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ANALYSIS OF PDZ BINDING MOTIFS*

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Using sequence homology searches, yeast two-hybrid assays and glutathione *S*-transferase (GST)-pull-down approaches we have identified a series of glutamate receptor subunits that interact differentially with the PDZ proteins GRIP, PICK1, and syntenin. GST-pull-down experiments identified more interactions than detected by yeast two-hybrid assays. We report several receptor-protein interactions, strong ones include: (i) GRIP and syntenin with mGluR7a, mGluR4a, and mGluR6; (ii) PICK1 and GRIP with mGluR3; and (iii) syntenin with all forms of GluR1–4 and mGluR7b. We further characterized the novel mGluR7a-GRIP interaction found both in yeast two-hybrid and GST-pull-down assays and observed that mGluR7a localization overlapped with GRIP with in hippocampal neurons. The wide range of targets for PICK1, GRIP, and syntenin suggests they may represent a molecular mechanism that can concentrate and/or regulate a number of different receptors at a common site on a synapse. These data also suggest that the structural determinants involved in PDZ interactions are more complex than originally envisaged.

Appropriate localization, cell surface expression, and activity-dependant regulation of neurotransmitter receptors in neurons are essential for their function. These are achieved and maintained via a complex network of protein-protein interactions, partly mediated by PDZ (PSD-95/discs large/ZO-1) domain containing adaptor proteins (1). PDZ domains comprise ~90 amino acids and bind proteins containing extreme carboxyl-terminal (ct)[1]-located PDZ binding motifs with the consensus sequence E(S/T)*X*(V/I) (type I), ΦXΦ (type II), and/or Φ/ΨXΦ (type III) (2, 3), where × is any amino acid, Φ is a hydrophobic residue, Ψ is a basic residue. A fourth class (type IV) has also being defined by Stricker and co-workers and corresponds to the *X*D*X*V ct sequence (4).

Glutamate is the main excitatory neurotransmitter in the vertebrate central nervous system, and it regulates a number of cellular signaling cascades and controls the excitability of central synapses pre-and postsynaptically (5). To date, three major classes of *N*-methyl-D-aspartate receptor subunits (NR1–3), four α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPAR) subunits (GluR1–4), five kainate receptor subunits (GluR5–7, KA1–2), and eight metabotropic receptors (mGluR1–8) have been reported (5).

Some PDZ domain-containing proteins expressed in neurons have been shown to interact with glutamate receptor subtypes, where they act as adaptor/scaffolding proteins that target, anchor, and spatially organize synaptic proteins to the membrane (2). These interactions are generally mediated by the cytoplasmic ct domains of the receptors. In the present study we focused on PICK1 and GRIP, two proteins that have been shown to dynamically regulate AMPAR cell surface expression (6). We also included syntenin as an additional protein containing PDZ domains (7).

Protein interacting with protein kinase C (PICK1) contains a single PDZ domain that interacts with type I (PKCα), type II (GluR2–4, ephrin ligands and receptors, ADP-ribosylation factor, dopamine transporter and mGluR7a) as well as atypical PDZ binding motifs (TIS21) (2, 8–13). In the yeast two-hydrid assay, PICK1 has also been shown to interact weakly with the type III PDZ binding motifs of mGluR4a and mGluR8a (2). In addition, PICK1 is a substrate for PKC phosphorylation and forms dimers at a site distinct from the PDZ domain, possibly at the coiled-coil motif (10, 11). It has therefore been proposed that PICK1 might serve as an adaptor that links transmembrane receptors to cytoplasmic PKC.

Glutamate receptor-interacting protein (GRIP) contains seven PDZ domains with the potential to bind a diverse array of partners. Like PICK1, GRIP (PDZ 4–5) interacts with GluR2–4 and GRIP (PDZ 6–7) with ephrin ligands and receptors (12, 14, 15). It also binds to the type I T/S*X*V motif of the huntingtin-associated protein HAP1-A (16). Dimerization of GRIP had been shown to increase its scaffolding abilities (17).

Syntenin was originally isolated as an interactor with syndecans (7). It contains two closely located PDZ domains that share some (ephrinB1, EphA7), but not all (EphB2), interacting proteins with PICK1 and GRIP. The PDZ domains of syntenin can recognize type I (neurofascin, ProTGFα), type II (syndecans, ephrin-B2, EphA7, and r-PTPη) and nonconserved PDZ binding motifs (Schwannomin-FEEL) (20–24). Syntenin can also dimerize, through a site that is currently unknown (18).

The present study was undertaken to investigate whether GRIP, PICK1, and syntenin could bind to a variety of glutamate receptor subtypes. Given the large number of AMPA,

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[1] The abbreviations used are: ct, carboxyl (or COOH)-terminal; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; AMPAR, AMPA receptor; PKC, protein kinase C; GRIP, glutamate receptor-interacting protein; GST, glutathione *S*-transferase; Ab, antibody; mAb, monoclonal antibody; GAD, glutamic acid decarboxylase.

kainate, and *N*-methyl-D-aspartate receptor subunits and the multiple COOH-terminal splice variants, it was not feasible to investigate all glutamate receptors. Therefore, as representative examples, we studied one class of ionotropic receptors, namely the AMPAR subunits GluR1–4 and all of the metabotropic receptors mGluR1–8. Using yeast two-hybrid assays we identified a previously unreported interaction between GRIP and mGluR7a, which was then further characterized. Using GST-pull-down assays we also found more novel receptor-PDZ protein interactions, suggesting general roles for PICK1, GRIP, and syntenin in glutamate receptors regulation. Finally, taking advantage of the number of PDZ binding motifs included in our study, we analyzed the structural determinants involved in PDZ interactions.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Yeast Two-hybrid Assays—cDNA fragments corresponding to the intracellular carboxyl-terminal domain of the receptors (with the exception of mGluR1 and mGluR5, where only the last 30 amino acids were fused) were amplified using PCR and subcloned into the pBTM bait vector. The entire coding region of PICK1 and syntenin and PDZ 4–7 of GRIP were subcloned into the pGAD10 fish vector (CLONTECH) as described previously (8). The receptor COOH-terminal domain was fused to GST by subcloning into pGEX-4T-1 (Amersham Biosciences), the Myc tag was added to the NH_2 terminus (nt) of GRIP PDZ domains 4–7 (residues 430–1112) by subcloning into pCMV-myc (CLONTECH), FLAG was tagged to the NH_2 terminus of PICK1 as described previously (8), and an HA tag was added to the NH_2 terminus of syntenin by subcloning into pCMV-HA (CLONTECH). Small scale yeast transformations were carried out as indicated elsewhere (19). Fish ($pGAD_{10}$ constructs of PICK1, GRIP, and syntenin) and bait plasmids (pBTM constructs of ct receptors) were cotransformed into *Saccharomyces cerevisiae* L40 reporter strain and transformants were selected by growth on synthetic dropout Trp/Leu plates. The colonies were tested for activation of β-galactosidase reporter gene by filter assays.

GST-pull-down Experiments—For pull-down experiments, extracts of *Escherichia coli* strain BL21 were prepared using BugBuster (Novagen) in the presence of a Complete® protease inhibitor mixture (Roche Molecular Biochemicals) and used as a source of GST or GST fusion proteins. Lysates of COS-7 cells transiently transfected with cDNA using FUGENE 6 (Roche Molecular Biochemicals) were used as a source for FLAG-PICK1, Myc-GRIP(PDZ 4–7), or HA-syntenin. GST-pull-downs were performed as described previously (20). After GST-pull-down, the samples were processed for Western blotting. Each interaction was verified by at least three independent experiments. GST-ct-$GluR2_{short}$ was included as positive control and used as reference interaction (*i.e.* 100%) in the experiments. The intensity of bands on immunoblots was quantified by densitometry using the Gel-Doc system (Bio-Rad).

Western Blotting and Immunocytochemistry—Samples were separated on 10% SDS-polyacrylamide gels (SDS-PAGE), and Western blotting was performed. Primary antibodies (Abs) were as follows: anti-Myc monoclonal mouse Ab (mAb) (Oncogen), anti-FLAG M2 mAb (Sigma), and polyclonal rabbit anti-HA Ab (Santa Cruz Biotechnology). The horseradish peroxidase-conjugated secondary antibodies used were goat anti-rabbit IgG or goat anti-mouse IgG (Sigma). The signal was detected using enhanced chemiluminescence (Roche Molecular Biochemicals). COS-7 cells were grown and transfected as described elsewhere (20). Low density hippocampal cultures were prepared as described previously (20) and used after 16–18 days in culture for immunocytochemistry. GRIP was stained by anti-GRIP rabbit Ab (Upstate Biotechnology) followed by Oregon green-conjugated donkey anti-rabbit IgG (Chemicon). mGluR7a was stained by anti-mGluR7a guinea pig Ab (20) followed by Texas Red-X-conjugated goat anti-guinea pig IgG (Molecular Probes). All antibodies were used at dilutions recommended by the manufacturer. Protein concentrations were determined using the BCA protein assay kit using serum bovine albumin as a standard (Pierce).

RESULTS AND DISCUSSION

Alignment of Glutamate Receptor Subunit ct Motifs—PDZ-mediated interactions have generally been reported to occur via the recognition of a short motif that usually contains 3–7 critical residues located at the extreme ct domain (3). Therefore, we examined closely the COOH terminus of GluR1–4 (AMPA) and mGluR1–8 (metabotropic), of which some have already been shown to interact with PDZ proteins (Fig. 1). In the brain, the COOH termini of AMPARs are either long (GluR1 and GluR2/4, also called GluR2c/4c) or short (GluR2/3/4) (21). Type II PDZ binding motifs are found in the short versions of GluR2–4 (S(V/I)KI), whereas GluR1 (TGL) contains a type I binding motif. The other extreme ct motifs of GluRs are nonconserved. For mGluRs, the last three residues of group I and II subtypes contain a common type I PDZ binding motif (S(T/S)L). The sequences for group III subtypes are more divergent, presenting either type II (mGluR7a/b) or type III (mGluR4a and mGluR8a; H(A/S)I) PDZ binding motifs. A type IV (*XDX*L) is also observed at the COOH terminus COOH terminus of mGluR4b (Fig. 1). It is worth noting that mGluR7b presents an atypical proline at position −2 and that mGluR6 and mGluR8b are nonconserved. Since variations among these receptors occur at the extreme COOH terminus, within the PDZ binding motifs, we expected that proteins interacting with this region might be different. Fig. 1 shows the summary of AMPAR and mGluR carboxyl-terminal sequences (containing the ct motifs) and their previously reported PDZ domain-interacting proteins. It also gives the overview of newly identified receptor-PDZ protein interactions.

	ct-domain information			interacting proteins	
	full ct	last 20 residues of ct	motif	previous reports	this report
AMPARs					
GluR1-long	E827 - L907	MQSIPSMSHS SGMDLGATGL*	Type I	SAP97, NARP, Prosetin4.1, Stargazin	PICK1, Syntenin
GluR2-long	E833 - S901	HAVPIVSPGH GSMVSVTDLS*	NC	To date nothing found	PICK1, Syntenin, GRIP
GluR4-long	E834 - P902	PTGTAIRQSS GLAVIASDLP*	NC	To date nothing found	Syntenin, GRIP
GluR2-short	E833 - I883	QNPAIYKEGT NVIGIESVKI*	Type II	PICK1, GRIP, ABP, NSF	Syntenin
GluR3-short	E838 - I888	QNYAIYRHGY NVIGIESVKI*	Type II	PICK1, GRIP, ABP	Syntenin
GluR4-short	E834 - I884	RNLAIYRHGY NVIGIESIKI*	Type II	PICK1 GRIP, ABP	Not determined
mGluRs					
mGluR1a	A840 - P922	PNVTIYASVI. RDYKQSSSTL*	Type I	Homers, CaM, Sish	No interaction found
mGluR5	A839 - P918	SSPSKDDLII RDYIQSSSSL*	Type I	Homers, CaM, Sish	No interaction found
mGluR2	Q820 - L872	QPVPIVCNGR EVVDSITSSL*	Type I	To date nothing found	No interaction found
mGluR3	Q829 - L879	TSVPIVCNGR EVLDSTTSSL*	Type I	To date nothing found	PICK1, Syntenin, GRIP
mGluR4a	H848 - I912	LETPALAIKQ TTVTTTNNAI*	Type III	PICK1	GRIP, Syntenin, Not PICK1
mGluR4b	H848 - L883	KLALPANDTE FSANVFCDGL*	Type III	To date nothing found	Not determined
mGluR6	H840 - K871	LKATSTMAAP PQNENAEDAK*	NC	To date nothing found	GRIP, Syntenin
mGluR7a	H851 - I915	VDPNSDAAKK KIVSTNNLVI*	Type II	PICK1, CaM, G-protein	GRIP, Syntenin
mGluR7b	H851 - V922	PPVRKSVQKS VTMTTLPPVP*	Type II	PICK1, CaM, G-protein	GRIP, Syntenin
mGluR8a	H844 - I908	LETNTSSTKT TTISYADHSI*	Type III	PICK1	No interaction found
mGluR8b	H844 - S908	LETNSKSSVD FSMVDSGSTS*	NC	PICK1	Not determined

FIG. 1. **Alignment of AMPAR and mGluR COOH termini.** Proteins previously shown to interact with glutamate receptors are indicated. Novel receptor-protein interactions isolated in this study by yeast two-hybrid studies and/or GST-pull-down experiments are also listed.

Yeast Two-hybrid Assays Revealed a New Interaction between GRIP and mGluR7a—We initially isolated full-length PICK1 and GRIP comprising PDZ domains 4–7 in yeast two-hybrid screens using GluR2 or GluR3 as a bait. Full-length syntenin was found in an independent screen performed in our laboratory.[2] We then performed a matrix of experiments to test the ability of a wide range of AMPAR and mGluR ct domains to interact with the PDZ-domain containing proteins PICK1 (entire coding region), GRIP (PDZ 4–7), and syntenin (entire coding region). In the yeast two-hybrid assays syntenin did not interact with either AMPARs or mGluRs. However our results confirmed previously identified interactions (*i.e.* GluR2–3_{short} with GRIP or PICK1). In addition, GRIP (PDZ 4–7) and mGluR7a gave a strong β-galactosidase signal in the yeast two-hybrid assay (Fig. 2*A*, see *boldface* material). This mGluR7a-GRIP interaction is interesting in light of reports that have already shown an interaction between PICK1 and mGluR7a (22), and it is also analogous to the interaction of both PICK1 and GRIP with GluR2 (8, 13, 14).

Next, we demonstrated that PDZ motif-domain association mediates the mGluR7a-GRIP interaction. GluR2, mGluR7a, and mGluR7b shared the same binding site for interaction with PICK1. However, whereas ct-GluR2 interacted with GRIP PDZ 4–5 (Fig. 2*B*, see Refs. 8 and 20), ct-mGluR7a required the

[2] H. Hirbec and J. M. Henley, unpublished observations.

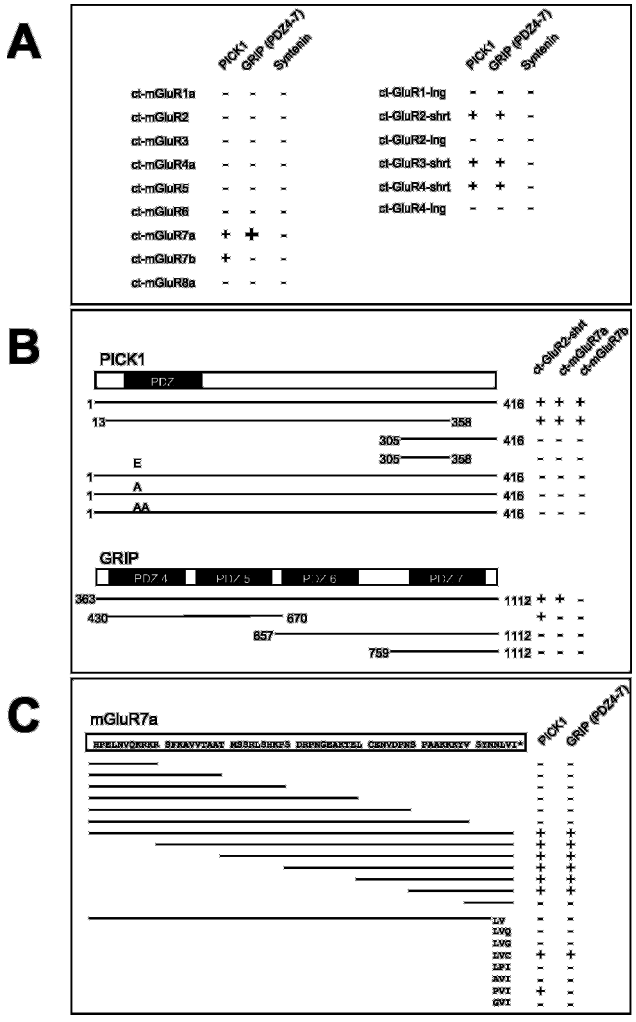

FIG. 2. **Characterization of the novel GRIP-mGluR7a interaction.** Positive interactions, as defined by filter β-galactosidase assays, are indicated as + and negative as −. *A*, a novel interaction between mGluR7a and GRIP is isolated and is indicated as + in *bold*. *B*, GluR2, mGluR7a, and mGluR7b use the PDZ domain of PICK1 for interaction. GluR2 can interact with PDZ 4–5 of GRIP, mGluR7a requires PDZ 4–7, and mGluR7b does not show any affinity for GRIP PDZ 4–7. *C*, both PICK1 and GRIP use the extreme 15 residues of ct-mGluR7a for interaction. Random mutations in the last three residues of ct-mGluR7a show the binding profiles of PICK1 and GRIP PDZ domains.

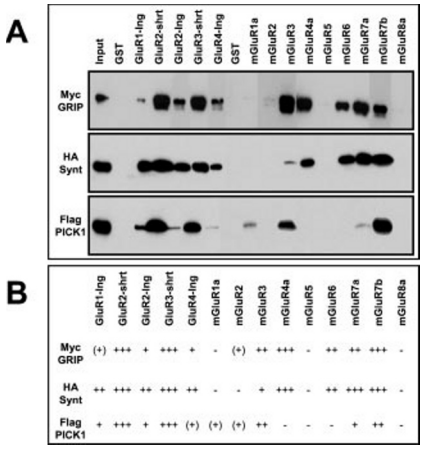

FIG. 3. **GST-pull-down experiments.** Experiments confirm the interaction between mGluR7a-GRIP. *A*, examples of Western blots using anti-FLAG, anti-Myc, and anti-HA Abs and indicating the levels of FLAG-PICK1, Myc-GRIP, and HA-syntenin retained, respectively. *B*, summary of GST-pull-down assays. The intensity of bands on immunoblots was compared by densitometry using the Gel-Doc system (Bio-Rad). Values are expressed as percent of FLAG-PICK1, Myc-GRIP (PDZ 4–7), or HA-syntenin retained by ct-GluR2$_{short}$. Ranking of the strength of the interactions found is as followed, in percent retained by ct-GluR2$_{short}$: −, no interaction; *(+)*, <10%; +, 10–25%; ++, 25–75%; +++, >75%.

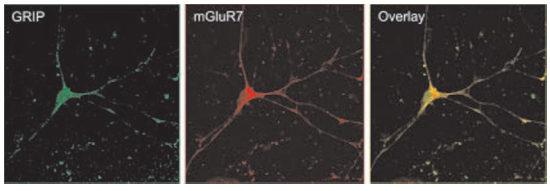

FIG. 4. **GRIP and mGluR7a distribution in rat hippocampal neurons.** Hippocampal neurons were immunostained for GRIP and mGluR7a proteins. GRIP was stained by anti-GRIP rabbit Ab followed by Oregon green-conjugated rabbit IgG, *green channel*. mGluR7a was stained by anti-mGluR7a guinea pig Ab followed by Texas Red-X-conjugated anti-guinea pig IgG, *red channel*. Overlay shows considerable overlap in GRIP and mGluR7a staining along neuron process and on cell bodies, *yellow channel*.

larger fragment GRIP PDZ 4–7. We found that ct-mGluR7b, which has a proline at position −2, did not bind GRIP (Fig. 2*B*). Generally one PDZ motif binds to one PDZ domain. However, GRIP requires multiple PDZ domains to work in combination, for example GluR2-GRIP (PDZ 4–5) (14) and Eph-GRIP (PDZ 6–7) (12). This suggests that the single PDZ domain in PICK1 is likely to have a broader binding capacity than single PDZ domain of GRIP and that PDZ domains in GRIP may act in concert possibly via conformation changes in the protein.

Similar to PICK1, the last 15 ct-located residues of mGluR7a were sufficient for the interaction with GRIP (Fig. 2*C*, see Ref. 20). Point mutations within the PDZ motif of ct-mGluR7a all resulted in a loss of interaction with GRIP and PICK1 (Fig. 2*C*). Exceptions occurred for NLVC, which had no effect on the interaction with GRIP or PICK, and for NPVI that (similar to mGluR7b, PPTV) showed interaction with PICK1 but not GRIP (Fig. 2*C*). A mutant based on ct-GluR2 (SVKI, wild type), namely EVKI, has been shown to interact with PICK1 but not GRIP (23, 24). Taken together, these results suggest that PDZ domains are able to bind a larger number of motifs than currently predicted by the classification of four types of PDZ ligands (types I, II, III, and IV). We suggest that there are more PDZ binding motif types, which, together with flexibility in PDZ domains, is likely to result in a number of PDZ motif-domain interactions that is beyond complete predictability.

GST-pull-down Experiments Confirmed Yeast Two-hybrid Results and Isolated Additional Novel Interactions—To biochemically confirm the mGluR7a-GRIP interaction found using yeast two-hybrid assays, direct interaction between FLAG-PICK1, Myc-GRIP, or HA-syntenin and the COOH termini of different glutamate receptors was assayed in GST-pull-down experiments. In agreement with the results of the yeast two-hybrid assay, GRIP was retained by GST-ct-mGluR7a (Fig. 3). Our densitometry results indicated that this interaction was comparable with that with GluR2/3$_{short}$. In our GST-pull-down assays we, as others previously, observed an interaction between PICK1 and mGluR7a. Although of weaker band intensity than that of GluR2$_{short}$, this PICK1-mGluR7a interaction is physiologically significant, being involved in the presynaptic clustering of mGluR7a (22).

Surprisingly, we identified more glutamate receptor-PDZ protein interactions in GST-pull-down experiments than in the yeast two-hybrid assays. In particular, we observed a robust interaction between syntenin and the COOH termini of GluR2/3$_{short}$, mGluR4a, and mGluR7a/b. We found that GRIP bound to mGluR4a with intensity similar to its interaction with GluR2/3$_{short}$. However, neither GRIP nor syntenin bound the

similar type III PDZ binding motif of mGluR8a. An interaction, ranging between 25 and 75% of intensity to $GluR2_{short}$, was revealed between syntenin and the long isoforms of AMPAR COOH terminus ($GluR1_{long}$, $GluR2_{long}$, $GluR4_{long}$) or mGluR6. We also found binding between GRIP and mGluR7b or mGluR6. Our GST-pull-down assays displayed virtually no interactions with group I mGluRs (QSSSSL). In contrast, at the very least, interactions of 10–25% intensity to $GluR2_{short}$ were detected with mGluR3 (STTSSL). Despite the high sequence homology between the COOH terminus of mGluR2 and mGluR3, the proteins, PICK, GRIP, and syntenin displayed virtually no interaction with mGluR2. Taken together, these data confirm the importance of the extreme COOH terminus but also show the critical structural requirements of amino acids located upstream of the PDZ binding motif.

We also observed weak intensity bands that indicated interactions not previously reported. We found interactions between ct-$GluR1–4_{long}$ and PICK1 or GRIP. Similar weak bands were also seen for mGluR7a-PICK1, an interaction that has been shown to be important for the receptor clustering (22). Whether, for example, a GluR1-PICK1 interaction has any functional roles is unclear. It has been shown that overexpression of PICK1 selectively alters the surface expression of GluR2 but not that of GluR1 (26). One possibility is that these interactions only become relevant in the absence of other competitive interacting proteins and are only important at certain developmental stages, cell states, or cell types. Assuming these interactions are functionally relevant, it would appear that PICK1, GRIP, and syntenin bind to a wider range of glutamate receptors than previously proposed. Previously, PICK1 has been implicated in the control of PKC-evoked receptor phosphorylation and receptor membrane insertion and internalization (2, 6, 20). Syntenin may play a role in the targeting, trafficking, or recycling of its interacting receptors (27). GRIP anchors receptors at the synapse in a phosphorylation-dependent manner (6, 23). Whether PICK1, GRIP, and syntenin each have different roles depending on their glutamate receptor partners remains to be resolved (6, 28).

GRIP Overlaps with mGluR7a at Excitatory Synapses—We focused on the further characterization of mGluR7a-GRIP interaction, because it was robust in yeast two-hybrid and GST-pull-down studies. We examined by immunofluorescence the cellular localization of both proteins in cultured hippocampal neurons. As shown in Fig. 4, there was considerable overlap of GRIP and mGluR7clusters on the dendritic shafts and cell bodies of hippocampal pyramidal neurons. Previously, GRIP has been found co-localized at excitatory glutamatergic synapses with GluR2 and at inhibitory GABAergic terminals with glutamic acid decarboxylase (GAD) (14, 17, 29). However, because mGluR7a (and PICK1) does not overlap with GAD (22), we conclude that the staining found here is localized at excitatory glutamatergic and not inhibitory GABAergic neurons (Refs. 2, 13, and 20, but also see Ref. 21). Taken together, these results suggest that mGluR7a and GRIP (in addition to PICK1) are spatially co-localized such that they could form complexes in hippocampal neurons. The removal of the PDZ motif on mGluR7a alters its surface expression, an event that has been associated with mGluR7a-PICK1 interaction (22). Whether PICK1 and GRIP work in combination to dynamically regulate mGluR7a cycling, as they do for GluR2, remains to be established. The rapid turnover of not only ionotropic but also metabotropic glutamate receptor numbers by PDZ proteins could play an important molecular mechanism for regulating synaptic strength and neuronal function.

In this present study we have used yeast two-hybrid assays to isolate a novel PDZ-mediated interaction between mGluR7a and GRIP. Direct interaction was confirmed by GST-pull-down assays that additionally reveal a number of new interactions. Furthermore, we suggested that the mGluR7a-GRIP interaction is likely to occur *in vivo*, because both proteins appeared highly co-localized in hippocampal neurons. This is relevant to the recent study, which shows that removal of the PDZ binding motif from mGluR7a prevents its interaction with PICK1 at its clustering at synapses (22). Our new results suggest that GRIP, in addition to PICK1, may also play a role in these effects (22). As a whole, our findings suggest that the current classification for PDZ binding motifs and PDZ domains is incomplete and that flanking amino acids residues and structural considerations can play a crucial role in determining the specificity of PDZ motif-domain interactions (4, 31).

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