The PDZ Proteins PICK1, GRIP, and Syntenin Bind Multiple Glutamate Receptor Subtypes

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 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-dependent 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/disks 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)(V/I) (type I), ΦXΦ (type II), and/or Ψ(YΦXΦ) (type III) (2, 3), where X is any amino acid, Ψ is a hydrophobic residue, Ψ is a basic residue. A fourth class (type IV) has also been defined by Stricker and co-workers and corresponds to the XDXV 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 (PKCo), type II (GluR2–4, ephrin ligands and receptors, ADP-riboylation factor, dopamine transporter and mGluR7a) as well as atypical PDZ binding motifs (TIS21) (2, 8–13). In the yeast two-hybrid 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/SXV 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-PTPα) and nonconserved PDZ binding motifs (Schwannomin-FEE1) (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, 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 mGluR7a–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—DNA 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 pBTH 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 Biochemicals), the Myc tag was added to the NH2 terminus of PICK1 as described previously (8), and an HA tag was added to the NH2 terminus of syntenin by subcloning into pCMV-myc (CLONTECH). FLAG was tagged to the NH2 terminus of PICK1 (as described previously), and an HA tag was added to the NH2 terminus of syntenin by subcloning into pCMV-HA (CLONTECH). Small scale yeast transformations were carried out as indicated elsewhere (19). Fish (pGADm, constructs of PICK1, GRIP, and syntenin) and bait plasmids (pBTH 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 washing of the GST beads, the samples were processed for Western blotting. Each interaction was verified by at least two independent experiments. GST-cT-GluR2short was included as a 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 Orygen 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/4e) or short (GluR2/3/4) (21). Type II PDZ binding motifs are found in the short versions of GluR2 4–7 (S/V/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/L) PDZ binding motifs. A type IV (XDXL) is also described 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 carboxy-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.

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. GluR2–3short 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. 2A, 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-dominant 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. 2B, see Refs. 8 and 20), ct-mGluR7a required the

![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 are listed.](http://www.jbc.org/)

mGluR7a and GRIP is isolated and is indicated as /H11001 and mGluR7b does not show any affinity for GRIP PDZ 4–5 of GRIP. mGluR7a requires PDZ 4–7, and mGluR7b does not show any affinity for GRIP PDZ 4–7. We found that ct-mGluR7b, which has a proline at position −2, did not bind GRIP (Fig. 2B).

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. 2C, see Ref. 20). Point mutations within the PDZ motif of ct-mGluR7a all resulted in a loss of interaction with GRIP and PICK1 (Fig. 2C). Exceptions occurred for NLVC, which had no effect on the interaction with GRIP or PICK1 and for NPVI that (similar to mGluR7b, PPTV) showed interaction with PICK1 but not GRIP (Fig. 2C). 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.

**GSP-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 ct-GluR2short. Ranking of the strength of the interactions found is as followed, in percent retained by ct-GluR2short: −, no interaction; (+), <10%; +, 10–25%; ++, 25–75%; ++++, >75%.
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 (GluR1long, GluR2long, GluR4long) or mGluR6. We also found binding between GRIP and mGluR7b or mGluR6. Our GST-pull-down assays displayed virtually no interactions with group I mGLRs (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-GluR1–4long 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 interactions 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 partner 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 play a crucial role in determining the specificity of PDZ motif-domain interactions (4, 31).

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