The Carboxyl Terminus of the Prolactin-Releasing Peptide Receptor Interacts with PDZ Domain Proteins Involved in α-Amino-3-hydroxy-5-methylisoxazole-4-propionic Acid Receptor Clustering

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ABSTRACT

PDZ domain proteins use the PDZ domain binding motif to bind to the C-terminal sequence of membrane proteins to help scaffold them and spatially organize the components of the intracellular signaling machinery. We have identified a sequence at the C terminus of a G protein-coupled receptor, the PrRP receptor, that shares similarities with the C-terminal sequence of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptor (AMPA-R) subunits that interact with PDZ domain proteins. When coexpressed in human embryonic kidney 293 cells, PrRP receptor was able to coimmunoprecipitate the three PDZ domain proteins known to interact with AMPA receptors: glutamate receptor interacting protein (GRIP), AMPA binding protein (ABP), and protein that interacts with C-kinase (PICK1), but not the PDZ domain protein PSD-95, which does not interact with AMPA receptors. These interactions are sequence-selective as determined by mutagenesis. Furthermore, we show that PrRP receptor forms intracellular clusters when coexpressed with PICK1, and that this clustering effect is dependent on the interaction between the PICK1 PDZ domain and the last four amino acids of PrRP receptor. We found that PrRP receptor interaction with GRIP is not protein kinase C-regulated but may be regulated by other unidentified kinase because okadaic acid dramatically reduced GRIP interaction. By in situ hybridization, we show that the PrRP receptor is expressed in neurons that also express these PDZ domain proteins. We thus demonstrate that PrRP receptor interacts with the same PDZ domain proteins as the AMPA-Rs, raising the possibility that these two proteins could be scaffolded together at the synapse. These results may help to gain important insights into PrRP functions within the central nervous system.

G protein-coupled receptors (GPCRs) belong to a superfamily of genes that encode proteins that interact with a variety of small extracellular ligands such as peptides, lipids, amino acids, and their derivatives (Strader et al., 1995). Although these receptors recognize a diverse range of ligands, the different classes of GPCRs share many of the same downstream signaling pathways. What accounts for the signaling specificity of a particular receptor? Recent evidence suggest that besides G protein coupling, GPCRs mediate signaling and activation of diverse downstream pathways through G protein-independent means (Heuss and Gerber, 2000). GPCRs contain intracellular loops and a C-terminal tail that, upon ligand binding, are involved in receptor activation, desensitization, and activation of downstream signaling events (Casey and Gilman, 1988; Wall et al., 1995; Lefkowitz, 1998). The C-terminal tails of GPCRs have recently been shown to also be involved in protein-protein interactions with proteins containing the PSD-95/Discs-large/ZO-1 (PDZ) domains (Bockaert and Pin, 1999). PDZ domain proteins play a role in the scaffolding and targeting of proteins to specific subcellular domains and help organize signal transduction machinery in

ABBREVIATIONS: GPCR, G protein-coupled receptor; PDZ, PSD-95, Discs-large, ZO-1; PrRP, prolactin-releasing peptide; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; PCR, polymerase chain reaction; PrRP-R, prolactin-releasing peptide receptor; HEK, human embryonic kidney; GRIP, glutamate receptor interacting protein; ABP, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid binding protein; PBS, phosphate-buffered saline; PMA, phorbol-12-myristate-13-acetate; PICK1, protein that interacts with C-kinase 1; PKC, protein kinase C; DMSO, dimethyl sulfoxide; NHE, Na$^+$/H$^+$ exchange.

the vicinity of a receptor (Craven and Bredt, 1998). Such protein-protein interactions linking GPCRs to a particular pathway through PDZ domain interactions may direct signaling specificity.

Of the hundreds of cloned GPCRs, there is a large group of so-called orphan GPCRs whose functions have remained a mystery because of the lack of our knowledge of their natural ligands (Wilson et al., 1998). Recent efforts using the orphan receptor strategy (Civelli, 1998) have led to identification of the natural ligands of an increasing number of orphan GPCRs and elucidated the functions of few of these orphan receptors. GPR10 was an orphan GPCR (Marchese et al., 1995; Welch et al., 1995) until a 31-amino-acid peptide (prolactin-releasing peptide; PrRP) was identified to be its endogenous ligand (Hinuma et al., 1998). PrRP was found to cause prolactin release from pituitary cells by binding to receptors expressed in the anterior pituitary, therefore earning its name. However, the receptor is also highly expressed in a few discrete locations in the brain (Roland et al., 1999; Ibata et al., 2000). Some recent studies have demonstrated PrRP effects on the secretion of various hypothalamic hormones such as corticotropin-releasing hormone (Matsumoto et al., 2000), luteinizing hormone/follicle-stimulating hormone (Seal et al., 2000), as well as on food intake (Lawrence et al., 2000). In view of its exquisite receptor localization, however, PrRP must have alternative central roles that remain undefined.

In this article, we identify in the C terminus of PrRP receptor a sequence that closely resembles a PDZ domain binding motif found in GluR2 and GluR3 of AMPA receptors (Kornau et al., 1997). We provide evidence suggesting that the PrRP receptor interacts with this subset of PDZ domain proteins and that these interactions alter receptor distribution within the cell. Our results therefore raise the possibility that the PrRP receptors may regulate AMPA receptor dynamics via PDZ domain proteins, which may constitute a novel role of the PrRP receptor in regulating central nervous system function.

Materials and Methods

Receptor Tagging and Mutagenesis. Flag-tag and C-terminal mutations were introduced by PCR. PrRP receptor (PrRP-R) cDNA in pcDNA (Brian O'Dowd, University of Toronto, Toronto, ON, Canada) was used as the template. Sequence was confirmed by dideoxy cycle sequencing containing deaza-dGTP on an ALF-Express automated sequencer (Amersham Pharmacia Biotech, Arlington Heights, IL).

Transient Transfection/Coimmunoprecipitation. HEK 293T cells (3.2×10^6) were seeded on 10-cm plates 1 day before transfection. Calcium phosphate transfections were done using 20 μg of DNA. Transfection efficiencies were determined to be ~50%. GRIP cDNA in pRK/CMV (gift of Richard Huganir, Johns Hopkins University, Baltimore, MD), PSD-95 myc (gift of Morgan Sheng, Harvard University, Boston, MA), and ABP-c-myc (gift of Edward Ziff, New York University, New York, NY) were used in different ratios to mutant and wild-type tagged PrRP receptor. Forty-eight hours after transfection, cells were washed once in PBS and lysed with IP buffer [25 mM Tris, pH 7.4, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, and protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN)]. Immunoprecipitation was performed using 2 μg of anti-Flag M2 antibody (Sigma, St. Louis, MO) or 1 μg of anti-myc antibody (Roche Molecular Biochemicals) followed by 25 μl of protein G-agarose (Sigma, St. Louis, MO). Immunoprecipitation

was carried out overnight at 4°C. Immunoprecipitated proteins were resolved on SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane, and Western blot was performed to detect GRIP using anti-GRIP antibody (1:1000). ABP-myc, PSD-95-myc, and PICK1-myc were detected using the monoclonal anti-myc antibody (1:500; CLONTECH, Palo Alto, CA).

Immunocytochemistry. COS-7 cells were seeded at 1.8×10^6 on 10-cm dishes 1 day before transfection. Cells were transfected with LipofectAMINE (Invitrogen, Carlsbad, CA) using 6 μg of DNA. Transfection efficiency was determined to be around 15%. Twenty-four hours after transfection, cells were trypsinized and seeded onto poly-D-lysine coated glass coverslips. All immunocytochemical analysis was done 48 h after transfection. Anti-PICK1 antibody (Jeff Staudinger, Department of Pharmacology, Toxicology, and Therapeutics, University of Kansas Medical Center, Kansas City, KS) (1:500) were incubated overnight at 4°C, whereas anti-myc monoclonal antibody (1:500; CLONTECH) were incubated for 2 h at room temperature. All chromophore conjugated secondary antibodies were incubated for 1 h at room temperature. Cells on coverslips were removed from incubator, washed once with PBS, and fixed with 4% paraformaldehyde for 30 min at room temperature. After three washes with PBS, permeabilization was done with 0.25% Triton X-100 for 5 min at room temperature, and blocked with 5% bovine serum albumin for 1 h at room temperature. All washing steps between antibody incubation were done three times with PBS. Coverslips were mounted on Vectashield mounting media (Vector Laboratories, Burlingame, CA).

Confocal Imaging. Fluorescently labeled samples were examined with the use of an inverted laser scanning microscope (LSM 410; Carl Zeiss, Oberkochen, Germany). The objective used was oil-immersed, 100× magnification, Plan-Neofluar, Phase 3, numerical aperture 1.3 (Carl Zeiss, Oberkochen, Germany). Thin optical sections were obtained for each sample. The 488 nm and 543 nm lines of the argon laser was used for detection of fluorescein isothiocyanate and Texas Red fluorophores, respectively. Blue channel was used for a nonconfocal phase-contrast image acquisition. Fluorescent images were pseudocolored red and green and overlaid using Adobe Photoshop 5.5 (Adobe Systems, San Jose, CA).

Testing the Effect of PMA and Okadaic Acid on PrRP-R and GRIP Interaction. Forty-eight hours after transfection, cells in 10-cm plates were washed once with treatment buffer (Dulbecco's DMEM + 20 mM HEPES, pH 7.2) and drugs (1 μM PMA or 1 μM okadaic acid) or an equivalent amount of DMSO resuspended in treatment buffer was added to the cell monolayer and placed back into the 37°C incubator for 30 min. After incubation, the plates were placed on ice water, the buffer was aspirated, and the cells were scraped off with cell scraper into ice-cold PBS. Cells were centrifuged 3 min at 800g and IP buffer was added to the pellet to carry out the immunoprecipitation procedure (see above).

In Situ Hybridization. Adult (250–300 g) Sprague-Dawley rat brains were quickly removed and placed in methylbutane cooled to −20°C for 1 min. Frozen sections (20 μm) were thaw-mounted onto poly-L-lysine–coated glass slides, fixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.4, desiccated, and stored at −20°C until prehybridization. Pretreated sections were incubated overnight at 60°C in hybridization buffer (50% formamide, 10% dextran sulfate, 500 ug/ml tRNA, 10 mM dithiothreitol, 0.3 M NaCl, 10 mM Tris, pH 8.0, and 1 mM EDTA, pH 8.0) with ^{35}S-labeled PrRP sense and antisense cRNA probes (10^7 cpm/ml). GRIP1 and ABP probes were prepared by PCR-amplifying a region of 350 base pairs in which the two genes have the least homology. PICK1 probe was prepared by PCR amplifying the region of 1 to 330 bases of the coding region. Sections were washed, dehydrated in graded ethanol, and exposed to HyperFilm βmax (Amersham Pharmacia Biotech) with ^{14}C standards of known radioactivity.

Results

PrRP Receptor Interacts with PDZ Domain Proteins GRIP, ABP (GRIP2), and PICK1 through Its C-Terminal Tail. The PrRP receptor has a C-terminal tail that terminates in a sequence of four amino acids (-SVVI) that resembles those found in the GluR2 and GluR3 subunits of the AMPA receptor (-SVKI) (Kornau et al., 1997) (Fig. 1A). These last four amino acids in AMPA receptors are required to interact with several PDZ domain proteins found to be important for targeting receptors to the synapse and controlling receptor biogenesis (Chung et al., 2000; Garner et al., 2000). To determine whether PrRP receptor may also interact with these proteins in intact cells, increasing amounts of DNA encoding Flag-tagged PrRP receptor were cotransfected with a fixed amount of GRIP [the first of the AMPA interacting proteins cloned (Dong et al., 1997)] in HEK 293T cells. Immunoprecipitation of the PrRP receptor with anti-Flag anti-

A

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PrRP-R tail  - FREELRKLLVAWPRKIAPHGQNMTVSVVI.
GluR2 tail   - NPSSSQNSQNFAATYKEGYNYYGIESVKI.
GluR3 tail   - FKPAPATNTQNYATYREGYNVYGTESVKI.
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B

C

D

Fig. 1. PrRP-R interacts with GRIP-like proteins through its cytoplasmic tail. A, alignment of amino acid sequence of the cytoplasmic tail of PrRP-R and AMPA receptor subunits GluR2 and -3. B, transient cotransfection and coimmunoprecipitation of PrRP-R and GRIP in HEK 293T. Top, immunoprecipitates using anti-Flag antibody. Bottom, crude lysate controlling for GRIP expression. Note: A low level of GRIP-like protein was detectable in untransfected HEK 293T cells. C, GRIP, myc-tagged ABP and myc-tagged PSD95 were expressed in 293T cell lines stably expressing wild-type or del6 forms of Flag-tagged PrRP-R (F-PrRP-R), respectively. Bottom, crude lysates controlling for expression of the corresponding proteins. D, same amount of DNA for myc-tagged PICK1 was cotransfected with varying amounts of wild type and mutant F-PrRP-R. Upper blot corresponds to immunoprecipitated protein and bottom blot shows the crude myc-PICK1 expression in the cell. Both blots were probed with the anti-myc antibody.

body caused a concomitant coprecipitation of GRIP (Fig. 1B). In addition to GRIP, several other proteins, ABP (Srivastava et al., 1998) (also known as GRIP2) and PICK1, a protein originally isolated as a PKC-α interacting protein (Staudinger et al., 1997), are known to interact with the C terminus of GluR2 and GluR3 subunits of AMPA receptors. We therefore repeated the experiment, coexpressing these proteins with Flag-tagged PrRP receptor. As expected, PrRP receptors were successfully coimmunoprecipitated with these proteins (Fig. 1C). The specificity of these interactions was tested using a receptor engineered to lack the last six amino acids. This mutant receptor was unable to immunoprecipitate with GRIP, ABP, or PICK1, showing that the last part of the C-terminal tail of PrRP receptor is required to bind to these PDZ domain proteins (Fig. 1, C and D). These interactions were found specific to Class II PDZ binding ligands (those with the consensus -ϕXϕ, ϕ = hydrophobic residue, X = any amino acid) and not to class I PDZ binding ligands (those with the consensus S/T X V/L) (Songyang et al., 1997; reviewed in Sheng and Sala, 2001), because the PrRP receptor was unable to interact with PSD-95 (Fig. 1C), a PDZ domain protein that interacts with the class I C-terminal motif of N-methyl-D-aspartate receptors and a subset of K^+ channels (Gomperts, 1996).

Residues and Domains Involved in the Association between PrRP Receptor and GRIP. To determine which key amino acids within the C terminus of PrRP receptor are critical for PDZ domain interaction, we carried out mutagenesis of the full-length receptor at its C-terminal tail (Fig. 2A, left). Deletion and substitution of the last six amino acids of the PrRP receptor completely eliminated its binding to GRIP (Fig. 2A, right). Alanine-scan mutagenesis of the last six residues revealed that only three of the last four residues are necessary for binding to GRIP (Fig. 2A), consistent with the proposed consensus sequence (-SVXI) for GRIP binding (Dong et al., 1997).

GRIP contains seven PDZ domains, of which domains 4 and 5 are important for interaction with AMPA GluR2/3 subunits (Dong et al., 1997). These two domains of GRIP contain a methionine at the B1 position (first residue of helix B of the PDZ motif) that interacts with a hydrophobic residue at position −2 of the class II binding peptide (Srivastava et al., 1998). The other domains of GRIP interact with other neuronal proteins. The class B ephrins, ligands for the Eph receptors that control various developmental processes in the nervous system, interact with PDZ domain 6 of GRIP (Lin et al., 1999), whereas the recently identified protein GRASP-1, interacts with PDZ domain 7 of GRIP (Ye et al., 2000). We wanted to determine which GRIP domains in GRIP interact with the PrRP receptor. PDZ domains 1 to 3, 4 to 6, and 7 were N-terminally tagged with a hexahistidine epitope and coexpressed with Flag-tagged PrRP receptor and immunoprecipitation were carried out using the anti-Flag antibody. Figure 2B shows that domains 4 to 6 of GRIP were specifically coprecipitated with PrRP receptor but not domains 1 to 3 or 7. To determine whether domain 6 of GRIP is involved in binding to PrRP-R, we made an additional construct with domains 4 and 5. We found that removing domain six did not alter the PrRP receptor's ability to bind to these domains (Fig. 2C), indicating that domain 6 is not involved in binding to PrRP receptor. These results suggest that the same GRIP
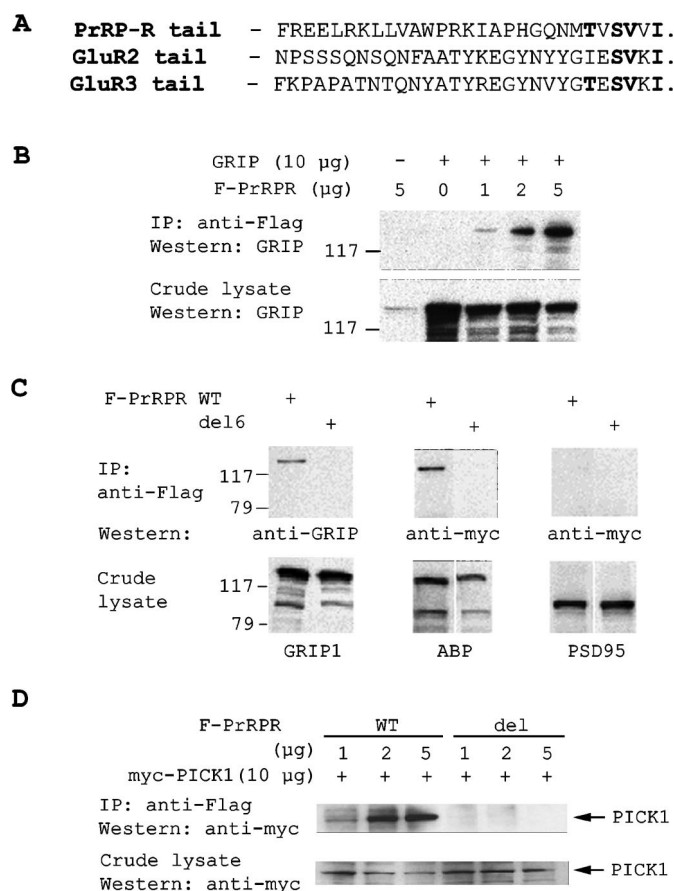
PDZ domains may be involved in binding PrRP-R as they are with GluR2 and GluR3.

Because PrRP receptor interacts with the same domains as AMPA receptors, it seems that the interactions between the GPCR and the channel protein to GRIP-like PDZ domain proteins are mutually exclusive. We tested this possibility by cotransfecting GRIP, Flag-PrRP receptor, and either GluR1 (as a control because this GluR lacks the appropriate PDZ binding motif to interact with GRIP) or GluR2 to test whether the GluR2 interaction with GRIP could compete off Flag-PrRP receptor interaction. Compared with GluR1, GluR2 was able to compete off PrRP receptor binding to

Fig. 2. PrRP-R interaction with GRIP relies on the –SVXI motif and PDZ domains 4–5. A, left, the mutant receptors altered in the last few amino acids. These numbers correspond to the lanes in the corresponding blot on the right. Top, western blot of immunoprecipitates showing how the mutations affect GRIP interaction. Middle, crude lysate controlling for GRIP expression. Bottom, blot from top was stripped and reprobed with anti-Flag antibody to determine Flag-tagged PrRP-R expression. This experiment is representative of five experiments. B, fragments of GRIP PDZ domains were fused in frame with pcDNA3.1 HIS vector as demonstrated in the schematic and coexpressed with wild-type and del6 mutant of Flag PrRP-R. Left half of the blot was loaded with immunoprecipitates and the right half was used to load crude lysate to control for fusion protein expression. C, hexa-histidine tagged fragment of GRIP PDZ 4–5 was ligated into pcDNA3.1 HIS and coexpressed and detected in the same manner as in B. "c" represents control vector. D, competition experiment was performed by triply transfecting GRIP, F-PrRP receptor, and GluR1 or -R2. Two micrograms each of GRIP and F-PrRP receptor were previously determined to be the concentration needed to generate ~50% of the signal seen on a Western blot compared with saturating levels (5–10 μg of GRIP, 2 μg of F-PrRPR). This ratio was fixed while increasing amounts of DNA for GluR1 and R2 were included in the cotransfection. Only with increasing amounts of GluR2 was the coimmunoprecipitation of GRIP with Flag-PrRP receptor concomitantly diminished. This experiment is representative of two separate experiments, with identical results.

GRIP (Fig. 2D), indicating that the PrRP receptor interacts with the same PDZ domains in GRIP as GluR2.

PrRP Receptor Is Coclustered with PICK1 in Cells. Of the three PDZ domain proteins found to interact with GluR2 and GluR3, the only one known to form intracellular and surface clusters with GluR2 and 3 subunits is PICK1. This has been shown by the fact that GluR2 or R3 subunits cocluster with PICK1, whereas cells expressing either proteins alone do not form intracellular aggregates (Dev et al., 1999; Xia et al., 1999). We therefore tested to see whether the PrRP receptor would also form intracellular aggregates with PICK1 using immunocytochemistry and laser scanning confocal microscopy. When wild-type Flag-PrRP receptor was expressed alone, the receptor seems to be distributed along filamentous structures intracellularly and perinuclearly (Fig. 3A) in permeabilized cells. The deletion mutant of Flag-PrRP receptor also exhibited the same pattern as its wild-type counterpart when expressed alone (Fig. 3B). PICK1, on the other hand, has a homogenous perinuclear distribution within the cytoplasm (Fig. 3C), an expression pattern that did not differ from a mutant form of PICK1, which has mutations in the PDZ domain (K27A/D28A; Staudinger et al., 1997) (Fig. 3D). However, when the wild-type forms of both proteins are coexpressed in the same cell, aggregates of both proteins are formed intracellularly, possibly as Golgi or ER vesicular pools (Fig. 3E). These results indicate that the PrRP receptor exhibits the same ability as AMPA receptors in clustering or to be clustered by PICK1.

PrRP Receptor/PICK1 Aggregates Are Dependent on PDZ Domain Interaction. To test whether the observed clustering of PrRP-R with PICK1 is dependent on PDZ domain interactions, we repeated the experiments using mutant PrRP-R lacking the last six amino acids. Compared with wild-type receptors (Fig. 4A), C-terminally deleted receptors were incapable of forming aggregates with PICK1 as shown by the fact that both proteins exhibit patterns identical with those obtained when expressed alone (Fig. 4B). Furthermore, when we used a mutant of PICK1 known to be incapable to interact with the C-terminal tail of GluR2 (Xia et al., 1999) to cotransfect with wild-type PrRP receptor, no intracellular clustering was observed (Fig. 4C). These results demonstrate that the C terminus of PrRP receptor as well as the PDZ domain of PICK1 is necessary to generate the clustering event.

Okadaic Acid Attenuates GRIP Binding to PrRP-R. Because several studies have shown that PKC phosphorylation of the –3 serine residue (serine 880) in GluR2 attenuates GRIP interaction and modulates AMPA receptor function in neurons (Matsuda et al., 1999; Chung et al., 2000; Matsuda et al., 2000), we were interested to see if phosphorylation of PrRP-R would also regulate GRIP association. Treatment of cells coexpressing PrRP-R and GRIP with a PKC activator PMA at 1 μM failed to alter GRIP coimmunoprecipitation with PrRP-R (Fig. 5), although this lot as well as the dose of PMA used were found to significantly activate PKCα translocation from the cytosol to the membrane fraction, as well as increasing phosphorylation of GluR2 at serine 880 (data not shown). However, treatment of cells with okadaic acid (1 μM), a potent inhibitor of PP1 and PP2A protein phosphatases, dramatically reduced the coimmunoprecipitation of GRIP with PrRP-R compared with DMSO treatment control (Fig. 5). These results suggest that PrRP-R interaction with
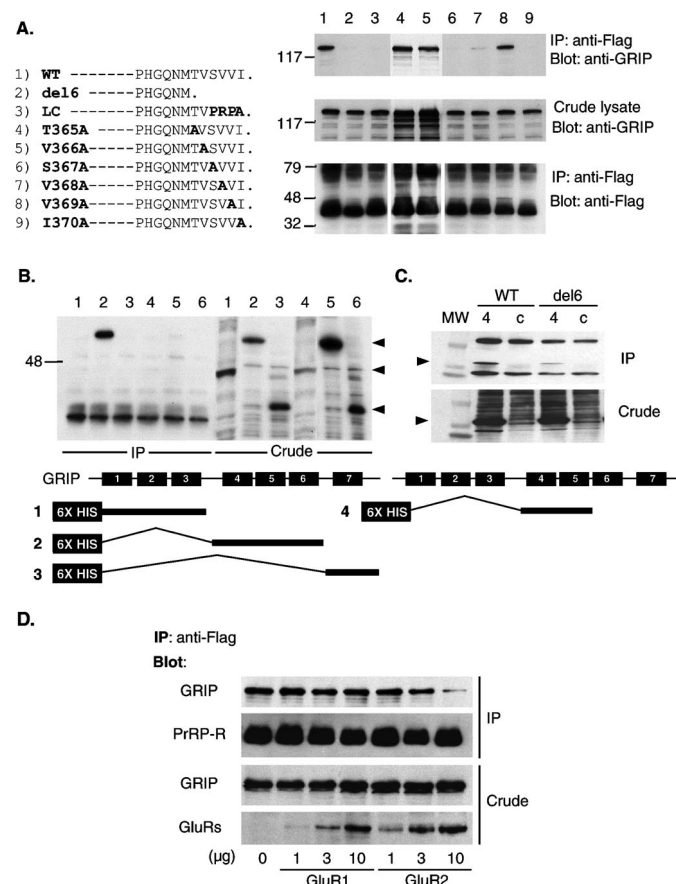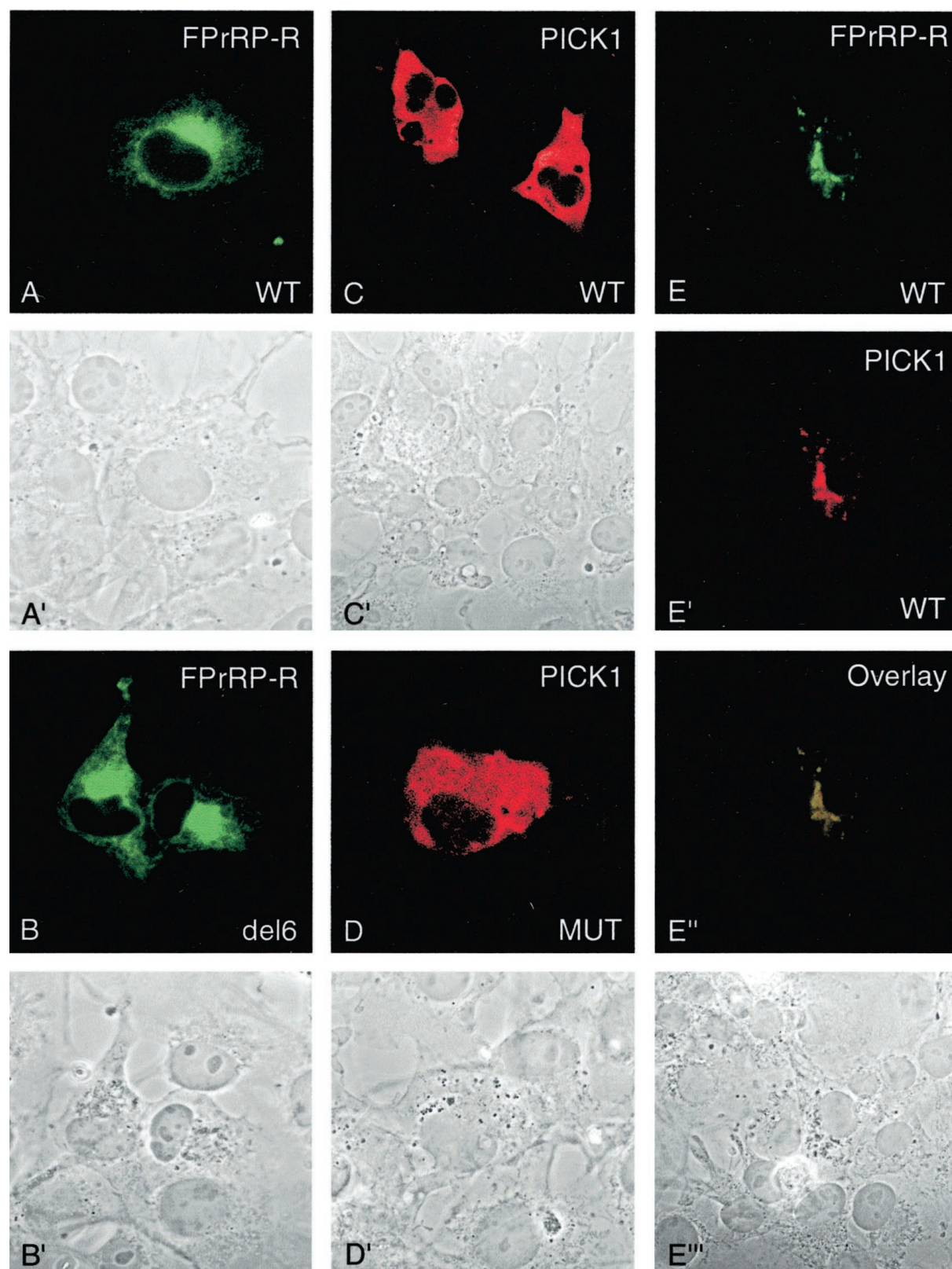
Fig. 3. PrRP receptor interacts with and is clustered by PICK1. A, the staining pattern of COS-7 cells expressing wild-type flag-tagged PrRP receptor (FPrRP-R) alone. A′, along with all other X′ plates, shows phase contrast images of the corresponding plate to show the morphology of the cells between expressing and the surrounding nonexpressing cells. No difference was observed between these cell groups. B, the staining pattern of mutant (del6) flag-tagged PrRP receptor. Note: no difference in staining patterns was observed compared with wild-type receptors. C, the staining pattern of COS-7 cells expressing wild-type PICK1 alone. D, COS-7 expressing mutant PICK1 alone. Again, no difference was noted between wild-type and mutant proteins. E, a cell coexpressing both proteins. Note the extensive overlap in the clustering seen in permeabilized cells (E″).
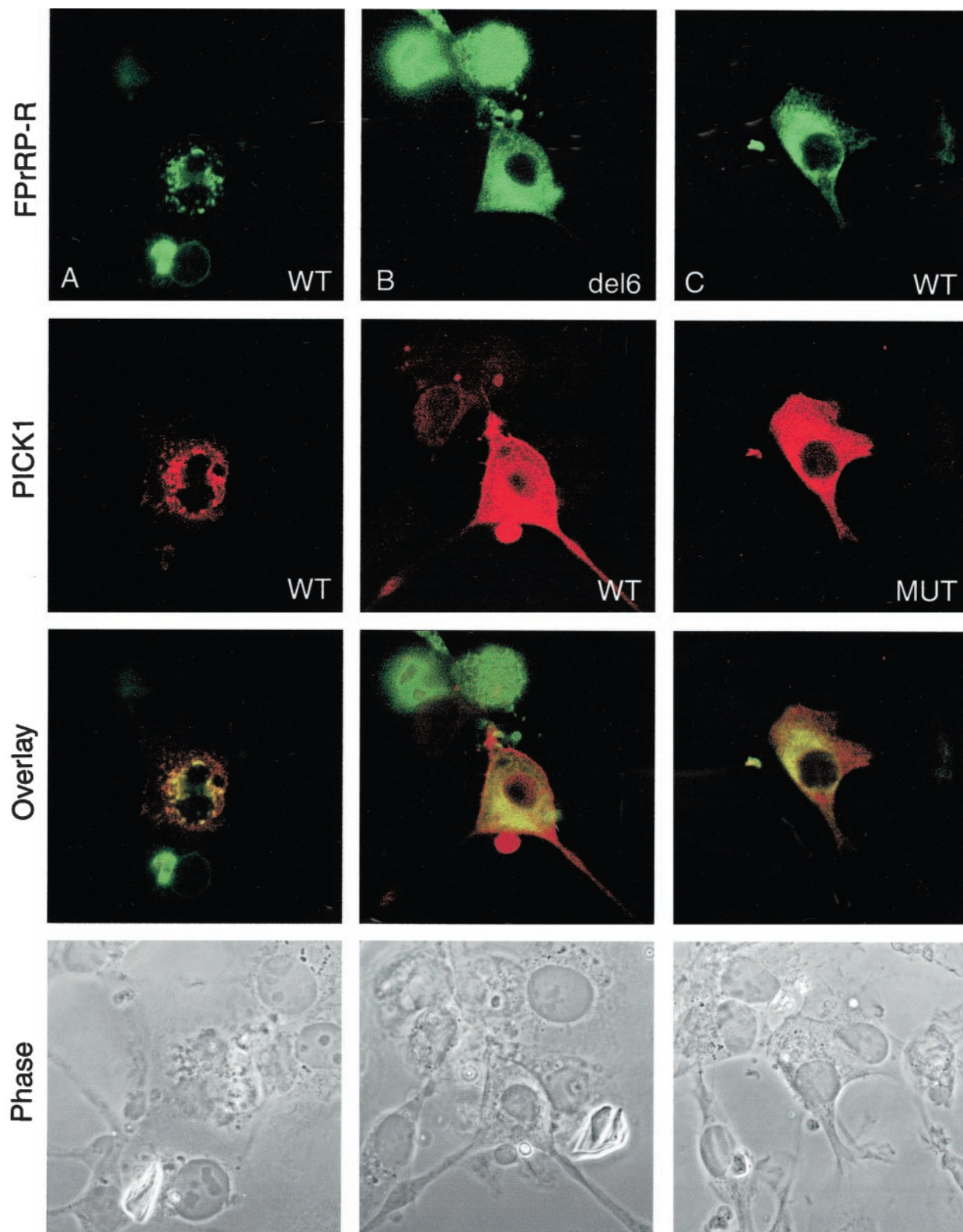
Fig. 4. The PrRP receptor/PICK1 coclustering is contingent on PDZ domain interaction. A, wild-type Flag-tagged PrRP receptor coexpressed with wild-type PICK1. Note the cell expressing PICK1 alone shows a homogenous cytoplasmic distribution within the cell. B, COS-7 cells coexpressing mutant form of PrRP-R with a wild-type PICK1 protein. The clustering seen when wild-type proteins were coexpressed was abolished. C, COS-7 cells coexpressing wild-type PrRP-R with a mutant PICK1 (MUT=K27D28AA PICK1 mutant) protein. Again, no clustering is observed in these cells. Notice the cell not transfected with PICK1 (in A) does not exhibit the clustering seen with PrRP-R coexpressed with PICK1.

GRIP may be regulated by phosphorylation, but not by PKC phosphorylation.

PrRP Receptor Are Coexpressed with GRIP, ABP, and PICK1 in Brain Nuclei. Since there are no available antibody for the PrRP receptor, to ascertain whether the PrRP receptor-PDZ domain protein interactions may occur in vivo we determined whether the mRNA of these proteins are

Fig. 5. GRIP interaction with PrRP-R can be regulated. Samples were treated and prepared as described under *Materials and Methods*. Compared with DMSO control, coimmunoprecipitation of cells coexpressing FPrRP-R with GRIP shows that 1 μM PMA treatment did not alter GRIP coimmunoprecipitation with FPrRP-R, whereas 1 μM okadaic acid (OA) caused a significant attenuation of GRIP appearing in the CoIP. A, IP fraction probed for GRIP. B, crude fraction probed for GRIP to normalize GRIP expression. C, IP fraction probed with anti-FLAG antibody to assess expression and efficiency of immunoprecipitation of FPrRP-R.
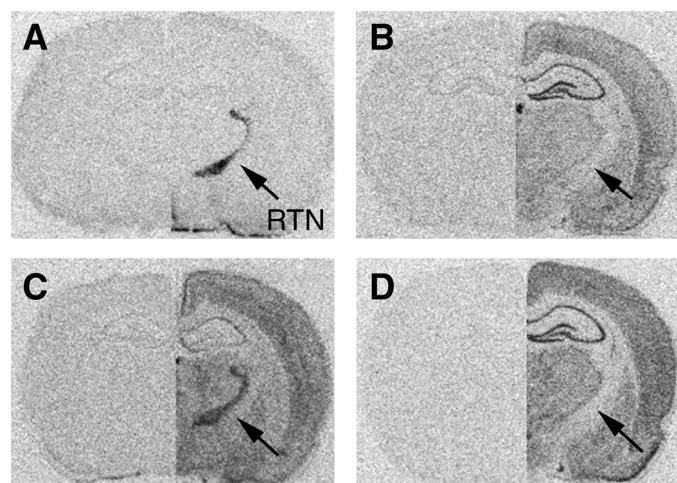
Fig. 6. GRIP1, ABP, and PICK1 are coexpressed in vivo with PrRP receptor. In situ hybridization was carried out on adult coronal brain slices as detailed under *Materials and Methods*. Each section was exposed to film for 6 days. The antisense is depicted on the right half of each section and the sense control is depicted on the left half. A, the expression of PrRP receptor in the reticular thalamic nucleus (RTN). The PrRP receptor is scantly expressed in other nuclei in the brain, such as various hypothalamic nuclei. B, GRIP1, using a fragment within the coding region that does not cross hybridize with ABP (GRIP2), was used in the experiment. GRIP1 is expressed in the RTN; however, the predominant expressing regions are in the hippocampus and thalamus, regions without PrRP receptor expression. C, ABP expression was detected using a probe made from a cDNA region extremely divergent from the GRIP1 sequence. Note that the hippocampus is lightly stained compared with GRIP1 (see B) and PICK1 (see D) and the majority of the signal comes from the RTN. D, PICK1 expression.

coexpressed in the brain using in situ hybridization. One of the predominant PrRP receptor-expressing nuclei is the reticular thalamic nucleus (RTN) [Fig. 6A, (Roland et al., 1999; Ibata et al., 2000)]. We found by in situ hybridization that this nucleus also specifically expresses GRIP1 (Fig. 6B), ABP (Fig. 6C), and PICK1 (Fig. 6D) compared with the sense strand control (left half of each section of Fig. 6). Interestingly, ABP has relatively high expression within the RTN, suggesting possibly that this isoform may be the endogenous form associated with PrRP receptor.
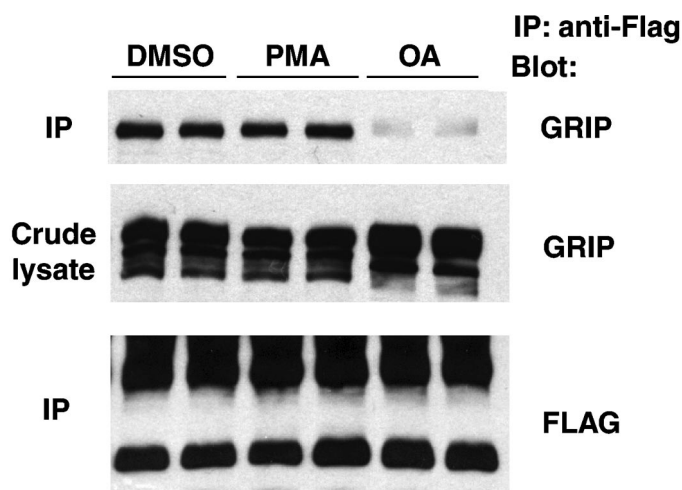
Discussion

We report in this manuscript that the PrRP receptor has a PDZ domain binding motif in its C-terminal tail (-SVVI). We demonstrate that this sequence can specifically direct PrRP receptor binding to GRIP1, ABP (GRIP2), and PICK1. We further show that the interaction between PrRP receptor and the PDZ domain of PICK1 is sufficient to confer clustering of these two proteins within cells. We also show that these interactions may be regulated, possibly by phosphorylation, and that these gene products are expressed within the same subset of PrRP receptor-expressing nuclei, thereby pointing to the in vivo relevance for the interaction. We conclude that PrRP receptor associates with the PDZ domain proteins that also interact with AMPA receptors.

It has been estimated that about 30% of GPCRs contain a PDZ domain binding motif (Ranganathan and Ross, 1997), but only a few of these have been demonstrated to interact with PDZ domain proteins. PrRP receptor is the first GPCR found to have a C-terminal sequence that closely resembles that present in AMPA receptors, leading to the prediction that PrRP receptor would share the same interacting proteins as those of the AMPA receptors. Our data shows that the PrRP receptor interacts with the same subset of PDZ domain proteins that are critical for the transport and retention of AMPA receptors at the postsynaptic location (Dong et al., 1997; Osten et al., 2000) and raises the possibility that PrRP receptor may also be targeted to and scaffolded at the postsynaptic membrane with AMPA receptors. Future efforts to generate antibody to PrRP receptor or transfection of tagged receptors into neurons will be needed to address these issues.

If PrRP receptor could be targeted to dendritic synapses through its association with GRIP-like PDZ domain proteins, the activation of PrRP receptor could modulate the postsynaptic neuron through an indirect interaction with AMPA receptors. Whether there exists a link between AMPA receptor function and PrRP receptor activation is being addressed presently in our laboratories. However, it is not unprecedented for a GPCR to regulate physiological function by modulating a channel protein through C-terminal tail interactions with PDZ domain proteins. For instance, the $\beta2$ adrenergic receptor ($\beta2R$) in the kidney regulates cellular pH by modulating Na^+/H^+ exchange through a G protein-independent pathway (Hall et al., 1998). This regulation was found to occur through the interaction of C-terminal four amino acids of $\beta2R$ with a protein containing a single PDZ domain called the Na^+/H^+ exchange (NHE) regulatory factor (Hall et al., 1998). Activation of $\beta2R$ leads to association with NHE regulatory factor, which normally binds and inhibits the NHE type 3 channel. The association with $\beta2R$ relieves the inhibition and restores NHE functioning. In *Drosophila* species

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photoreceptor cells, the rhodopsin protein activates a calcium transient through a transient receptor potential calcium channel via a Gq/phospholipase C/eyePKC pathway (Tsunoda et al., 1997). The PDZ domain protein inaD with five PDZ domains interacts with components of this photo-transduction cascade (TRP channel, phospholipase C, and PKC) to form a macromolecular machinery of proteins to which rhodopsin efficiently couples once it is activated by a photon (Tsunoda et al., 1997).

It is also interesting to note that both the PrRP receptor and GluR2 and GluR3 contain a serine residue at the –3 position (-SVVI versus –SVKI, respectively) which was shown to be critical for GRIP interaction (Dong et al., 1997) (Fig. 2, A and B). This data raises the question for the role of the serine at the –3 position. The last four amino acids of GluR2 and 3 (-SVKI) conforms to the consensus required for PKC phosphorylation sites. Indeed, it was shown recently that PKC phosphorylation at the serine residue in GluR2 attenuates binding to GRIP1 (Matsuda et al., 1999) and this phosphorylation event is critical for regulating the internalization and surface expression dynamics of AMPA receptors containing GluR2 subunits (Chung et al., 2000; Matsuda et al., 2000). PrRP receptor lacks the critical basic amino acid at the –1 position (-SVVI). We predict, therefore, that the binding between the C terminus of PrRP receptor and GRIP and ABP should not be modulated by PKC. Consistent with this prediction, experiments using the phorbol ester PMA to activate PKC did not change the ability of the PrRP receptor to interact with GRIP, although okadaic acid, which increases the overall phosphorylation states of proteins by inhibiting PP1/PP2A phosphatases, did dramatically attenuate binding of GRIP to PrRP receptor. Whether the interaction between PrRP receptor and these PDZ domain proteins is regulated by phosphorylation at the specific serine residue in PrRP receptor, and whether PrRP receptor activation alters this interaction by the activation of other kinases, such as the G protein-coupled receptor kinases, is currently under investigation.

In conclusion, we have found that a GPCR can associate with the same PDZ domain proteins that interact with AMPA receptors. These data could be important to help further understand the PrRP receptor function in the central nervous system by suggesting that PrRP receptor may modulate neurotransmission specifically at glutamatergic synapses.

Acknowledgments

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