GRASP-1: A Neuronal RasGEF Associated with the AMPA Receptor/GRIP Complex

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Summary

The PDZ domain–containing proteins, such as PSD-95 and GRIP, have been suggested to be involved in the targeting of glutamate receptors, a process that plays a critical role in the efficiency of synaptic transmission and plasticity. To address the molecular mechanisms underlying AMPA receptor synaptic localization, we have identified several GRIP-associated proteins (GRASPs) that bind to distinct PDZ domains within GRIP. GRASP-1 is a neuronal rasGEF associated with GRIP and AMPA receptors in vivo. Overexpression of GRASP-1 in cultured neurons specifically reduced the synaptic targeting of AMPA receptors. In addition, the subcellular distribution of both AMPA receptors and GRASP-1 was rapidly regulated by the activation of NMDA receptors. These results suggest that GRASP-1 may regulate neuronal ras signaling and contribute to the regulation of AMPA receptor distribution by NMDA receptor activity.

Introduction

Glutamate receptors are the major excitatory neurotransmitter receptors in the central nervous system (CNS) (Seeburg, 1993; Hollmann and Heinemann, 1994). The ionotropic glutamate receptors are composed of three distinct groups, namely N-methyl-D-aspartic acid (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), and kainate receptors. NMDA receptors are essential for the induction of many forms of activity-dependent synaptic plasticity in the adult as well as in the developing animal, while AMPA receptors mediate rapid excitatory synaptic transmission. These receptors are critical for the induction and maintenance of long-term potentiation (LTP) and long-term depression (LTD), two cellular models of learning and memory (Collingridge, 1987; Bliss and Collingridge, 1993). Antagonists of the NMDA receptor, such as APV and MK801, completely blocked the induction of LTP and LTD (Coan et al., 1987; Dudek and Bear, 1992; Mulkey and Malenka, 1992). The deletion of the NR1 subunit, an essential subunit of the NMDA receptor, in hippocampal CA1 region results in the abolishment of LTP in this region and impaired spatial learning (Tsien et al., 1996). AMPA-type glutamate receptors are crucial for the expression of LTP and LTD. LTP and LTD have been proposed to be mediated, at least in part, by changes in AMPA receptor function (Raymond et al., 1993b; Roche et al., 1994).

A variety of studies have observed changes in AMPA receptor responses after LTP induction (Kauer et al., 1988; Muller et al., 1988; Davies et al., 1989; Isaac et al., 1995; Liao et al., 1995; Benke et al., 1998). Moreover, recently, it has been reported that targeted deletion of the GluRA/GluR1 gene of the AMPA receptor results in impaired hippocampal LTP (Zamanillo et al., 1999), further supporting the required involvement of AMPA receptors in synaptic plasticity.

AMPA receptors have been proposed to be regulated by at least two distinct mechanisms during the expression of LTP/LTD. Protein phosphorylation/dephosphorylation of AMPA receptors has long been thought to be a critical factor in the expression of LTP and LTD (Raymond et al., 1993a; Roche et al., 1996). Recent studies demonstrating that the phosphorylation state of AMPA receptors correlates with the formation of LTP and LTD in hippocampal slices have provided support for this hypothesis (Barria et al., 1997; Kameyama et al., 1998; Lee et al., 1998). More recently, it has been proposed that the postsynaptic localization of AMPA receptors may be regulated by synaptic activity (Isaac et al., 1995; Liao et al., 1995). According to this hypothesis, the induction of LTP results in the insertion or targeting of AMPA receptors at the postsynaptic plasma membrane, whereas LTD-inducing stimuli cause the internalization or dispersion of AMPA receptors from the postsynaptic plasma membrane. A recent report has provided evidence for the synaptic insertion of the GluR1 subunit of AMPA receptors during LTP induction in hippocampal slices (Shi et al., 1999). Moreover, a decrease in the number of AMPA receptor clusters at synapses, but not NMDA receptor clusters, has recently been observed during the induction of NMDA receptor–dependent LTD in cultured neurons (Carroll et al., 1999).

The molecular mechanisms underlying the synaptic targeting and the activity-dependent redistribution of glutamate receptors have been an area of recent interest. Several proteins that directly interact with glutamate receptors such as PSD-95 and GRIP (glutamate receptor interacting protein) have been isolated and proposed to be involved in glutamate receptor synaptic targeting (Kornau et al., 1995; Dong et al., 1997). A common feature of these two proteins is that they are adaptor proteins containing multiple protein–protein interaction motifs called PDZ domains. PSD-95 contains three PDZ domains, one SH3 domain and one guanylate kinase homology domain. These domains interact with various proteins including NMDA receptors, potassium channels, nitric oxide synthase (NOS), neuroligin, synGAP, and GKAP (Kornau et al., 1995; Brenman et al., 1996; Irie et al., 1997; Kim et al., 1997, 1998; Chen et al., 1998). Among these proteins, synGAP is a rasGAP highly enriched at excitatory synapses that has been suggested to link NMDA receptors to synaptic ras signaling (Chen et al., 1998; Kim et al., 1998). GRIP1 and GRIP2/ABP are adaptor proteins for the AMPA receptor and contain seven PDZ domains, which presumably link AMPA receptors to other GRIP-associated proteins. Overexpression of the C-terminal domain of GluR2 that interacts with GRIP in neurons disrupts the synaptic targeting of AMPA receptors, suggesting that GRIP or

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A

Bait 1 Bait 2 Bait 3

1 2 3 4 5 6 7

B

MAQALSEEEFQRMQTQLLELRTNNYQLSDELRKNGVELSSLRQKVAYLDKEFSKA
QKALSKSKKAQEVEVLLSEKEMLQAKLHSQEEDFRLQNS**TLMAEFSKLCSQLEQL**
ELENRQLKEGVPGAAGPHVDGELLRLQAENTALQKNMAAL**QERYGKEAVRPSA**
VSEGQGDPPGDVLPISLSPMPLAEVELKWEMEREEKKLLWEQLQGLESSKQAET
SRLQEELAKLSEKLKKKQESFCRLQTEKETLFNDSRNKIEELQQRKEADLKAQLART
QKLQQELEAANQSLAELRDQRQGERLEHAAALRALQDQVSSQSADAQEQVEGLLA
ENNALRTSLAALEQIQTAKTQELNMLREQNTELAAELKHRQADYEELMGQKDDLN
SQLQESLRANSRLLEQLQEMGQEKEQLIQ**DLQEARKSAEKRKVMLDELAME**TLQ
EKSQHKEELGAVRLRHEKEMLGVRARYERELRELHEDKKRQEEELRGQIREEKART
RELENLQHTVEELQAQVHSMDGAKGWFERRLKEAEESLLQQEQEQEETLKQCREQ
HAAELKGKEEELQNVRDQLQQAQEERDGHVKTISNLKQEVK DTVD GQRILEKKG
SAVLKDLKRQLHLERKRADKLQERLQEILTNSKSRTGLEELVLSEMNSPSRTQTGDS
SSVSSFSYREILKEKESSAIPARSLSSSPQAQPPRPAELSDEEVAELFQRLAETQQEKW
MLEEKVKHLEVSSASMAEDLCRKSAIIETYVMDSRID_VSVAAGHTDRSGLGSVLRD_
_LVKPGDENLREMNKKLQNMLEEQLTKNMHLHKDMEVLSQEIVRLSKECVGS_PDPD
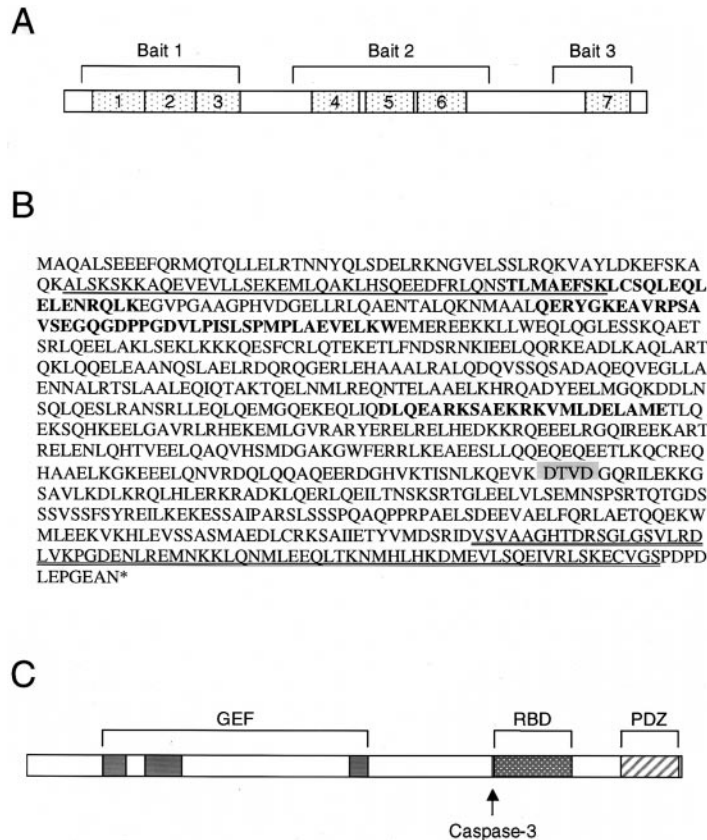LEPGEAN*

C

GEF RBD PDZ

Caspase-3

Figure 1. Schematic Structure of GRIP1 and the Sequence and Domain Structure of GRASP-1

(A) Schematic structure of GRIP1 showing the regions used in the yeast two-hybrid screens.
(B) Amino acid sequence of GRASP-1. The three sequence conserved regions (SCRs) of the rasGEF domain are shown in bold. The PDZ-like domain is doubly underlined. The caspase-3 cleavage site is shaded. The underlined region is alternatively spliced.
(C) GRASP-1 has a rasGEF domain, a caspase-3 cleavage site, a region homologous to ras binding domains (RBDs), and a PDZ-like domain.

related PDZ domain–containing proteins may be required for synaptic targeting (Dong et al., 1997). However, the molecular mechanisms underlying the regulation of AMPA receptor function by GRIP remain unclear.

To address the regulation of AMPA receptor localization, we set out to identify proteins in the GRIP/AMPA receptor complex. We performed yeast two-hybrid screens using different PDZ domains of GRIP1 and isolated multiple cDNAs encoding GRIP-associated proteins (GRASPs). Here, we characterize the GRIP1-associated protein, GRASP-1, which specifically interacts with the seventh PDZ domain of GRIP. GRASP-1 is a neuron-specific guanine nucleotide exchange factor (GEF) for the ras family of the small G proteins and is associated with the GRIP/AMPA receptor complex in brain. Overexpression of GRASP-1 dramatically inhibits AMPA receptor synaptic targeting in cultured neurons. Moreover, we found that the distribution of both GRASP-1 and AMPA receptors is rapidly regulated by NMDA receptor activity. These results suggest that GRASP-1 signaling may be involved in the regulation of neuronal ras signaling and AMPA receptor synaptic targeting.

Results

Isolation of GRASPs
The yeast two-hybrid system was employed to screen for molecules that interact with the various PDZ domains of GRIP1 (Figure 1A). A hippocampal cDNA library was independently screened with constructs containing the PDZ domains 1, 2, and 3 together (PDZ 1–3), 4, 5, and 6

together (PDZ 4–6), and 7 alone (PDZ 7). Positive clones were isolated and confirmed by back-transformation into yeast. Using PDZ domains 1–3, we obtained three novel genes, designated GRASP-2, -3, and -4 (Table 1). Using PDZ domains 4–6, we obtained seven genes that fall into three distinct classes of PDZ ligands. The first class of GRASPs contained type I PDZ domain ligand motifs (T/SXV) at the C terminus of the interacting proteins (Songyang et al., 1997) and included the huntingtin-associated protein HAP1-A (Li et al., 1995). The second class of proteins contained type II T/SXV motifs (ϕXϕ, where ϕ is a hydrophobic amino acid) (Songyang et al., 1997) and included Numb-like (Zhong et al., 1997), P140 (gene accession number AF040944), G2 (gene accession number U10991, and two novel genes (GRASP-5 and -6). The third class of GRASP, isolated over ten times, was GRIP1 itself, confirming that GRIP1 associates with itself most likely through a PDZ–PDZ interaction (Srivastava et al., 1998; Dong et al., 1999). Finally, using the last PDZ domain (PDZ 7) of GRIP1 as bait, which is alternatively spliced in GRIP2/ABP gene (Srivastava et al., 1998; Dong et al., 1999), we identified a novel gene designated GRASP-1, which will be the focus of this paper.

Molecular Cloning of GRASP-1 and Examination of Its Interaction with GRIP1
The seventh PDZ domain of GRIP1 was used as bait to screen two rat hippocampal cDNA yeast two-hybrid libraries. Approximately 3.1×10^6 clones in a random-primed library and 3.0×10^6 clones in an oligo-dT-primed library were screened. Eight and twenty-four

Table 1. GRIP-Associated Proteins Identified through Yeast Two-Hybrid Screening

Bait	Prey		Homology	Remark
PDZ 1, 2, 3 (bait 1)	GRASP-3	T/SXV*	Novel, SNARE	Vesicle transport
	GRASP-2		Novel, NAB domain	
	GRASP-4		Novel, BTB domain	
PDZ 4, 5, 6 (bait 2)	HAP1-A	T/SXV*		Huntington's disease
	Numblike	$\phi X \phi$*		Cortical neurogenesis
	P140	$\phi X \phi$*		Enriched in postnatal nervous system
	G2	$\phi X \phi$*		
	GRASP-5	$\phi X \phi$*	Novel, Rap2 interacting protein	
	GRASP-6	$\phi X \phi$*		
	GRIP	PDZ		
PDZ 7 (bait 3)	GRASP-1	PDZ	Novel, neuronal rasGEF	

The PDZ domains 1–3, 4–6, and 7 were used as baits to screen a random-primed rat hippocampal cDNA library. Positive clones were selected on selective media and assayed for β-galactosidase activity. The clones containing either T/SXV* motifs or PDZ domains are indicated in the table. The homology to known genes is listed for novel genes.
Asterisks indicate stop codons.

positives, respectively, were isolated from the two libraries, and all of them encoded the same gene, designated GRASP-1. To obtain the full-length gene encoding GRASP-1, a CapFinder rat hippocampal cDNA library and an oligo-dT-primed rat hippocampal cDNA λ ZAP library were screened with the SalI/SacI fragment (about 300 bp) of yeast clone D7-D-12 (the last 1.4 kb of the whole cDNA) as a probe. The longest clone obtained from the dT-primed library was ~2.8 kb. More than ten clones of ~3 kb were obtained from the CapFinder library. The GRASP-1 cDNA encoded an 838 amino acid protein (Figure 1B). Two splicing isoforms were observed. The full-length sequence of GRASP-1 was confirmed by comparing the size of in vitro–translated and heterologously expressed proteins with the endogenous protein in brain (data not shown). Sequence analysis revealed that GRASP-1 has a rasGEF catalytic domain, a potential caspase-3 cleavage site, a region weakly homologous to the ras binding domain (RBD) of ralGDS (Hofer et al., 1994; Spaargaren and Bischoff, 1994), and a PDZ domain (Figure 1C). This overall domain structure is similar to ralGDS, which contains an N-terminal GEF catalytic domain and a C-terminal regulatory domain (Hofer et al., 1994; Spaargaren and Bischoff, 1994).

Unlike most PDZ domain–interacting proteins, GRASP-1 does not contain a characterized type I or II PDZ ligand at its C terminus (Kornau et al., 1995; Saras and Heldin, 1996; Songyang et al., 1997). To identify the region of GRASP-1 required for interaction with GRIP1, we analyzed the interaction of various GRASP-1 constructs with GRIP1 using the yeast two-hybrid system (Figure 2A). These experiments demonstrated that the last 100 amino acids encoding the PDZ domain were required and sufficient for the interaction, suggesting that the interaction between GRASP-1 and GRIP1 is most likely mediated through a PDZ–PDZ interaction similar to the previously described interaction between PSD-95 and NOS (Brenman et al., 1996). To further confirm the GRIP1-GRASP-1 association, we analyzed the interaction of GRIP1 and GRASP-1 in transfected fibroblasts. QT-6 cells were cotransfected with a Myc-tagged GRASP-1 construct with a full-length GRIP1 cDNA, and immunoprecipitations were performed from cell lysates with anti-Myc antibody or anti-GRIP1 antibody (Figure 2B). These experiments demonstrated that a GRASP-1-GRIP1 complex could be isolated from the cotransfected cells using either the anti-GRIP1 or anti-Myc antibodies. Lysates from cells transfected with GRIP1 only,

Myc-GRASP-1 only, or an empty vector were used as negative controls. Since QT-6 cells do not express endogenous AMPA receptor, this result also suggested that the interaction between GRASP-1 and GRIP1 does not require AMPA receptors. GRASP-1 was also found to associate with GRIP2 using similar coimmunoprecipitation experiments from transfected HEK293 cells (Figure 2B).

Generation of GRASP-1 Antibodies
To analyze the GRASP-1 protein, we generated an antibody against a fusion protein containing the C-terminal PDZ (anti-GP1-PDZ) and an antibody against a peptide containing the C-terminal 18 amino acids (anti-GP1-Pep). Both of these antibodies recognized a major protein in brain lysates at 110 kDa and a minor protein around 30 kDa (Figure 2C). Antibody recognition of both the 110 kDa and the 30 kDa proteins was blocked when the antibodies were preincubated with antigen (data not shown). The 30 kDa protein is a caspase-3 proteolytic product of the full-length protein (see below).

GRASP-1, GRIP1, and AMPA Receptors Form a Protein Complex In Vivo
To see if GRIP1 and GRASP-1 are associated in neurons, coimmunoprecipitation experiments were performed with detergent extracts from cultured cortical neurons (Figure 2D). Anti-GRIP1 antibody, but not anti-Myc or anti-SAP102, immunoprecipitated GRASP-1 from cultured neuron lysates, suggesting that GRIP1 and GRASP-1 interact in neurons. GRIP1 contains seven PDZ domains, which have been proposed to serve as adaptor modules to link AMPA receptors to other neuronal proteins (Dong et al., 1997). To explore whether GRASP-1 forms a complex with GRIP1 and AMPA receptors in vivo, the interaction of these three proteins in solubilized brain plasma membrane extracts was analyzed using coimmunoprecipitation techniques. Isolation of AMPA receptors from brain lysates using a GluR2 monoclonal antibody coimmunoprecipitated GRASP-1, together with GRIP1 (Figure 2D). In contrast, control monoclonal antibodies against Myc or the NR1 subunit of NMDA receptors did not coimmunoprecipitate either GRASP-1 or GRIP1. These results indicate that GRASP-1 specifically forms a complex with GRIP1 and AMPA receptors in the brain.
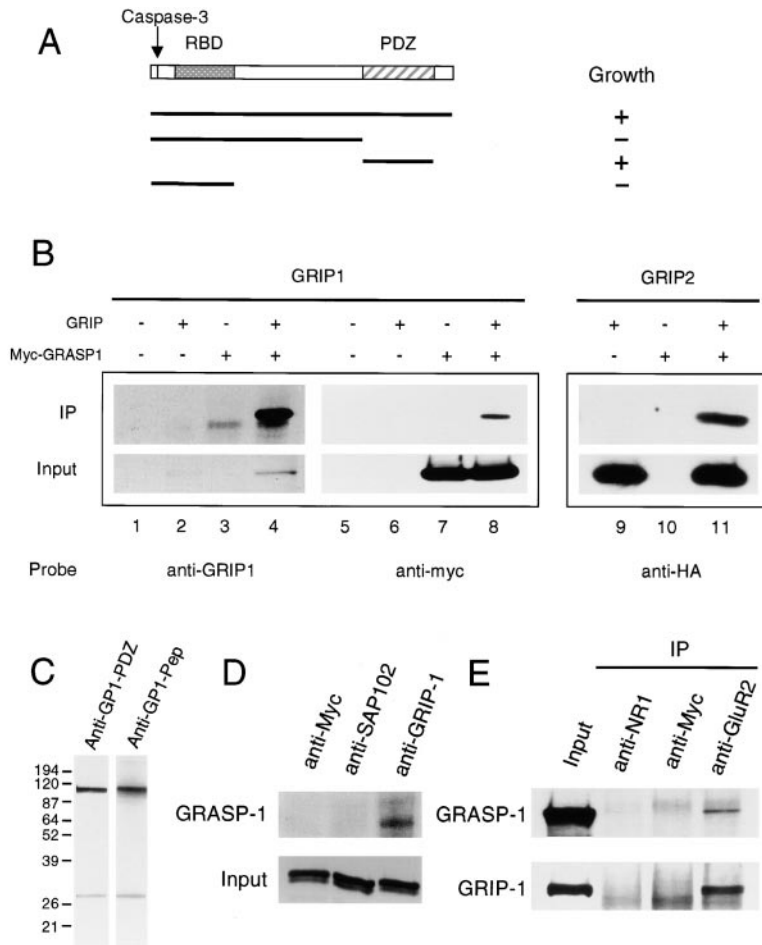
Figure 2. Association of GRASP-1 and GRIP1 In Vitro and In Vivo

(A) Mapping of the region in GRASP-1 that interacts with GRIP1 using the yeast two-hybrid system. Wild-type and deletion constructs of the C-terminal domain downstream of the caspase cleavage site were transformed into yeast HF7C together with GRIP1 PDZ 7 and assayed for growth on media deficient of leucine, tryptophan, and histidine.

(B) Coimmunoprecipitation of GRIP1 and GRASP-1 from transfected heterologous cells. For coimmunoprecipitation of GRIP1 and GRASP-1, QT-6 cells were cotransfected with Myc-tagged GRASP-1 and a full-length GRIP1 construct (lanes 4 and 8), with Myc-GRASP-1 alone (lanes 3 and 7), or with GRIP1 alone (lanes 2 and 6) or were mock transfected (lanes 1 and 5). The cell lysates were immunoprecipitated with either anti-Myc antibody (lanes 1–4) or anti-GRIP1 antibody (lanes 5–8). The immunoprecipitates were analyzed by Western analysis with either anti-GRIP1 antibody (lanes 1–4) or anti-Myc-antibody (lanes 5–8). For coimmunoprecipitation of GRIP2 and GRASP-1, HEK293T cells were cotransfected with HA-tagged GRIP2 PDZ 7 and Myc-GRASP-1 or these two constructs separately. Anti-Myc antibody was used for immunoprecipitation and anti-HA antibody was used for Western analysis.

(C) Western analysis on brain lysates with two anti-GRASP-1 antibodies, anti-GP1-PDZ, and anti-GP1-Pep.

(D) Coimmunoprecipitation of GRASP-1 and GRIP1 from solubilized cortical cultures. Rabbit polyclonal antibodies against Myc and SAP102 as controls or GRIP1 were incubated with 2% Triton X-100-solubilized high-density cortical cultured neurons and protein A-Sepharose. After washes, the immunoprecipitates were eluted with Laemmli sample buffer, separated by 7.5% SDS–PAGE, and transferred to PVDF membrane. The PVDF membrane was then probed with anti-GP1-Pep to detect GRASP-1.

(E) Coimmunoprecipitation of GRASP-1, GRIP1, and AMPA receptors from rat brain total membrane fraction. Mouse monoclonal antibodies against GluR2, NR1, or Myc were incubated with deoxycholate-solubilized rat brain membrane extracts and protein G-Sepharose, and the isolated immunoprecipitates were separated by 7.5% SDS–PAGE and transferred to PVDF membrane. The PVDF membranes were then probed with anti-GRASP-1 (anti-GP1-PDZ) or anti-GRIP1 antibodies (anti-GRIP1C).

RasGEF Activity of GRASP-1

By analyzing the amino acid sequence with BLAST search and MACAW program (Schuler et al., 1991), we found that GRASP-1 contained regions homologous to the catalytic domain of guanine nucleotide exchange factors (GEFs) for the ras family of small G proteins (Figure 3A). The rasGEF catalytic domains share homology in three structurally conserved regions (SCRs) separated by variable regions (Boguski and McCormick, 1993; Boriack-Sjodin et al., 1998), and GRASP-1 contains all three SCRs (Figure 3A). To examine whether GRASP-1 has rasGEF activity, we generated a bacterially expressed GST fusion protein (GST-C) containing the GEF catalytic domain upstream of the caspase cleavage site. The release of GDP from ras was then measured in the presence or absence of this fusion protein (Albright et al., 1993). H-ras was loaded with [^{3}H]GDP and then incubated with either GST or GST-C. After incubating with GST-C, H-ras loaded with [^{3}H]GDP rapidly released 80% of its radioactivity within 1 min, while [^{3}H]GDP-loaded H-ras incubated with GST was stable for at least 20 min (Figure 3B). This result confirms that GRASP-1 has rasGEF activity.

GRASP-1 Is a Substrate of Caspase-3

Both anti-GRASP-1 antibodies against the C-terminal portion of GRASP-1 recognize a predominant band at 110 kDa and an additional band at about 30 kDa (Figure 2C). Sequence analysis of GRASP-1 revealed a putative cleavage site for caspase-3 (D^{591}TVD^{594}) (Figure 4A) that would produce a C-terminal proteolytic fragment of about 30 kDa, close to the size of the 30 kDa protein recognized by the GRASP-1 antibodies. To examine whether caspase-3 could cleave this site, purified caspase-3 was incubated with in vitro–translated ^{35}S-methionine-labeled GRASP-1. Caspase-3 was found to rapidly generate a GRASP-1 cleavage product of about 30 kDa (Figure 4B). To confirm the site of caspase-3 cleavage, the aspartate residues (D591 and D594) within this sequence that have been shown to be required for caspase-3 cleavage in other proteins were mutated to glutamates (E) to eliminate cleavage. The resulting mutant GRASP-1 (D/E mutant) was not cleaved in vitro by caspase-3, confirming this as the site of cleavage (Figure 4C). To examine whether the 30 kDa protein observed in Western blots of brain lysates was in fact the cleavage product of the 110 kDa full-length protein, we incubated
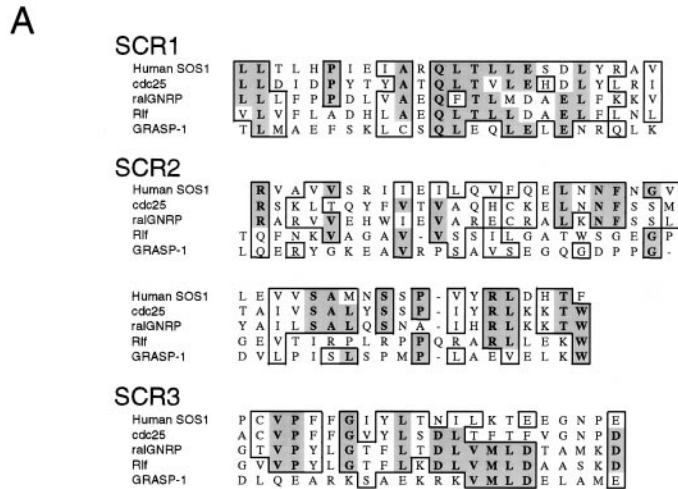
A

SCR1

Human SOS1	L	L	T	L	H	P	I	E	I	A	R	Q	L	T	L	L	E	S	D	L	Y	R	A	V
cdc25	L	L	D	I	D	P	Y	T	Y	A	T	Q	L	T	V	L	E	H	D	L	Y	L	R	I
ralGNRP	L	L	L	F	P	P	D	L	V	A	E	Q	F	T	L	M	D	A	E	L	F	K	K	V
Rlf	V	L	V	F	L	A	D	H	L	A	E	Q	L	T	L	L	D	A	E	L	F	L	N	L
GRASP-1	T	L	M	A	E	F	S	K	L	C	S	Q	L	E	Q	L	E	L	E	N	R	Q	L	K

SCR2

Human SOS1	R	V	A	V	V	S	R	I	I	E	I	L	Q	V	F	Q	E	L	N	N	F	N	G	V
cdc25	R	S	K	L	T	Q	Y	F	V	T	V	A	Q	H	C	K	E	L	N	N	F	S	S	M
ralGNRP	R	A	R	V	V	E	H	W	I	E	V	A	R	E	C	R	A	L	K	N	F	S	S	L
Rlf	T	Q	F	N	K	Y	A	G	A	V	-	V	S	S	I	L	G	A	T	W	S	G	E	G	P
GRASP-1	L	Q	E	R	Y	G	K	E	A	V	R	P	S	A	V	S	E	G	Q	G	D	P	P	G	-

Human SOS1	L	E	V	V	S	A	M	N	S	S	P	-	V	Y	R	L	D	H	T	F
cdc25	T	A	I	V	S	A	L	Y	S	S	P	-	I	Y	R	L	K	K	T	W
ralGNRP	Y	A	I	L	S	A	L	Q	S	N	A	-	I	H	R	L	K	K	T	W
Rlf	G	E	V	T	I	R	P	L	R	P	P	Q	R	A	R	L	L	E	K	W
GRASP-1	D	V	L	P	I	S	L	S	P	M	P	-	L	A	E	V	E	L	K	W

SCR3

Human SOS1	P	C	V	P	F	F	G	I	Y	L	T	N	I	L	K	T	E	E	G	N	P	E
cdc25	A	C	V	P	F	F	G	V	Y	L	S	D	L	T	F	T	F	V	G	N	P	D
ralGNRP	G	T	V	P	Y	L	G	T	F	L	T	D	L	V	M	L	D	T	A	M	K	D
Rlf	G	V	V	P	Y	L	G	T	F	L	K	D	L	V	M	L	D	A	A	S	K	D
GRASP-1	D	L	Q	E	A	R	K	S	A	E	K	R	K	V	M	L	D	E	L	A	M	E

Figure 3. The RasGEF Activity of GRASP-1

(A) Alignment of GRASP-1 rasGEF domain with the rasGEF domains of human SOS1, cdc25, ralGNRP, and Rlf by ClustalW program. The identical amino acids are shown in black boxes and the similar residues are shown in gray boxes.

(B) Purified GRASP-1 catalytic domain (GST-C) promotes dissociation of GDP from ras. GST-H-ras was loaded with [³H]GDP and then incubated with either purified GST (open squares) or GST-C (open triangles). Aliquots of these two reactions were taken out at different time points and were filtered on HA filters. The [³H]GDP that remained on H-ras was measured by liquid scintillation counting. Note that after incubating with GST-C, H-ras loaded with [³H]GDP quickly lost 80% of its radioactivity within 1 min, while H-ras incubated with GST retained its original radioactivity for at least 20 min.

B
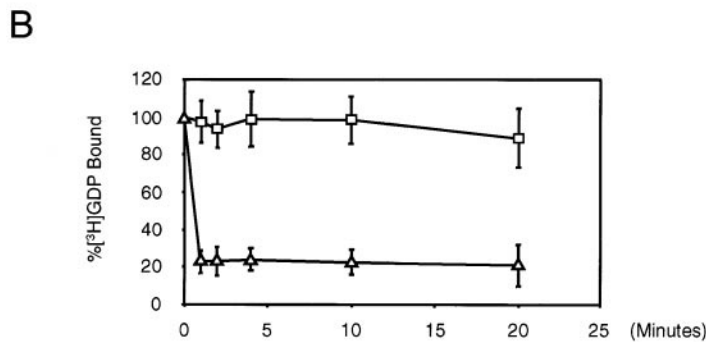
brain lysate with purified caspase-3. With increasing incubation times, the amount of the full-length 110 kDa protein decreased, while the 30 kDa protein increased (Figure 4D). These results demonstrate that the native 30 kDa protein in brain comigrates with the product from in vitro caspase-3 cleavage and suggests that this protein is likely to be an in vivo caspase-3 cleavage product of GRASP-1. Interestingly, this cleavage results in the separation of the N-terminal catalytic domain from the potential C-terminal regulatory domain.

Neuronal Expression of GRASP-1

To examine the tissue distribution of GRASP-1, Western analysis was performed on cell lysates from multiple tissues using the anti-GP1-PDZ fusion protein antibody (Figures 5A and 5B). GRASP-1 (110 kDa) was specifically expressed in all tissues of the nervous system including cortex, cerebellum, hippocampus, olfactory bulb, thalamus, spinal cord, and brainstem and was not detected in muscle, lung, heart, liver, kidney, and spleen (Figures 5A and 5B). Northern analysis showed a similar tissue distribution and identified a 3.0–3.5 kb GRASP-1 mRNA (data not shown). The distribution of GRASP-1 is similar to that of GRIP1 and -2, which has previously been shown to be specifically expressed in neurons (Dong et al., 1997).

To further investigate the cellular distribution of GRASP-1, immunohistochemistry was performed with the GP1-PDZ fusion protein antibody (Figure 5C). GRASP-1 was specifically expressed in neurons in the

soma and dendritic processes. GRASP-1 was not observed in glia. Consistent with results from Western analysis, GRASP-1 was widely localized in various brain regions including cortex, hippocampus, thalamus, striatum, and brainstem cerebellum (Figure 5C). In both cortex and hippocampus, the most prominent staining was observed in pyramidal cells, although other neurons were also stained. In cerebellum, Purkinje cells and granule cells were positive for GRASP-1 immunoreactivity. The cellular distribution of GRASP-1 is similar to that of GRIP1 and AMPA receptors (Petralia et al., 1994, 1997; Wyszynski et al., 1998; Dong et al., 1999).

GRASP-1 Is Distributed to Punctate Structures in Neurons and Is Partially Localized to Synapses

GRIP1 partially colocalizes with AMPA receptors at excitatory synapses and is enriched in postsynaptic density (PSD) fraction of brain homogenate (Dong et al., 1997, 1999; Wyszynski et al., 1998). To determine the subcellular distribution of GRASP-1 in neurons, embryonic hippocampal neuronal cultures (embryonic day 18, 3 weeks in vitro) were stained with Fluo-EX-labeled anti-GP1-PDZ antibody, and then analyzed using immunocytofluorescence techniques (Figure 6Aa). GRASP-1 was distributed throughout the cell soma (Figure 6Ab) and dendrites (Figure 6Ac) as small fluorescent puncta. The specificity of the staining was confirmed by both blocking with the antigen (data not shown) and the high degree of colocalization of the punctate staining by anti-GP1-PDZ and anti-GP1-Pep, two different antibodies against GRASP-1 (Figures 6Ba and 6Bb).

A

GRASP-1	VKD**T**V**D**GQ
MEKK 1	SED**T**L**D**GQ
PARP	KGDEVDGT
MDM2	GFDVPDCK
Procaspase-6	PLDVVDNQ
Procaspase-9	TFDQLDAI

B

[^{35}S]Met
Labeled

W.t.   W.t.

194
120
87
64
52
39

26

21

C

Western

W.t.   D/E
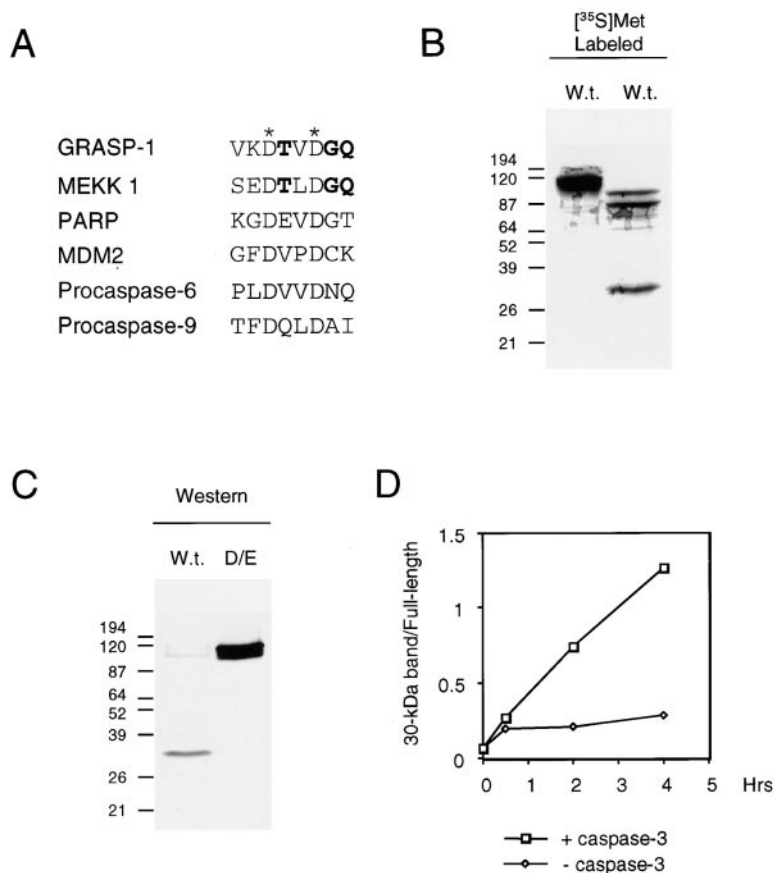
194
120
87
64
52
39

26

21

D

Figure 4. GRASP-1 Is a Substrate for Caspase-3 In Vivo and In Vitro

(A) Comparison of the amino acid sequence of the putative caspase-3 cleavage site in GRASP-1 (D^{591}TVD^{594}) with other identified caspase-3 cleavage sites. The identical amino acids between the sites of GRASP-1 and MEKK1 are shown in bold.

(B) Cleavage of in vitro–translated ^{35}S-methionine-labeled GRASP-1 by purified caspase-3. Left lane, cleavage reaction without purified caspase-3. Right lane, cleavage reaction with purified caspase-3.

(C) Caspase-3 cleaves at D591 and D594. The putative caspase-3 cleavage site was mutated in GRASP-1 (D/E mutant) and translated in vitro and used as substrate for in vitro caspase-3 cleavage reactions followed by Western analysis using anti-GR1-PDZ antibody. The wild-type protein was cleaved (left lane), whereas the D/E mutant was not (right lane).

(D) The in vitro cleavage product comigrates with the 30 kDa band recognized by GRASP-1 antibodies. Brain lysates (2 μg) were incubated in the presence or absence of purified caspase-3 (200 ng) for variable times. Note that with increasing incubation times, the ratio of the 30 kDa band over the full-length 110 kDa protein increased in the presence of caspase-3, while the ratio was relatively stable in the absence of caspase-3.

To examine whether GRASP-1 was distributed to synapses, the cultured neurons were triple-labeled with antibodies against GRASP-1, GRIP1, and synaptophysin, a marker for synapses. A subset of the GRASP-1 puncta colocalized with GRIP1 and synaptophysin, suggesting GRASP-1 and GRIP1 can be colocalized at synapses (Figure 6C). To further investigate the subcellular distribution of GRASP-1, we performed biochemical subcellular fractionation of rat brain lysate. GRASP-1 (110 kDa) was distributed in every fraction examined with a modest enrichment in the PSD1 fraction as well as in the cytosol (S2) (Figure 5D). Neither 0.5% Triton X-100 nor 3% sarcosyl solubilized the GRASP-1 in the PSD fraction. Interestingly, the 30 kDa GRASP-1 caspase-3 cleavage product was enriched in cytosolic fractions (S2 and SC) but not the PSD fraction and was readily solubilized by 3% sarcosyl (Figure 5D). The distribution of GRASP-1 is distinct from soluble protein lactate dehydrogenase (LDH) and the synaptic GluR2/3 subunit (Figure 5D). The partial distribution of GRASP-1 between cytosol and membrane is in agreement with the finding that many rasGEFs are partially localized to the membrane fraction, reflecting their dynamically regulated membrane association (Quilliam et al., 1994, 1995).

Overexpression of GRASP-1 Decreases the Synaptic Localization of AMPA Receptors

The results presented so far show that GRASP-1 is a novel neuronal rasGEF that interacts with GRIP and exists in a protein complex with GRIP1 and AMPA receptors in vivo. Together with other AMPA receptor–interacting proteins such as Pick-1 and NSF, GRIP1 has been implicated in the synaptic localization of AMPA receptors. To explore the possibility that GRASP-1 regulates AMPA receptor distribution, we overexpressed a Myc-tagged full-length GRASP-1 in primary neuronal cultures. Low-density hippocampal neurons were transfected at 3–5 days in vitro with constructs containing either Myc-GRASP-1 or GFP. The transfected coverslips were cultured for another 9–11 days and then labeled with a polyclonal antibody against GluR1 or NR1 (Liao et al., 1999). The neurons that were transfected with Myc-GRASP-1 were identified by double labeling the neurons with a Myc staining, while the neurons transfected with GFP were detected with GFP fluorescence.

Strikingly, we observed that overexpression of Myc-GRASP-1 in neurons dramatically reduced the number of synaptic AMPA receptor clusters (Figures 7A and 7C) compared to cells overexpressing GFP or untransfected cells on the same coverslip (Figures 7B and 7C). In contrast, overexpression of GRASP-1 had no significant effect on the number of NMDA receptor clusters (Figures 7A–7C). Consistent with previous findings, approximately half of the synaptic AMPA clusters colocalized with NMDA clusters in untransfected cells (Figure 7C) (Liao et al., 1999). This result suggests that the overexpression of GRASP-1 specifically disrupts AMPA receptor synaptic targeting.

To study the domain requirement for disruption of AMPA receptor targeting, we generated specific deletion mutants of GRASP-1. To inactivate the GEF domain, we deleted the second SCR (ΔSCR; Δ amino acids 150–192) in the catalytic domain. Deletion of this region eliminated the disruption of the synaptic targeting of the
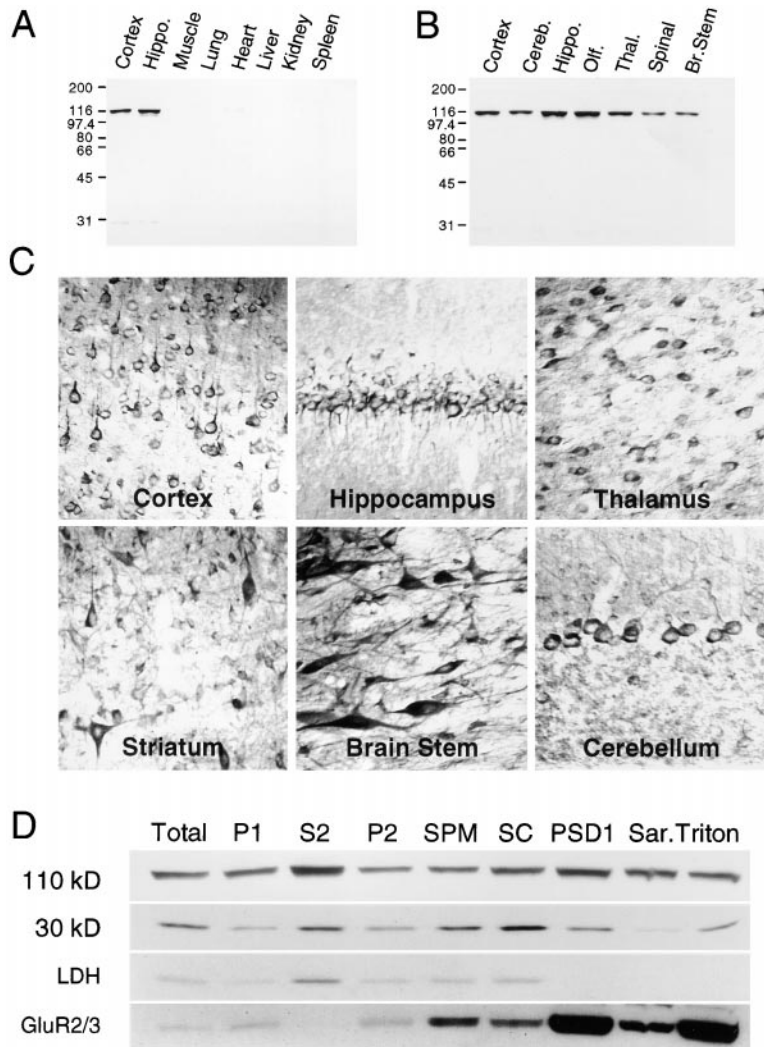
Figure 5. Neuronal Expression of GRASP-1

(A and B) Western analysis of GRASP-1 distribution. GRASP-1 was specifically expressed in brain tissues represented by cortex and hippocampus and was absent in muscle, lung, heart, liver, kidney, and spleen (A). GRASP-1 was expressed in various regions in brain including cortex, cerebellum, hippocampus, olfactory bulb, spinal cord, and brainstem (B).

(C) Immunohistochemistry of GRASP-1 in different brain regions.

(D) Western analysis of the localization of GRASP-1 in subcellular fractions of rat brain homogenate. Ten micrograms of protein from each fraction were separated on a SDS-polyacrylamide gel. The same PVDF membrane was probed with anti-GRASP-1 (anti-GP1-PDZ), anti-LDH (1:1000, Chemicon), and anti-GluR2/3 (JH1684, 1:500) sequentially after stripping.

AMPA receptors by GRASP-1 overexpression (Figures 8A and 8B). In addition, deletion of the C-terminal "regulatory" domain (ΔR; Δ amino acids 592–827) eliminated the effect of GRASP-1 overexpression on AMPA receptor targeting (Figures 8A and 8B). These results suggest that the rasGEF activity is required but not sufficient for disruption of AMPA receptor synaptic targeting and indicate that the C-terminal "regulatory" domain may be required for the proper regulation or localization of the GEF activity.

NMDA Receptor–Induced AMPA Receptor and GRASP-1 Redistribution

Recent studies have indicated that the redistribution of synaptic AMPA receptors may be critical for the expression of LTP and LTD (Davies et al., 1989; Isaac et al., 1995; Liao et al., 1995; Malenka and Nicoll, 1997). Experiments in cultured neurons have recently shown that low-frequency stimulation or glutamate treatment rapidly depresses AMPA receptor responses and decreases the number of AMPA receptor synaptic clusters (Carroll et al., 1999; Lissin et al., 1999). To address whether GRASP-1 may be involved in this process, we activated glutamate receptors in high-density cultured cortical neurons (embryonic day 17, 3 weeks in vitro) with 40 μM glutamate for 5 min and then stained for

GluR1 or GRASP-1 together with NR1. Consistent with a previous study (Lissin et al., 1999), we observed that glutamate treatment caused a pronounced reduction of the synaptic AMPA receptor clusters (Figures 9Aa and 9Ad) while exhibiting no significant effects on NMDA receptor localization (Figures 9Ab and 9Ae). The effect of glutamate did not appear to be due to excitotoxicity, as the synaptic targeting of AMPA receptors recovered after several hours in normal media (data not shown). The glutamate-induced redistribution of AMPA receptors was NMDA receptor–dependent since the NMDA receptor antagonist APV (200 μM) completely blocked the effects of glutamate, while the AMPA receptor antagonist CNQX (20 μM) had no effect (data not shown). Intriguingly, glutamate-treatment also caused a very dramatic dispersal of the GRASP-1 immunoreactive puncti in the soma and dendritic processes (Figures 9Ba and 9Bb). The disappearance of GRASP-1 immunoreactive puncti was not due to degradation or cleavage of GRASP-1, since the amount of GRASP-1 protein in the cultures was stable for at least 1 hr after the transient glutamate treatment (Figure 9C). As with the redistribution of the AMPA receptors, the glutamate-induced redistribution of GRASP-1 was blocked by APV, while CNQX had no effect (Figures 9Bc and 9Bd). These results, combined with the GRASP-1 overexpression data pre-
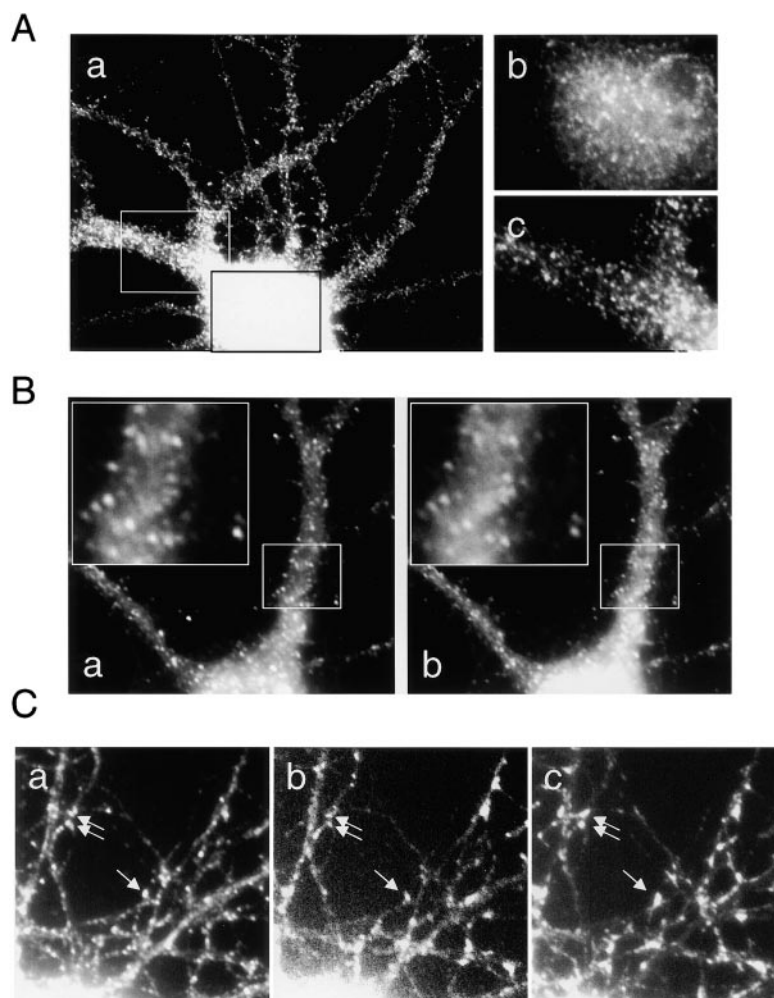
Figure 6. GRASP-1 Is Partially Localized to Synapses

(A) Immunocytochemistry of GRASP-1.
(a) Low-density hippocampal cultured neurons were immunostained with Cy3-labeled anti-GRASP-1 antibody (anti-GP1-PDZ).
(b) Close-up of a region of the soma.
(c) Close-up of the dendrites.
(B) Two different anti-GRASP-1 antibodies stained the same structure.
(a) Staining with Cy3-labeled anti-GP1-PDZ.
(b) Staining with the Cy2-labeled anti-GP1-Pep.
(Insets) Close-up of the boxed regions.
(C) Colocalization of GRASP-1, GRIP1, and synaptophysin. Low-density hippocampal neurons were triply stained with Cy3-labled anti-GRASP-1 (a), Cy2-labled anti-GRIP1 (b), and mouse monoclonal anti-synaptophysin antibodies (c). Arrows show colocalized GRASP-1, GRIP1, and synaptophysin.

sented above, suggest that GRASP-1 may be important in the regulation of AMPA receptor synaptic targeting.

Discussion

GRIP1 and -2 are multi–PDZ domain–containing proteins that appear to serve as adaptor proteins to link AMPA receptors to other neuronal proteins through the interaction of its PDZ domains with specific protein ligands. In this study we describe the isolation of several proteins that specifically interact with distinct PDZ domains in GRIP. We characterize one of these proteins in detail and report that GRASP-1 is a novel protein that interacts with PDZ 7 of GRIP1 and -2 and is associated with AMPA receptors and GRIP1 in vivo in rat brain. GRASP-1 is a neuronal specific rasGEF that is a member of a novel rasGEF family. GRASP-1 has a complex domain structure that has some similarities to the ralGDS family of rasGEFs (Albright et al., 1993; Hofer et al., 1994; Kikuchi et al., 1994) and includes a rasGEF domain in the N-terminal region and RBD and PDZ domains in the C-terminal region. GEFs positively regulate the activation of the ras, rho, rab, ran, and ARF families of small G proteins (Bourne et al., 1991; Boguski and McCormick, 1993; Quilliam et al., 1995).

Ras was the first identified small G protein and is involved in a variety of functions including cell growth, differentiation, and transformation. However, recent studies have shown that ras signaling is critical for NMDA-dependent synaptic plasticity in the brain. NMDA receptor activation leads to the activation of ras and the MAPK kinase pathway (Yun et al., 1998). Moreover, using genetically engineered mice several laboratories have suggested that distinct ras signaling molecules may be critical for synaptic plasticity in several different brain regions (Orban et al., 1999). Mice deficient in rasGRF, a neuronal GEF for ras, exhibit impaired amygdala LTP and impaired fear conditioning, a form of amygdala-dependent learning (Brambilla et al., 1997). In contrast, hippocampal CA1 LTP and spatial learning appeared normal in these mice, suggesting that rasGRF is specifically involved in synaptic plasticity in the amygdala. However, heterozygous mice with targeted deletions in the NF1 gene, a rasGAP gene, have been shown to have defects in spatial learning (Silva et al., 1997), suggesting that hippocampal synaptic plasticity also requires the regulation of ras signaling. The ras subfamily includes several members including ras, rap, ral, rin/rit, and rheb proteins that vary in their biochemical properties and cellular and tissue distributions, suggesting that they each have distinct physiological roles. For example, rin and rheb are enriched in neurons (Yamagata et al., 1994; Lee et al., 1996; Wes et al., 1996), while ras, rap, ral, and rit are expressed ubiquitously. Rin and rit can bind to
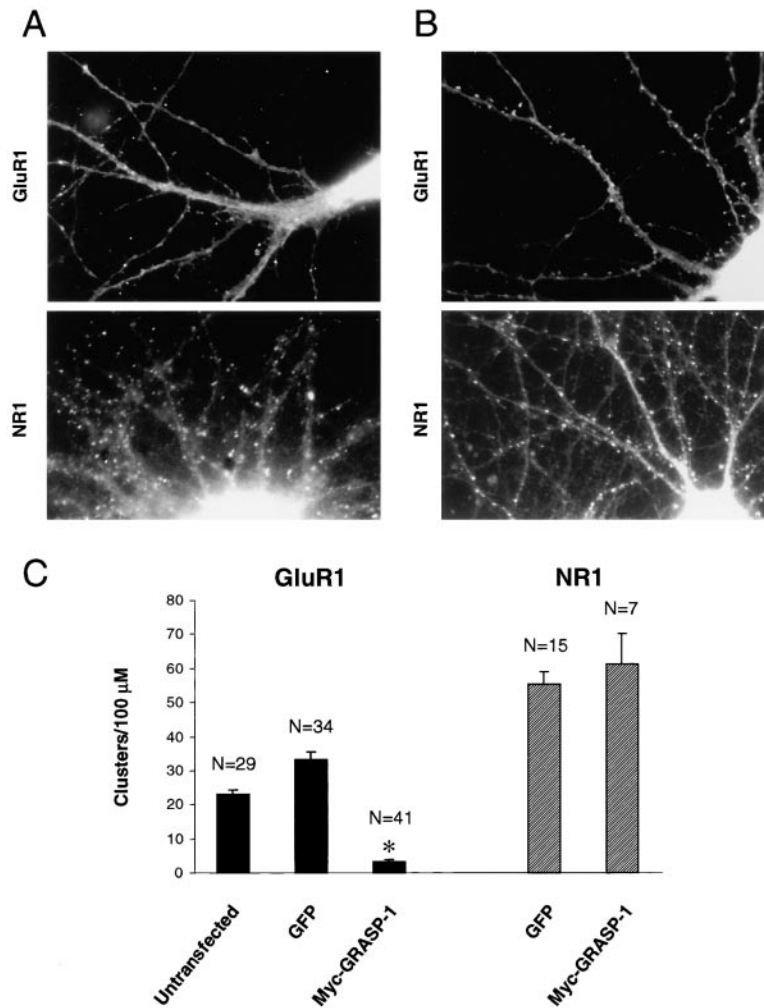
Figure 7. Overexpression of GRASP-1 Specifically Reduces AMPA Receptor Clusters

Low-density hippocampal neurons cultured on coverslips were transfected with either Myc-GRASP-1 or pEGFP. After 9–11 days, the neurons were immunostained to examine NMDA and AMPA receptor distribution.
(A) GluR1 and NR1 staining in neurons overexpressing Myc-GRASP-1.
(B) GluR1 and NR1 staining in neurons overexpressing GFP.
(C) Statistical analysis of the transfection experiments. Data represent the mean $\pm$ SD of the number of cells indicated by n; *p $<$ 0.01 compared with GFP-transfected cells using t test.

calmodulin, suggesting that they might link membrane calcium channels, including NMDA receptors, to ras-like signaling pathways (Lee et al., 1996; Wes et al., 1996), while rheb is an immediate-early gene that appears to be involved in neuronal plasticity (Yamagata et al., 1994; Yee and Worley, 1997). Although GRASP-1 can serve as a rasGEF in vitro, it is still not clear which ras is the in vivo target of GRASP-1.

The functional roles of the other identified domains of GRASP-1 are not clear, although they most likely play a role in the regulation of the GEF activity of GRASP-1. RBD domains have previously been observed in combination with GEF domains in the ralGDS family of GEFs. In ralGDS, the binding of ras to the RBD activates its GEF activity toward its target small G protein ral (Hofer et al., 1994; Spaargaren and Bischoff, 1994; Urano et al., 1996). It is likely that binding of ras-like proteins to the RBD region of GRASP-1 may also regulate its GEF activity. Our results demonstrate that the PDZ domain of GRASP-1 binds to the seventh PDZ domain of GRIP, suggesting that GRIP may serve as an adaptor protein to link GRASP-1 with other GRIP-associated proteins. Previous studies in other systems have shown that the recruitment of rasGEFs to signal transduction complexes plays a critical role in the activation of G proteins and downstream signal transduction (Quilliam et al.,

1994, 1995). For example, the rasGEF SOS1 is recruited to signal transduction complexes containing receptor tyrosine kinases by the adaptor molecule Grb2. The association of SOS1 with the receptor brings it to plasma membrane where it is able to activate ras (Buday and Downward, 1993; Chardin et al., 1993; Li et al., 1993; Olivier et al., 1993; Rozakis-Adcock et al., 1993). GRIP1 has seven PDZ domains, which mediate protein–protein interactions, allowing the recruitment of GRASP-1 to a large signal-transducing complex (Dong et al., 1997). Previous studies have shown that GRIP1 interacts with the GluR2 subunit of AMPA receptors and with the Eph receptor tyrosine kinases and their ligands, such as ephrin-B2 (Torres et al., 1998; Bruckner et al., 1999). The association with ephrin-B2 results in the recruitment of GRIP to raft membrane microdomains, a highly organized membrane region that has been proposed to be important for various signal transduction pathways. Moreover, stimulation of ephrin-B resulted in the recruitment of GRIP to large raft patches on the cell surface, presumably bringing a signaling complex into close proximity to ephrin-B. These results suggest that GRASP-1 in addition to regulating AMPA receptor function may also be important in Eph and ephrin signaling.

Recent structural analysis of the PDZ–PDZ interaction of nNOS and syntrophin revealed that the interaction is
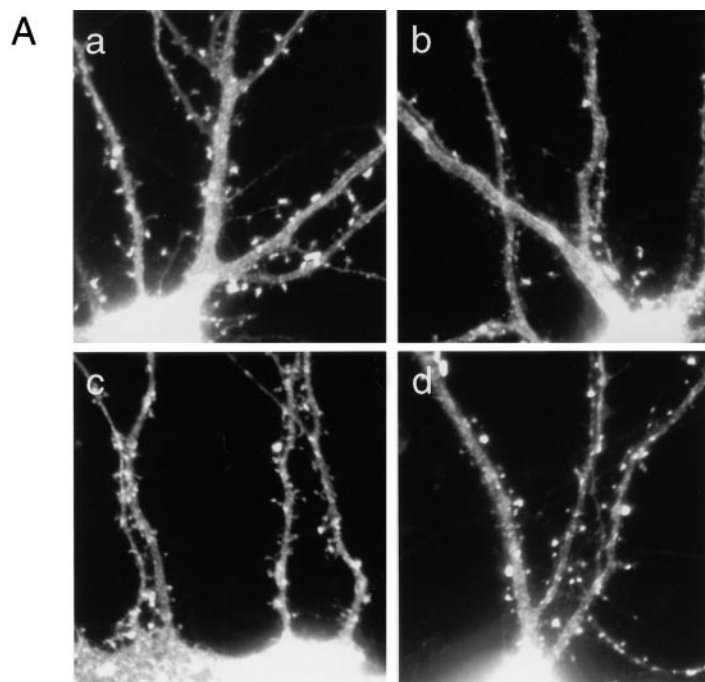
Figure 8. GEF and Regulatory Domains of GRASP-1 Required to Disrupt Synaptic Targeting

Low-density hippocampal neurons cultured on coverslips were transfected with the indicated DNA plasmids at 3–5 days in vitro and were immunostained after 14 days.
(A) GluR1 staining in neurons overexpressing GFP (a), Myc-GRASP-1 (b), Myc-GRASP-1ΔSCR2 (c), and Myc-GRASP-1-ΔR (d).
(B) Statistical analysis of the transfection experiments. The level of expression of the various deletion mutants was similar as detected by staining with anti-Myc antibody (not shown). Data represent the mean ± SEM of the number of cells indicated by n; *p < 0.01 analyzed by ANOVA.
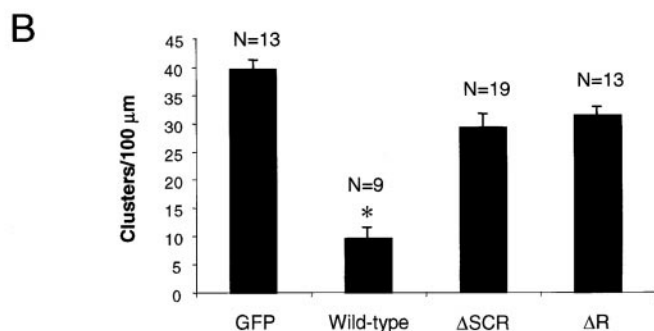
mediated by a β finger flanking the nNOS PDZ domain which mimics a COOH-terminal T/SXV PDZ ligand motif. Analysis of the secondary structure of GRIP1 and -2 by Chou-Fasman and Robson-Garnier methods suggests that a similar β sheet–β turn–β sheet structure may be conserved in the seventh PDZ of GRIP1 and -2. This structure has a pseudo-T/SXV motif (DLVI) in the first β sheet that may be important for the PDZ–PDZ interaction.

Interestingly the rasGEF domain of GRASP-1 is separated from the RBD and PDZ domains by a functional caspase-3 cleavage site. Caspase-3 is a critical protease in the protease cascade central to programmed cell death (reviewed by Thornberry and Lazebnik, 1998). Caspase-3 cleavage of substrate proteins regulates their function and results in the inactivation of apoptosis inhibitors (Bcl-2 and I^{CAD}), the disassembly of cell structures (lamins), and the deregulation of enzymes through the separation of regulatory and catalytic domains (gelsolin, DNA-PKcs, and MEKK1) (reviewed by Thornberry and Lazebnik, 1998). Recently, it has been shown that caspase-3 activation occurs after nontoxic depolarization (30 mM and 100 mM K^+) of cultured hippocampal neurons, and, moreover, intraventricular administration

of a caspase inhibitor (zVAD-fmk) to adult rats impairs learning ability in the Morris water maze (Chan and Mattson, 1999). These data suggest that caspase-3 may play nonapoptotic roles in neuronal function. Although the consequence of caspase cleavage of GRASP-1 is unclear, it is possible that it may uncouple the C-terminal "regulatory" domain of GRASP-1 from the rasGEF domain, disrupting the proper regulation or targeting of GEF. In fact, overexpression of the N-terminal GEF catalytic domain (ΔR) was not sufficient to downregulate AMPA receptor synaptic targeting. This idea is consistent with the effect of caspase-3 on MEKK1. Caspase-3 cleavage of MEK kinase 1 (MEKK1) results in its constitutive activation and redistribution of MEKK1 kinase domain from membranes to cytosolic fractions (Widmann et al., 1998).

LTP and LTD have been proposed to be mediated, in part, by changes in AMPA receptor function. Increases in AMPA receptor responses have been observed during the expression of LTP (Kauer et al., 1988; Muller et al., 1988; Davies et al., 1989; Liao et al., 1995). Recently, it has been shown that a high proportion of synapses in hippocampal CA1 region contains only NMDA receptors and acquires AMPA receptors only after the induction
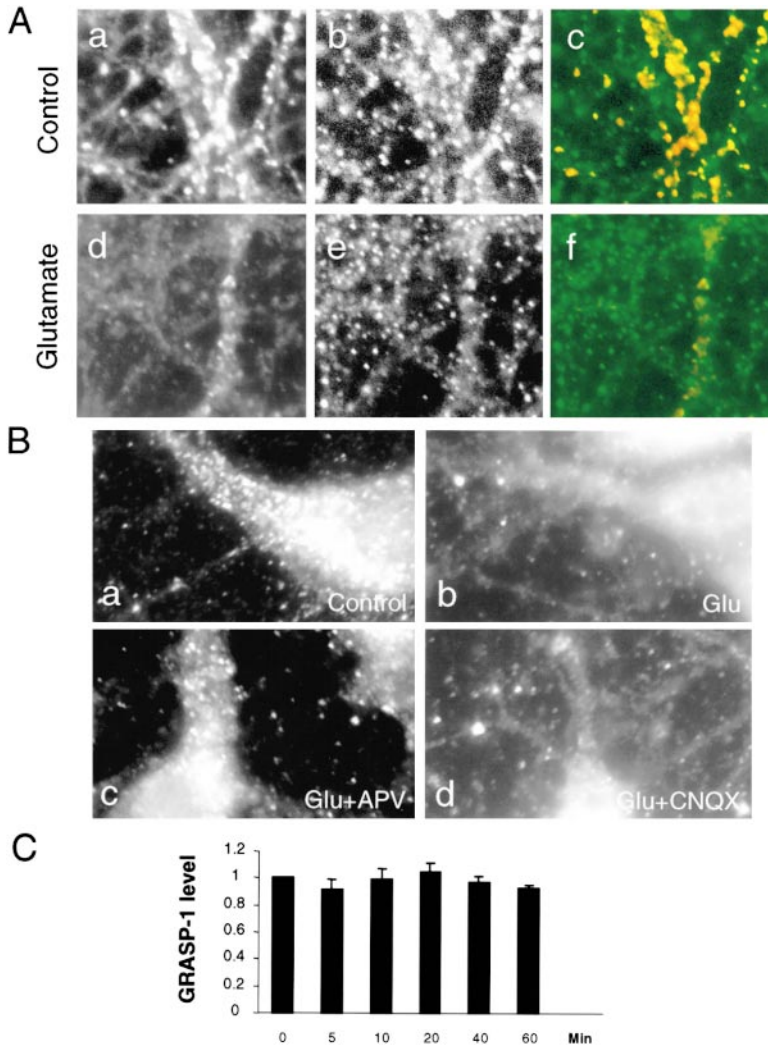
Figure 9. Glutamate Induces Redistribution of GRASP-1 and AMPA Receptors

(A) Glutamate (40 μM) treatment caused a dramatic loss of AMPA receptor clusters compared to cortical cells (a and d) but had no effect on NMDA receptor localization (b and e). (c) and (f) show overlap of the NMDA receptor staining (b or e) and the AMPA receptor staining (a or d). The AMPA receptor (GluR1/2/3) staining is indicated in red. The NMDA receptor (NR1) staining is indicated in green, and the overlapping regions are indicated in yellow. High-density cortical neuronal cultures on coverslips were stained with anti-NR1 (NMDA receptors) or anti-GluR1 plus anti-GluR2/3 (AMPA receptors) antibodies.

(B) Glutamate induced redistribution of GRASP-1 (a and b). Note that the bright puncta of GRASP-1 immunofluorescence in the soma and processes disappeared and became more diffuse after the glutamate treatment. Two hundred micromolar APV (c) but not twenty micromolar CNQX (d) blocked the effect of glutamate on GRASP-1 distribution.

(C) Western analysis of the GRASP-1 protein amount at different time points after the transient glutamate treatment. The same type of cultured neurons was used except that the neurons were plated on coated dishes rather than coverslips.

of LTP (Isaac et al., 1995; Liao et al., 1995). This emergence of AMPA receptor current seems due to the appearance of synaptic AMPA receptors (Liao et al., 1999; Shi et al., 1999). Moreover, NMDA receptor–dependent LTD in cultured neurons has recently been observed to correlate with a decrease in the levels of synaptic AMPA receptors (Carroll et al., 1999). Previous studies have suggested that AMPA receptor–associated proteins, such as GRIP, are involved in the synaptic targeting of AMPA receptors (Song et al., 1998; Xia et al., 1999). In this study, we have added GRASP-1 to this complex and provided evidence that it may also be important in regulation of AMPA receptor function and play a role in AMPA receptor synaptic targeting. Overexpression of GRASP-1 in neurons downregulated synaptic AMPA receptor clusters, while it had no effect on synaptic NMDA receptor synaptic targeting. Both the rasGEF catalytic domain and the C-terminal "regulatory" domain were required for this activity. Activation of NMDA receptors dramatically induced the redistribution of both GRASP-1 and AMPA receptors from punctate membrane structures to a more diffuse pattern. Together with the GRASP-1 overexpression data, these results suggest that the overall spatial distribution of GRASP-1, as well as the absolute levels, may be important for AMPA receptor targeting. These results suggest that GRASP-1

and possibly ras signaling may play a role in the regulation of AMPA receptor synaptic targeting and its regulation by NMDA receptor activity.

Experimental Procedures

Yeast Two-Hybrid Screening and Full-Length cDNA Cloning
The PDZ domains 1, 2, and 3 together, 4, 5, and 6 together, and 7 alone were subcloned into yeast expression vector pPC97 in a fusion with the GAL4 DNA binding domain. These constructs were transformed into yeast strain Y190 by the lithium acetate method (Straus and Ausubel, 1990). Transformants were selected on leucine-deficient (Leu⁻) medium and subsequently transformed with a rat hippocampal cDNA library subcloned into vector pPC86. For screening with bait 1 (PDZ 1, 2, 3) and bait 2 (PDZ 4, 5, 6), random-primed rat hippocampal library was used. For screening with bait 3 (PDZ 7), both random-primed and oligo-dT-primed libraries were used (gifts of Dr. Anthony Lanahan and Dr. Paul Worley). Positive clones were selected on triple-deficient plates (Leu⁻, Trp⁻, His⁻) containing 50 mM 3-aminotriazole and assayed for β-galactosidase activity. Those clones that both grew on triple-deficient plates and turned blue in the β-galactosidase assay were selected, and their plasmids were isolated and transformed into *E. coli* DH10B. The *E. coli* clones containing the pPC86 construct were rescued and their plasmids were isolated. The positive clones were cotransformed into yeast Y190 with either the bait vector or the original pPC97 vector to confirm the interaction.

To obtain the full-length cDNA of GRASP-1, the SalI/SacI fragment

(about 300 bp) of yeast clone D7-D-12 (1.4 kb) was used as a probe to screen rat hippocampal cDNA libraries. Both a CapFinder library and an oligo-dT-primed library subcloned in phage λ ZAP were used (both are gifts of Dr. Anthony Lanahan and Dr. Paul Worley). The CapFinder library was constructed with the CapFinder PCR cDNA library Construction Kit (Clontech Laboratories, CA). This library construction method takes advantage of the 7-methylguanosine cap structure, which is present on the 5′ end of all eukaryotic mRNAs to enrich the full-length clones in the resulting library.

Antibody Generation and Purification and Western Analysis

The cDNA encoding the last 156 amino acids of GRASP-1 was subcloned into vector pTrcHisB (Invitrogen, CA) in a fusion with an N-terminal polyhistidine (His$_6$) tag and transformed into *E. coli* BL21. The His$_6$-tagged fusion protein was purified in denaturing condition according to the procedure provided by the manufacturer of the pTrcHis protein purification system (Invitrogen, CA). To generate a peptide antigen, a peptide containing the last 20 amino acids of GRASP-1 was synthesized (RH157) and cross-linked to thyroglobulin. The fusion protein and the peptide were then used to immunize two New Zealand White rabbits (JH 2377 and JH 2730, respectively) (Hazleton Research Products).

Various organs and different regions of the nervous systems of Sprague-Dawley rats were homogenized and sonicated. The homogenates were centrifuged at 1000 × g for 15 min. The supernatant (10 μg) from each tissue was lysed in Laemmli buffer (Laemmli, 1970) and loaded onto 7.5% SDS–PAGE gels and transferred to PVDF membranes. Anti-GP1-PDZ antibody (JH2377, 1:5000) or anti-GP1-C (JH2730, 1:5000) was used as the primary antibody for Western analysis.

Immunohistochemistry

Male Sprague-Dawley rats (200–350 g) were anesthetized with pentobarbital (50 mg/kg) and perfused transcardially with 100 ml of cold 0.1 M phosphate-buffered saline (PBS), followed by cold 4% paraformadehyde/0.1 M phosphate buffer (pH 7.3). Brains were removed and postfixed for 2 hr at 4°C and cryoprotected in 30% sucrose/PBS overnight at 4°C. Coronal and sagittal sections (40 μm) were made with a sliding microtome and washed with PBS followed by incubation in 0.6% H$_2$O$_2$/10% ethanol/PBS for 15 min at room temperature. After being blocked and permeabilized in 10% normal goat serum/2% skim milk/0.2% Triton X-100/PBS for 1 hr at room temperature, the sections were incubated with GP1-PDZ antiserum (JH2377) at a dilution of 1:10,000 in 2% normal goat serum/2% skim milk/PBS overnight at 4°C. Preimmune serum at the same dilution was used as negative control. The sections were then washed in PBS, incubated with 1:300 biotinylated goat anti-rabbit antibody in PBS for 1 hr at room temperature, and subsequently incubated with avidin-biotin-peroxidase complex for 1 hr at room temperature (Vector Labs, CA). After incubation with 55% 3,3-diaminobenzidine tetrahydrochloride (DAB)/0.1% H$_2$O$_2$/50 μM Tris-HCl for 5–15 min, the sections were dehydrated and mounted.

Subcellular Fractionation

Subcellular fractionation was performed according to Carlin et al. (1980) with the following modifications. The synaptosomal fraction was solubilized in 0.5% ice-cold Triton X-100 for 15 min and centrifuged at 32,000 × g for 20 min to obtain PSD1 fraction. PSD1 was resuspended and solubilized either in 0.5% Triton X-100 or in 3% sarcosyl (ice cold) for 10 min and then centrifuged at 200,000 × g for 1 hr to obtain the PSD2 or PSD3 fractions, respectively. After the fractions were resuspended in 40 mM Tris-HCl (pH 8.0), the protein concentrations were determined by BCA assay (Pierce Chemical, IL). Equal amounts of proteins were then solubilized in Laemmli buffer and separated by SDS–PAGE.

In Vitro Caspase-3 Cleavage Assay, In Vitro Translation, and Generation of the Cleavage-Deficient Mutant

Coupled in vitro transcription and translation was performed with the TNT Coupled Reticulocyte Lysate System (Promega, WI). In brief, 25 μl of TNT rabbit reticulocyte lysate, 2 μl of TNT reaction buffer, 1 μl of TNT T7 RNA polymerase, 1 μl of 1 mM amino acid mixture minus methionine, [^{35}S]methionine (1,000 Ci/mmol) at 10 mCi/ml (NEN), 1 μl of 40 μg/μl Rnasin ribonuclease inhibitor (Promega, WI), and 1 μg of GRASP-1 cDNA subcloned in vector pCDNA3.1(−) were mixed in a volume of 50 μl at 30°C for 1 hr.

To perform the caspase-3 cleavage reaction in vitro, 1 μl of in vitro–translated protein sample or 2 μg of brain lysate was incubated with 1 μl of purified active recombinant human caspase-3 (Pharmingen) in 18 μl caspase cleavage reaction buffer (20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% CHAPS, and 10% sucrose [pH 7.2]) at 37°C for 2 hr.

To generate caspase cleavage-deficient mutants, amino acids D591 and D594 were point mutated to glutamates with the Quik-Change Site-Directed Mutagenesis kit (Stratagene, CA).

GDP Dissociation Assay

To load ras with [^{3}H]GDP (NEN), 0.5 μg GST-ras (Upstate Biotechnology) was incubated with 10 μCi [^{3}H]GDP in 50 μl nucleotide loading buffer (50 mM Tris with 10 mM EDTA, 5 mM MgCl$_2$, 1 mM DTT, and 1 mg/ml BSA) at 37°C. Twenty minutes later 60 μl of nucleotide loading stopping buffer (50 mM Tris-HCl [pH 7.4] with 5 mM MgCl$_2$, 1 mM DTT, and 1 mg/ml BSA) was added. The 110 μl sample was divided in two, and each sample was added to 500 μl dissociation reaction buffer (25 mM Tris-HCl [pH 7.4] with 2 mM MgCl$_2$, 1 mM DTT, 1 mg/ml BSA, and 0.1 mM GDP) containing 1 μg GST-GRASP-1-C and 1 μg GST, respectively. The two reactions were incubated at 37°C. A sample (100 μl) was then removed from each mixture at specific time points and mixed with 200 μl ice-cold dissociation reaction stopping buffer (50 mM Tris-HCl [pH 7.4] with 10 mM MgCl$_2$). Filtration was performed on HA filters (Millipore, 0.45 μm), and the radioactivity was measured by liquid scintillation counting.

Coimmunoprecipitation

For coimmunoprecipitation in heterologous cells, quail fibroblast QT-6 cells were transfected with no DNA, full-length GRIP1 alone, Myc-tagged GRASP-1 clone D7-4 (C-terminal 1.2 kb) subcloned in vector pRK5, or full-length GRIP1 and Myc-tagged D7-4 together. Two days later, the cells were lysed in immunoprecipitation buffer (IPB: 1× PBS with 100 μM phenylmethylsulfonylfluoride [PMSF] and 20 μg/ml aprotinin) containing 1% Triton X-100. The soluble fraction was incubated with either anti-GRIP1 antibody (JH2260, purified) or anti-Myc antibody (monoclonal) and protein A-Sepharose (1:1 slurry) for 2 hr at 4°C. The mixture was then washed once with IPB containing 1% Triton X-100, twice with IPB containing 1% Triton X-100 and 500 mM NaCl, and three times with IPB. The proteins were eluted with Laemmli sample buffer (Laemmli, 1970), and loaded onto 12% SDS-polyacrylamide gels. Western analysis was performed with either anti-Myc antibody, if anti-GRIP1 antibody was used for immunoprecipitation, or with anti-GRIP1, if anti-Myc antibody was used for immunoprecipitation.

For coimmunoprecipitation of the endogenous proteins from cultured neurons, cortical cultured neurons (embryonic day 17, 2–3 weeks in vitro) were solubilized with 2% Triton X-100 in IPB (1× PBS with 100 μM phenylmethylsulfonylfluoride [PMSF] and 20 μg/ml aprotinin). Soluble fraction after centrifugation at 14,000 × g for 10 min was used as input for immunoprecipitation. 1 μg of either anti-GRIP1 (C8399, gift of Dr. M. Sheng), anti-Myc, or anti-SAP102 was added to the input lysates together with 50 μl of protein A-Sepharose (1:1 slurry) and incubated for 3 hr at 4°C. The mixture was then washed in the same way as the coimmunoprecipitation in heterologous cells and eluted with 50 μl Laemmli sample buffer. Twenty microliters of the elute were loaded onto 7.5% SDS-polyacrylamide gels and Western analysis was performed with anti-GRASP-1 antibody (anti-GP1-Pep, 1:5000).

For coimmunoprecipitation of the endogenous proteins from rat brain, the total membrane fraction (P2) was prepared from rat brain cortex homogenate and solubilized with sodium deoxycholate (Fisher Scientific, NJ) according to Luo et al. (1997). The solubilized P2 fraction (200–500 μg) was incubated with 25 μl of protein A-Sepharose (bed volume) and monoclonal antibodies against GluR2 (Chemicon), NR1 (S3C11), or Myc (9E10) at 4°C for 2 hr. The immunoprecipitate was then washed three times with binding buffer and eluted with 50 μl of 2× Laemmli sample buffer and boiled for 5 min before loading onto SDS-polyacrylamide gels.

Neuron Cultures, Immunocytochemistry, Transfection, and Drug Treatment

Low-density hippocampal neurons were cultured following the procedure described by Goslin and Banker (1991). For immunocytochemistry, cultured neurons growing on coverslips (3 weeks in vitro) were sequentially fixed in 4% paraformaldehyde/4% sucrose for 30 min and cold methanol ($-20°C$) for 20 min and then were permeabilized in 0.2% Triton X-100 for 20 min at 4°C. After washing three times in $1\times$ PBS, the neurons were first incubated with 10% normal donkey serum and then incubated with fluorescent dye-labeled antibodies at 37°C for 2 hr. After washing in $1\times$ PBS three times, the coverslips were mounted onto glass slides with PermaFluor Aqueour (Lipshaw Immunon, Pittsburgh, PA) containing 0.25% 1,4-diazabicyclo-[2.2.2]-octane (DABCO) (Aldrich Chemical, WI).

For transfection studies, low-density hippocampal neurons were transfected at 3–5 days in vitro with pEGFP-C1, which was an empty vector encoding green fluorescent protein (Clontech Laboratories, CA), Myc-GRASP-1, or mutants subcloned in mammalian expression vector pRK5. The transfections were carried out by the calcium phosphate method (Xia et al., 1996; Threadgill et al., 1997). The neurons were then stained at about 2 weeks after the transfection with the method described above.

Cortical neurons from embryonic day 17 Sprague-Dawley rats were cultured according to the procedure of Ghosh and Greenberg (1995), except that the cells were maintained in minimum essential medium (MEM) supplemented with 5% heat-inactivated horse serum. For glutamate treatment, cortical neurons were incubated in normal media containing 40 μM glutamate for 5 min and were then immediately fixed. For blocking by glutamate receptor antagonist, 200 μM APV or 20 μM CNQX was added together with 40 μM glutamate for 5 min.

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