Protein Science *9*, 2170-80.

Li, Y., Zhang, Y., and Yan, H. (1996). Kinetic and thermodynamic characterizations of yeast guanylate kinase. Journal of Biological Chemistry *271*, 28038-44.

Lim, W. A., Fox, R. O., and Richards, F. M. (1994). Stability and peptide binding affinity of an SH3 domain from the Caenorhabditis elegans signaling protein Sem-5. Protein Sci *3*, 1261-6.

Lim, W. A., and Richards, F. M. (1994). Critical residues in an SH3 domain from Sem-5 suggest a mechanism for proline-rich peptide recognition. Nature Structural Biology *1*, 221-5.

Lim, W. A., Richards, F. M., and Fox, R. O. (1994). Structural determinants of peptide-binding orientation and of sequence specificity in SH3 domains [see comments] [published erratum appears in Nature 1995 Mar 2;374(6517):94]. Nature *372*, 375-9.

Lue, R. A., Marfatia, S. M., Branton, D., and Chishti, A. H. (1994). Cloning and characterization of hdlg: the human homologue of the Drosophila discs large tumor suppressor binds to protein 4.1. Proc Natl Acad Sci U S A *91*, 9818-22.

MacArthur, M. W., and Thornton, J. M. (1991). Influence of proline residues on protein conformation. Journal of Molecular Biology *218*, 397-412.

Makino, K., Kuwahara, H., Masuko, N., Nishiyama, Y., Morisaki, T., Sasaki, J., Nakao, M., Kuwano, A., Nakata, M., Ushio, Y., and Saya, H. (1997). Cloning and characterization of NE-dlg: a novel human homolog of the Drosophila discs large (dlg) tumor suppressor protein interacts with the APC protein. Oncogene *14*, 2425-33.

Marfatia, S. M., Leu, R. A., Branton, D., and Chishti, A. H. (1995). Identification of the protein 4.1 binding interface on glycophorin C and p55, a homologue of the Drosophila discs-large tumor suppressor protein. Journal of Biological Chemistry *270*, 715-9.

Marrs, G. S., Green, S. H., and Dailey, M. E. (2001). Rapid formation and remodeling of postsynaptic densities in developing dendrites. [Comment In: Nat Neurosci. 2001 Oct;4(10):967-8 UI: 21458932]. Nature Neuroscience *4*, 1006-13.

Masuko, N., Makino, K., Kuwahara, H., Fukunaga, K., Sudo, T., Araki, N., Yamamoto, H., Yamada, Y., Miyamoto, E., and Saya, H. (1999). Interaction of NE-dlg/SAP102, a neuronal and endocrine tissue-specific membrane-associated guanylate kinase protein, with calmodulin and PSD- 95/SAP90. A possible regulatory role in molecular clustering at synaptic sites. J Biol Chem *274*, 5782-90.

Maximov, A., Südhof, T. C., and Bezprozvanny, I. (1999). Association of neuronal calcium channels with modular adaptor proteins. Journal of Biological Chemistry *274*, 24453-6.

Mayer, B. J., and Eck, M. J. (1995). SH3 domains. Minding your p's and q's. Current Biology *5*, 364-7.

McGee, A. W., and Bredt, D. S. (1999). Identification of an intramolecular interaction between the SH3 and guanylate kinase domains of PSD-95. Journal of Biological Chemistry *274*, 17431-6.

McGee, A. W., Topinka, J. R., Hashimoto, K., Petralia, R. S., Kakizawa, S., Kauer, F., Aguilera-Moreno, A., Wenthold, R. J., Kano, M., and Bredt, D. S. (2001). PSD-93 knock-out mice reveal that neuronal MAGUKs are not required for development or function of parallel fiber synapses in cerebellum. Journal of Neuroscience *21*, 3085-91.

Migaud, M., Charlesworth, P., Dempster, M., Webster, L. C., Watabe, A. M., Makhinson, M., He, Y., Ramsay, M. F., Morris, R. G., Morrison, J. H., O'Dell, T. J., and Grant, S. G. (1998). Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. Nature *396*, 433-9.

Moarefi, I., LaFevre-Bernt, M., Sicheri, F., Huse, M., Lee, C. H., Kuriyan, J., and Miller, W. T. (1997). Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement. Nature *385*, 650-3.

Moon, I. S., Apperson, M. L., and Kennedy, M. B. (1994). The major tyrosine-phosphorylated protein in the postsynaptic density fraction is N-methyl-D-aspartate receptor subunit 2B. Proceedings of the National Academy of Sciences of the United States of America *91*, 3954-8.

Morais Cabral, J. H., Petosa, C., Sutcliffe, M. J., Raza, S., Byron, O., Poy, F., Marfatia, S. M., Chishti, A. H., and Liddington, R. C. (1996). Crystal structure of a PDZ domain. Nature *382*, 649-52.

Mori, H., Manabe, T., Watanabe, M., Satoh, Y., Suzuki, N., Toki, S., Nakamura, K., Yagi, T., Kushiya, E., Takahashi, T., Inoue, Y., Sakimura, K., and Mishina, M. (1998). Role of the carboxy-terminal region of the GluR epsilon2 subunit in synaptic localization of the NMDA receptor channel. Neuron *21*, 571-80.

Muller, B. M., Kistner, U., Kindler, S., Chung, W. K., Kuhlendahl, S., Fenster, S. D., Lau, L.-F., Veh, R. W., Huganir, R. L., Gundelfinger, E. D., and Garner, C. C. (1996). SAP102, a novel postsynaptic protein that interacts with NMDA receptor complexes in vivo. Neuron *17*, 255-265.

Muller, B. M., Kistner, U., Veh, R. W., Cases-Langhoff, C., Becker, B., Gundelfinger, E. D., and Garner, C. C. (1995). Molecular characterization and spatial distribution of SAP97, a novel presynaptic protein homologous to SAP90 and the Drosophila discs-large tumor suppressor protein. J Neurosci *15*, 2354-66.

Murphy, C. (1974). Cell death and autonomous gene action in lethals affecting imaginal discs in Drosophila melanogaster. Developmental Biology *39*, 23-36.

Musacchio, A., Saraste, M., and Wilmanns, M. (1994). High-resolution crystal structures of tyrosine kinase SH3 domains complexed with proline-rich peptides. Nature Structural Biology *1*, 546-51.

Musacchio, A., Wilmanns, M., and Saraste, M. (1994). Structure and function of the SH3 domain. Progress in Biophysics and Molecular Biology *61*, 283-97.

Newcomer, M. E. (2001). Trading places. [Comment In: Nat Struct Biol. 2001 Apr;8(4):281 UI: 21173897 Comment On: Nat Struct Biol. 2001 Apr;8(4):316-20 UI: 21173909]. Nature Structural Biology *8*, 282-4.

Nguyen, J. T., Turck, C. W., Cohen, F. E., Zuckermann, R. N., and Lim, W. A. (1998). Exploiting the basis of proline recognition by SH3 and WW domains: design of N-substituted inhibitors. Science *282*, 2088-92.

Nix, S. L., Chishti, A. H., Anderson, J. M., and Walther, Z. (2000). hCASK and hDlg associate in epithelia, and their src homology 3 and guanylate kinase domains participate in both intramolecular and intermolecular interactions. Journal of Biological Chemistry *275*, 41192-200.

Nusser, Z., Mulvihill, E., Streit, P., and Somogyi, P. (1994). Subsynaptic segregation of metabotropic and ionotropic glutamate receptors as revealed by immunogold localization. Neuroscience *61*, 421-7.

Okabe, S., Kim, H. D., Miwa, A., Kuriu, T., and Okado, H. (1999). Continual remodeling of postsynaptic density and its regulation by synaptic activity. Nature Neuroscience *2*, 804-11.

Okabe, S., Miwa, A., and Okado, H. (2001). Spine formation and correlated assembly of presynaptic and postsynaptic molecules. Journal of Neuroscience *21*, 6105-14.

Otwinowski, Z., and Minor, W. (1997). Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. *276*, 307–326.

Pak, D. T., Yang, S., Rudolph-Correia, S., Kim, E., and Sheng, M. (2001). Regulation of dendritic spine morphology by SPAR, a PSD-95-associated RapGAP. [Comment In: Neuron. 2001 Aug 2;31(2):169-71 UI: 21394363]. Neuron *31*, 289-303.

Penzes, P., Johnson, R. C., Sattler, R., Zhang, X., Huganir, R. L., Kambampati, V., Mains, R. E., and Eipper, B. A. (2001). The neuronal Rho-GEF Kalirin-7 interacts with PDZ domain-containing proteins and regulates dendritic morphogenesis. Neuron *29*, 229-42.

Peters, A., Palay, S. L., and Webster, H. d. (1976). The Fine Structure of the Nervous System: The Neurons and Supporting Cells (Philadelphia: W.B. Saunders Co.).

Rao, A., Kim, E., Sheng, M., and Craig, A. M. (1998). Heterogeneity in the molecular composition of excitatory postsynaptic sites during development of hippocampal neurons in culture. Journal of Neuroscience *18*, 1217-29.

Ren, R., Mayer, B. J., Cicchetti, P., and Baltimore, D. (1993). Identification of a ten-amino acid proline-rich SH3 binding site. Science *259*, 1157-61.

Rousseau, F., Schymkowitz, J. W., Wilkinson, H. R., and Itzhaki, L. S. (2001). Three-dimensional domain swapping in p13suc1 occurs in the unfolded state and is controlled by conserved proline residues. Proceedings of the National Academy of Sciences of the United States of America *98*, 5596-601.

Satoh, K., Yanai, H., Senda, T., Kohu, K., Nakamura, T., Okumura, N., Matsumine, A., Kobayashi, S., Toyoshima, K., and Akiyama, T. (1997). DAP-1, a novel protein that interacts with the guanylate kinase-like domains of hDLG and PSD-95. Genes To Cells *2*, 415-24.

Sattler, R., Xiong, Z., Lu, W.-Y., Hafner, M., MacDonald, J. F., and Tymianski, M. (1999). Specific coupling of NMDA receptor activation to nitric oxide neurotoxicity by PSD-95 protein. Science *284*, 1845-1848.

Schlunegger, M. P., Bennett, M. J., and Eisenberg, D. (1997). Oligomer formation by 3D domain swapping: a model for protein assembly and misassembly. Advances in Protein Chemistry *50*, 61-122.

Schymkowitz, J. W., Rousseau, F., and Itzhaki, L. S. (2000). Sequence conservation provides the best prediction of the role of proline residues in p13suc1. Journal of Molecular Biology *301*, 199-204.

Sheng, M., and Sala, C. (2001). PDZ domains and the organization of supramolecular complexes. Annu. Rev. Neurosci. *24*, 1-29.

Shin, H., Hsueh, Y. P., Yang, F. C., Kim, E., and Sheng, M. (2000). An intramolecular interaction between Src homology 3 domain and guanylate kinase-like domain required for channel clustering by postsynaptic density-95/SAP90. Journal of Neuroscience *20*, 3580-7.

Sicheri, F., Moarefi, I., and Kuriyan, J. (1997). Crystal structure of the Src family tyrosine kinase Hck. Nature *385*, 602-9.

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

Sprengel, R., Suchanek, B., Amico, C., Brusa, R., Burnashev, N., Rozov, A., Hvalby, O., Jensen, V., Paulsen, O., Andersen, P., Kim, J. J., Thompson, R. F., Sun, W., Webster, L. C., Grant, S. G., Eilers, J., Konnerth, A., Li, J., McNamara, J. O., and Seeburg, P. H. (1998). Importance of the intracellular domain of NR2 subunits for NMDA receptor function in vivo. Cell *92*, 279-89.

Stehle, T., and Schulz, G. E. (1992). Refined structure of the complex between guanylate kinase and its substrate GMP at 2.0 A resolution. Journal of Molecular Biology *224*, 1127-41.

Steigerwald, F., Schulz, T. W., Schenker, L. T., Kennedy, M. B., Seeburg, P. H., and Köhr, G. (2000). C-Terminal truncation of NR2A subunits impairs synaptic but not extrasynaptic localization of NMDA receptors. Journal of Neuroscience *20*, 4573-81.

Stewart, M., Murphy, C., and Fristrom, J. W. (1972). The recovery and preliminary characterization of X chromosome mutants affecting imaginal discs of Drosophila melanogaster. Developmental Biology *27*, 71-83.

Stricker, N. L., Christopherson, K. S., Yi, B. A., Schatz, P. J., Raab, R. W., Dawes, G., Bassett, J., D. E. , Bredt, D. S., and Li, M. (1997). PDZ Domain of neuronal nitric oxide synthase recognizes novel C-terminal peptide sequences as determined by *in vitro* selection. Nature Biotechnology *15*, 336-342.

Takeuchi, M., Hata, Y., Hirao, K., Toyoda, A., Irie, M., and Takai, Y. (1997). SAPAPs. A family of PSD-95/SAP90-associated proteins localized at postsynaptic density. Journal of Biological Chemistry *272*, 11943-51.

Tejedor, F. J., Bokhari, A., Rogero, O., Gorczyca, M., Zhang, J., Kim, E., Sheng, M., and Budnik, V. (1997). Essential role for dlg in synaptic clustering of Shaker K+ channels in vivo. J Neurosci *17*, 152-9.

Terwilliger, T. C., and Berendzen, J. (1996). Correlated phasing of multiple isomorphous replacement data. Acta Crystallogr. *D 52*, 749–757.

Thomas, U., Ebitsch, S., Gorczyca, M., Koh, Y. H., Hough, C. D., Woods, D., Gundelfinger, E. D., and Budnik, V. (2000). Synaptic targeting and localization of discs-large is a stepwise process controlled by different domains of the protein. Current Biology *10*, 1108-17.

Van Duyne, G. D., Standaert, R. F., Karplus, P. A., Schreiber, S. L., and Clardy, J. (1993). Atomic structures of the human immunophilin FKBP-12 complexes with FK506 and rapamycin. Journal of Molecular Biology *229*, 105-24.

Walikonis, R. S., Jensen, O. N., Mann, M., Provance, D. W., Jr., Mercer, J. A., and Kennedy, M. B. (2000). Identification of proteins in the postsynaptic density fraction by mass spectrometry. Journal of Neuroscience *20*, 4069-80.

Walsh, M. J., and Kuruc, N. (1992). The postsynaptic density: constituent and associated proteins characterized by electrophoresis, immunoblotting, and peptide sequencing. Journal of Neurochemistry *59*, 667-78.

Wittekind, M., Mapelli, C., Farmer, B. T., 2nd, Suen, K. L., Goldfarb, V., Tsao, J., Lavoie, T., Barbacid, M., Meyers, C. A., and Mueller, L. (1994). Orientation of peptide fragments from Sos proteins bound to the N-terminal SH3 domain of Grb2 determined by NMR spectroscopy. Biochemistry *33*, 13531-9.

Woods, D. F., and Bryant, P. J. (1991). The discs-large tumor suppressor gene of Drosophila encodes a guanylate kinase homolog localized at septate junctions. Cell *66*, 451-64.

Woods, D. F., and Bryant, P. J. (1989). Molecular cloning of the lethal(1)discs large-1 oncogene of Drosophila. Developmental Biology *134*, 222-35.

Woods, D. F., and Bryant, P. J. (1993). ZO-1, DlgA and PSD-95/SAP90: homologous proteins in tight, septate and synaptic cell junctions. Mechanisms of Development *44*, 85-9.

Woods, D. F., Hough, C., Peel, D., Callaini, G., and Bryant, P. J. (1996). Dlg protein is required for junction structure, cell polarity, and proliferation control in Drosophila epithelia. J Cell Biol *134*, 1469-82.

Wyszynski, M., Kharazia, V., Shanghvi, R., Rao, A., Beggs, A. H., Craig, A. M., Weinberg, R., and Sheng, M. (1998). Differential regional expression and ultrastructural localization of alpha-actinin-2, a putative NMDA receptor-anchoring protein, in rat brain. Journal of Neuroscience *18*, 1383-92.

Wyszynski, M., Lin, J., Rao, A., Nigh, E., Beggs, A. H., Craig, A. M., and Sheng, M. (1997). Competitive binding of alpha-actinin and calmodulin to the NMDA receptor. Nature *385*, 439-42.

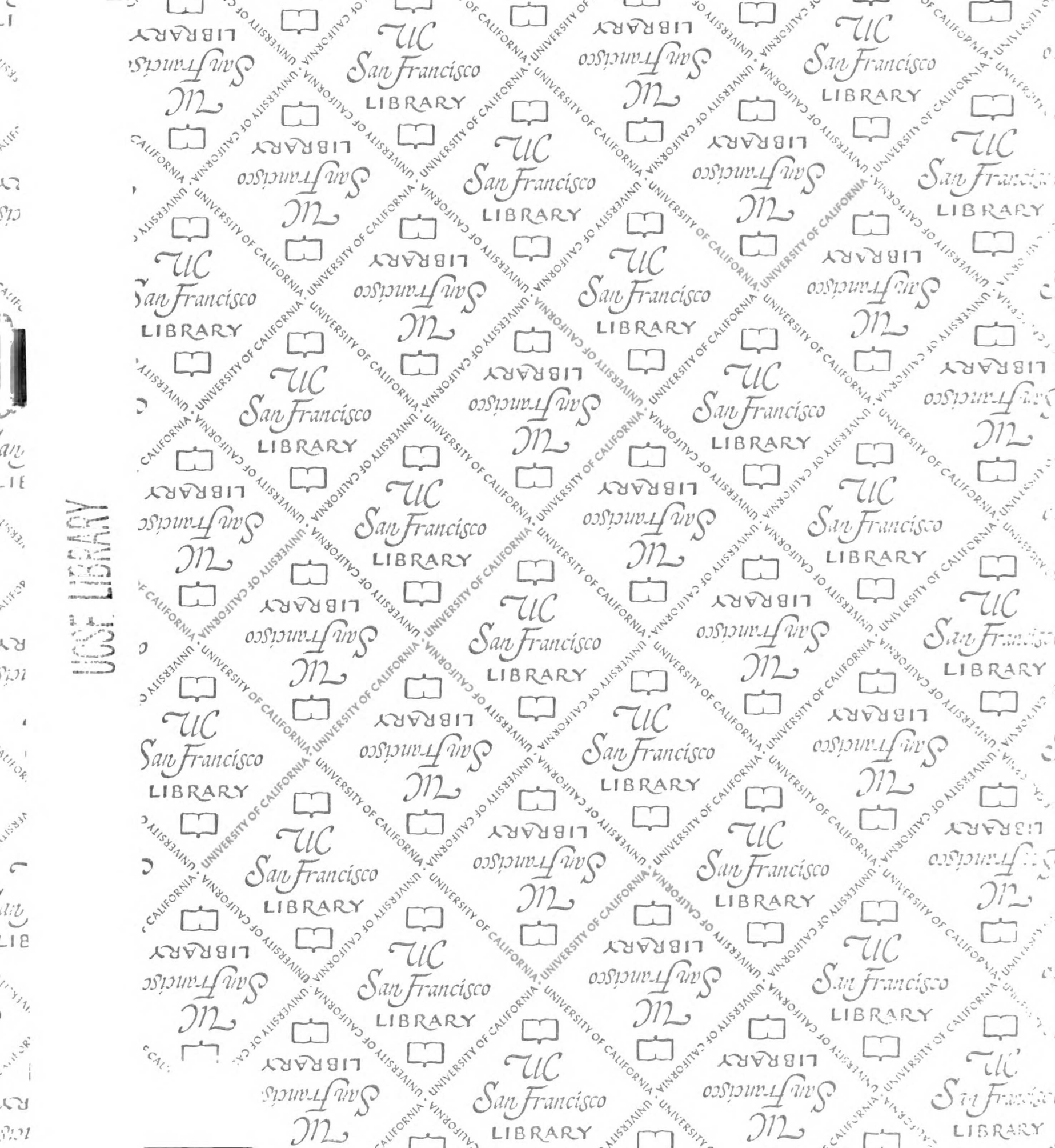
Xia, H., Winokur, S. T., Kuo, W. L., Altherr, M. R., and Bredt, D. S. (1997). Actinin-associated LIM protein: identification of a domain interaction between PDZ and spectrin-like repeat motifs. J Cell Biol *139*, 507-15.

Xu, W., Harrison, S. C., and Eck, M. J. (1997). Three-dimensional structure of the tyrosine kinase c-Src. Nature *385*, 595-602.

Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W., and Schreiber, S. L. (1994). Structural basis for the binding of proline-rich peptides to SH3 domains. Cell *76*, 933-45.

Ziff, E. B. (1997). Enlightening the postsynaptic density. Neuron *19*, 1163-74.

Zito, K., Fetter, R. D., Goodman, C. S., and Isacoff, E. Y. (1997). Synaptic clustering of Fascilin II and Shaker: essential targeting sequences and role of Dlg. Neuron *19*, 1007-16.


UC San Francisco LIBRARY
UNIVERSITY OF CALIFORNIA

For reference

Not to be taken from the room.

7065900

3 1378 00706 5900

UC San Francisco LIBRARY

UNIVERSITY OF CALIFORNIA