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PICK1 Interacts with ABP/GRIP to Regulate AMPA Receptor Trafficking

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Summary

PICK1 and ABP/GRIP bind to the AMPA receptor (AMPAR) GluR2 subunit C terminus. Transfer of the receptor from ABP/GRIP to PICK1, facilitated by GluR2 S880 phosphorylation, may initiate receptor trafficking. Here we report protein interactions that regulate these steps. The PICK1 BAR domain interacts intermolecularly with the ABP/GRIP linker II region and intramolecularly with the PICK1 PDZ domain. Binding of PKCα or GluR2 to the PICK1 PDZ domain disrupts the intramolecular interaction and facilitates the PICK1 BAR domain association with ABP/GRIP. Interference with the PICK1-ABP/GRIP interaction impairs S880 phosphorylation of GluR2 by PKC and decreases the constitutive surface expression of GluR2, the NMDA-induced endocytosis of GluR2, and recycling of internalized GluR2. We suggest that the PICK1 interaction with ABP/GRIP is a critical step in controlling GluR2 trafficking.

Introduction

AMPARs mediate the majority of fast excitatory neurotransmission in the mammalian brain. They are heterotetrameric cation channels composed of subunits GluR1-4 (GluRA-D) (Hollmann and Heinemann, 1994). AMPAR trafficking is a dynamic process that underlies the activity-dependent modification of synaptic strength, including long-term potentiation (LTP) and long-term depression (LTD) (Barry and Ziff, 2002; Bredt and Nicoll, 2003; Malinow and Malenka, 2002).

The C terminus of the GluR2 subunit functions in AMPAR trafficking through binding to proteins including ABP/GRIP and PICK1 (Dong et al., 1997; Srivastava et al., 1998; Wyszynski et al., 1999; Xia et al., 1999). ABP and GRIP are two closely related multi-PDZ domain-containing proteins that are thought to scaffold receptors. Both ABP and GRIP are synthesized in a palmitoylated form that is targeted to spines and a nonpalmitoylated form that is targeted to intracellular clusters (DeSouza et al., 2002; Yamazaki et al., 2001). During AMPAR trafficking, ABP/GRIP may anchor AMPARs at synaptic and intracellular membranes (Braithwaite et al., 2002; Daw et al., 2000; DeSouza et al., 2002; Fu et al., 2003; Hirbec et al., 2003; Osten et al., 2000; Seidenman et al., 2003). PICK1 contains a single PDZ domain (Staudinger et al., 1997) and a BAR (*B*in/amphiphysin/*R*vs) domain (Peter et al., 2004). BAR domains are crescent-shaped dimers that bind preferentially to curved, negatively charged membranes (Peter et al., 2004). The function of the PICK1 BAR domain in AMPAR trafficking is not known, but may be involved in association with curved membranes (Peter et al., 2004). The PDZ domain of PICK1 is required to bind GluR2 and PKCα (Dev et al., 1999; Perez et al., 2001; Staudinger et al., 1997; Xia et al., 1999). PICK1 may present PKCα to AMPARs, whereupon PKCα phosphorylates S880 of GluR2 (Chung et al., 2000; Matsuda et al., 1999; Perez et al., 2001).

Although ABP/GRIP and PICK1 bind the same site on GluR2, their interactions with GluR2 can be regulated independently by PKC. GluR2 phosphorylated at S880 by PKC loses its ability to interact with ABP/GRIP, but the interaction with PICK1 remains intact (Chung et al., 2000; Matsuda et al., 1999). In cerebellum, PKC phosphorylation of GluR2 S880 and regulation of interactions between GluR2 and PICK1 or ABP/GRIP by PKC are required for LTD (Chung et al., 2003; Matsuda et al., 2000; Xia et al., 2000). In hippocampus, GluR2 receptors (which refers hereafter to GluR2/3 heteromers and GluR2 homomers) undergo continuous cycling, and PICK1 and ABP/GRIP are involved in both receptor internalization and recycling (reviewed by Bredt and Nicoll, 2003; Malinow and Malenka, 2002). Synaptic ABP/GRIP functions in GluR2 anchorage at synapses, and the endocytosis of GluR2 requires dissociation of GluR2 from ABP/GRIP (Kim et al., 2001; Osten et al., 2000; Seidenman et al., 2003). PICK1 plays a role in hippocampal LTD by promoting GluR2 endocytosis (Kim et al., 2001; Perez et al., 2001) and NSF regulates PICK1-mediated AMPAR trafficking (Hanley et al., 2002; Luthi et al., 1999). Internalized GluR2 may be recycled back to the plasma membrane to maintain synaptic transmission (Daw et al., 2000; Ehlers, 2000; Hirbec et al., 2003). Receptor recycling back to the surface is proposed to require receptor dissociation from internal anchorage by ABP/GRIP (Braithwaite et al., 2002; Daw et al., 2000; DeSouza et al., 2002; Fu et al., 2003; Hirbec et al., 2003). The activation of PKC relieves the retention of internalized GluR2 by ABP/GRIP and thereby promotes receptor recycling (Daw et al., 2000). PICK1 also functions in recycling of AMPAR back to the plasma membrane in cerebellar stellate cells (Gardner et al., 2005; Liu and Cull-Candy, 2005). However, the specific role of PICK1 in receptor recycling is unclear. PICK1 has been hypothesized to target PKC to GluR2 to enable PKC to phosphorylate GluR2. However, how PICK1 can target PKC to GluR2 that is associated with ABP/GRIP and the molecular mechanisms that underlie the regulation of AMPAR trafficking by PICK1 and ABP/GRIP are largely unknown.

Here we report an interaction between PICK1 and ABP/GRIP, two important factors in organizing AMPAR trafficking. This interaction targets PICK1 to an ABP/GRIP-GluR2 complex to modulate receptor trafficking and may function in targeting of PKC to GluR2. The PICK1 PDZ domain also interacts intramolecularly with its BAR domain. Ligand binding to the PDZ domain disrupts this interaction, exposing the BAR domain and

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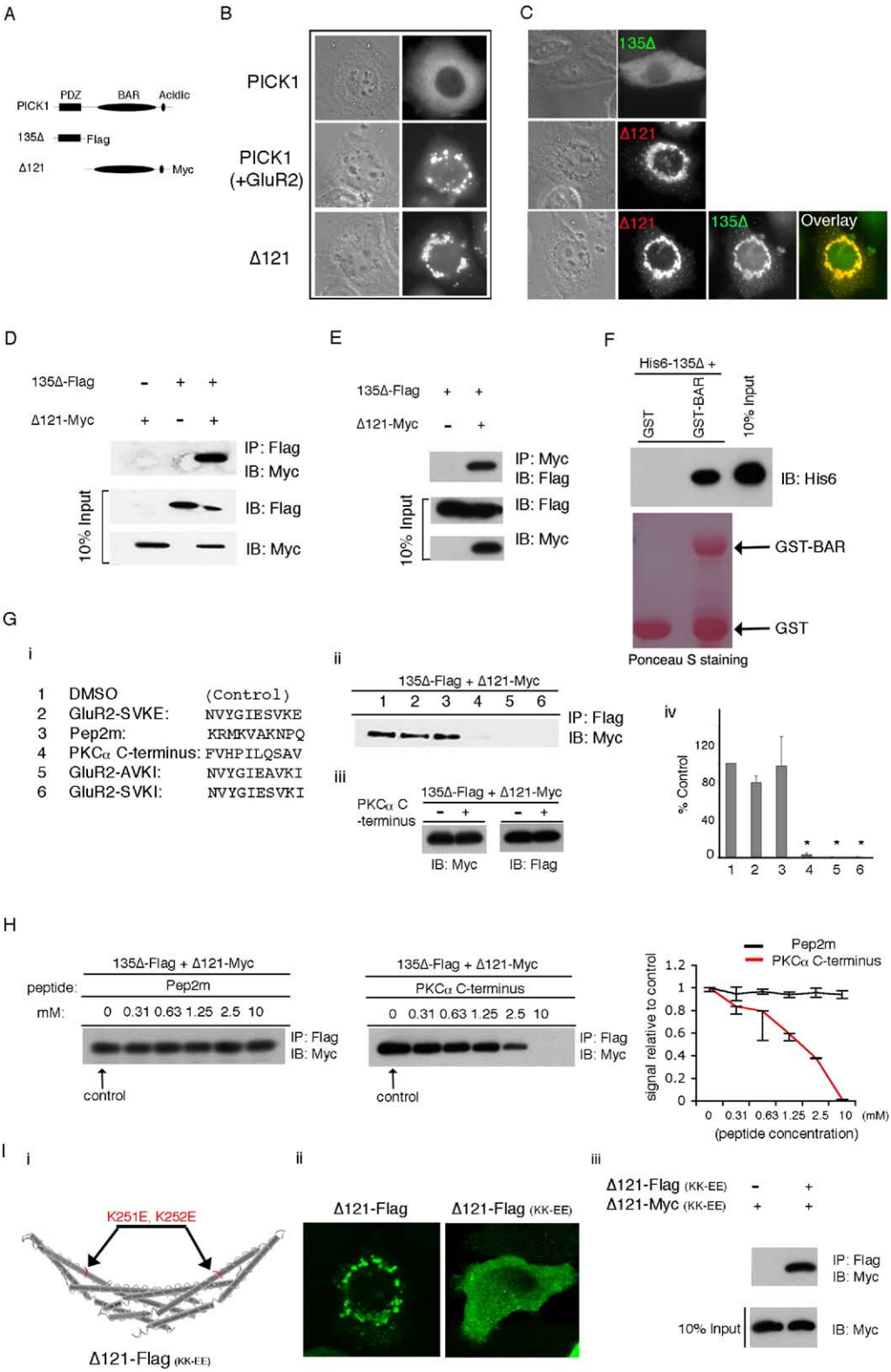


Figure 1. Regulation of the PICK1 Intramolecular Interaction

(A) Schematics of PICK1 and PICK1 mutants.

(B) Immunostaining (right panel) of HeLa cells expressing PICK1 (top), PICK1 plus GluR2 (GluR2 fluorescence not shown) (middle), or Δ121 (bottom). Left panel shows the transmission images.

(C) Colocalization of 135Δ and Δ121 in HeLa cells. Top and middle panels show the localization when 135Δ or Δ121 was transfected on their

facilitating the PICK1-ABP/GRIP association. Impairment of the PICK1-ABP/GRIP interaction decreases the phosphorylation of GluR2 at S880 by PKC, GluR2 surface expression, recycling, and the NMDA-induced endocytosis. The PICK1-ABP/GRIP interaction thus is a critical step in the regulation of GluR2 trafficking.

Results

Intramolecular Interaction of PICK1

We previously reported that either binding of the PICK1 PDZ domain to a ligand, such as GluR2 or PKCα, or deletion of the PICK1 PDZ domain induced an intracellular relocalization of PICK1 to perinuclear clusters (Figures 1A and 1B; Perez et al., 2001). We hypothesized that, in the absence of a ligand, the PDZ domain interacts with the coiled coil region (BAR domain, Peter et al., 2004) of PICK1 and suppresses PICK1 relocalization and clustering. PDZ domain ligand binding, or PDZ deletion, would disrupt this intramolecular interaction and allow PICK1 to enter into clusters. To test this hypothesis, we asked if the PDZ domain interacts with the BAR domain when the PICK1 PDZ domain (135Δ-Flag) or the PICK1 BAR domain (Δ121-Myc) were expressed in HeLa cells. Expressed individually, 135Δ was diffusely distributed in the cytoplasm, and Δ121 formed perinuclear clusters (Figure 1C, upper and middle panels, respectively). When coexpressed, 135Δ and Δ121 colocalized in large perinuclear clusters (Figure 1C, lower panel), suggesting that the two interact. In a coIP assay, Δ121 was coprecipitated with 135Δ from cells cotransfected with both constructs, but not from control cells transfected with either one (Figure 1D). The reverse coIP assay also showed that 135Δ was coprecipitated with Δ121 (Figure 1E). A purified GST-BAR domain fusion, but not the control, GST alone, pulled down the purified His6-tagged PDZ domain (Figure 1F), confirming a direct PICK1 PDZ-BAR domain interaction.

We next examined how ligand binding to the PICK1 PDZ domain affected the PDZ-BAR domain interaction. Peptides shown in Figure 1Gi and the vehicle, DMSO, were incubated with lysates from 293T cells cotransfected with Δ121 and 135Δ. CoIP and immunoblotting (IB) assays of the PDZ-BAR domain interaction were then performed as indicated. Peptides that bind to the PICK1 PDZ domain disrupted the PDZ-BAR domain interaction (Figures 1Gii and 1Giv). However, control peptides that do not bind to the PICK PDZ domain or DMSO did not disrupt the interaction. Neither the PDZ domain nor the BAR domain degraded upon peptide binding to the PDZ domain (Figure 1Giii). We further titrated the PDZ-BAR domain interaction with peptides. Incubation of lysates from cells coexpressing the PICK1 PDZ and BAR domains with PKCα C-terminal peptide, but not incubation with a control peptide, Pep2m, led to a dose-dependent disruption of the PDZ-BAR domain interaction (Figure 1H). These data suggest that the binding of a ligand to the PICK1 PDZ domain can indeed disrupt the PICK1 PDZ-BAR domain interaction.

To understand the function of the PICK1 BAR domain in membrane interactions better, we mutated two basic resides on the concave face of the PICK1 BAR domain (Δ121-FlagKK251/252EE) (Peter et al., 2004) (Figure 1Ii). While the wt PICK1 BAR was clustered in cells, the KK251/252EE mutants were diffusely distributed in the cytoplasm (Figure 1Iii). The KK251/252EE mutant is capable of forming dimers (Figure 1Iiii), which is a prerequisite for generating the BAR domain crescent shape. This suggests that PICK1 contains a functional BAR domain that employs basic residues to associate with membrane.

PICK1 Colocalizes with GRIP/ABP and Binds to GRIP/ABP In Vitro

To study the specificity of the PICK1 PDZ-BAR domain interaction, we coexpressed the PICK1 BAR domain (Δ121) with other PDZ proteins, ABP/GRIP and PSD-95, in HeLa cells and examined the distribution of ABP/GRIP or PSD-95 by immunofluorescence. When expressed alone, GRIP formed small clusters in the cytoplasm, and PSD-95 was targeted to the plasma mem-

own, respectively. Bottom panel shows the colocalization when Δ121 and 135Δ were cotransfected. Left panel shows the transmission images.

(D and E) CoIP of Δ121 with 135Δ from 293T cells. Lysates from cells transfected with 135Δ-Flag or/and Δ121-Myc were precipitated with an anti-Flag (D) or an anti-Myc (E) antibody. The IPs and 10% input were probed with indicated antibodies.

(F) A direct PICK1 PDZ-BAR domain interaction. Purified His6-135Δ was incubated with GST or GST-BAR. Bound proteins were detected by IB with an anti-His6 antibody. Bottom panel shows the Ponceau S staining, indicating GST species.

(G) Disruption of the PICK1 intramolecular interaction by peptides from PKCα and GluR2 C termini. (Gi) Peptides (2–6) used in the experiment. (Gii) Peptides that bind to the PICK1 PDZ domain disrupt the PICK1 PDZ-BAR domain interaction. Lysates from cells cotransfected with 135Δ and Δ121 were divided equally to incubate with peptides shown in (Gi) or the vehicle, DMSO, for 30 min at RT. IP and IB were then performed as indicated. (Giii) Peptide that binds to the PICK1 PDZ domain did not induce obvious degradation of the PICK1 PDZ and BAR domains. Equal amount of lysates from 293T cells expressing 135Δ and Δ121 was incubated with DMSO or PKCα C-terminal peptide for 30 min at RT. Lysates were then probed with indicated antibodies. (Giv) Data from three experiments were quantitated by scanning densitometry. The bars numbered 1–6 correspond to DMSO or peptide treatments shown in (Gi and Giii). Values are relative to DMSO control (n = 3, t test, *p < 0.0001). Bar graph shows mean ± SD.

(H) Dose-dependent disruption of the PICK1 PDZ-BAR domain interaction by peptide that binds to the PICK1 PDZ domain. Different amounts of peptide were incubated with aliquots of lysates from cells expressing 135Δ and Δ121 as described above. IP and IB were then performed as indicated.

(I) PICK1 contains a BAR domain. (Ii) The molecular model of the PICK1 BAR domain. This model is based on the analog to the arfaptin2 BAR domain. Two basic residues (K251, K252, red) in the concave face of the PICK1 BAR domain were mutated into glutamate. (Iii) Immunostaining of Δ121 or Δ121 (KK-EE) expressed individually in HeLa cells. Compared with the cluster distribution of Δ121 (left), Δ121 (KK-EE) was diffuse in the cytoplasm (right). (Iiii) The KK251/252EE mutant is capable of forming dimers. Δ121 (KK-EE) mutants tagged with Flag or Myc were cotransfected into 293T cells. CoIP and IB experiments were performed as indicated.

brane (Figure 2A). However, when coexpressed with the BAR domain (Δ121), GRIP translocated into perinuclear clusters and assumed a pattern that resembled the distribution of Δ121 (Figure 2A). This suggests that Δ121 can bring GRIP into clusters, possibly by direct interaction. In contrast, coexpression of Δ121 induced no change in PSD-95 distribution, indicating that the effect of Δ121 on ABP/GRIP was specific (Figure 2A).

We next determined the domain of GRIP necessary for the colocalization with Δ121. GRIP mutants containing linker II (LII) colocalized with Δ121 (Figure 2B). This suggests that GRIP LII harbors the sequence responsible for colocalization with Δ121, a conclusion that was later confirmed (see Figures 2C and 5A). To confirm a direct interaction between PICK1 and ABP/GRIP and to map more precisely the region of ABP/GRIP required for binding PICK1, we performed a GST pulldown assay. ABP mutants fused to GST were incubated with lysates from 293T cells expressing Δ121-Myc. After GST pulldown, bound proteins were eluted and subjected to IB assay. A 55 aa sequence (mutant 8 in Figure 2Ci) falling between aa 859 and 913 of ABP in LII (Br, *B*inding *r*egion) was required and sufficient for binding to Δ121 (Figure 2Cii and 2Ciii). In the same assay, a GRIP mutant fusion to GST containing the Br also pulled down Δ121 (Figure 2Cii, right). Alignment of the 55 aa Br of ABP with the same region of GRIP reveals an 82% identity (Figure 2D), although the overall identity between LII regions of ABP and GRIP is low (31.8%). These experiments indicate that both ABP and GRIP bind to PICK1 via the Br in LII.

PICK1 Associates with ABP/GRIP in 293T Cells and in Rat Brain

We next determined the ability of PICK1 to bind ABP/GRIP in vivo. In 293T cells, GRIP was coprecipitated with PICK1 or with PICK1 mutants containing the BAR domain, but not with a PICK1 mutant lacking the BAR domain, or, as a control, in the absence of PICK1 expression (Figure 3A). This showed the specificity of the coIP and a dependence of PICK1 on the BAR domain for the interaction with GRIP.

To determine if PICK1 and GRIP associate with each other in rat brain, we performed a coIP assay. An antiserum to GRIP, but not a control IgG, coprecipitated PICK1 with GRIP from rat brain lysate (Figure 3Bi). The reverse coIP experiment also showed the association of PICK1 and GRIP (Figure 3Bii). Thus, PICK1 and ABP/GRIP are associated with each other in native brain tissue.

PICK1 Forms Clusters with GRIP in Cultured Hippocampal Neurons

The ability of PICK1 and Δ121 to colocalize with GRIP in HeLa cells suggested that PICK1 and Δ121 might also colocalize with GRIP in neurons. Figures 3C–3E show localizations of Δ121, PICK1, and GRIP expressed individually from Sindbis viruses in cultured hippocampal neurons. Similar to our previous report (Perez et al., 2001), Δ121 formed intracellular clusters in neurons (Figure 3C), and PICK1 was diffuse and penetrated into spines (Figure 3D). GRIP in neurons formed large clusters in the soma and dendritic shafts (Figure 3E). When coexpressed with GRIP, both Δ121 and PICK1 colocalized with GRIP in dendritic shafts and in the soma (Figures 3F and 3G). The strong colocalization of PICK1 and GRIP in neurons suggests that in neurons either coexpression of the two proteins or binding to endogenous factors can disrupt the intramolecular interaction of PICK1 and facilitate the PICK1 BAR domain interaction with GRIP (see data from Figures 4C and 4D). These experiments recapitulate in neurons the localizations seen in HeLa cells and indicate that PICK1 may be recruited into GRIP/ABP complexes in neurons through the PICK1-GRIP/ABP interaction.

Δ121, GRIP, and GluR2 Colocalize in HeLa Cells and Δ121 Binds to the GRIP-GluR2 Complex In Vitro

We next examined if PICK1, ABP/GRIP, and GluR2 could form a triple complex. This triple complex may function in the presentation of PKC-PICK1 complexes to the ABP/GRIP-GluR2 complex. We assayed complex formation by Δ121 because PICK1 itself is capable of binding to GluR2. GluR2 coexpressed with Δ121 showed a diffuse staining in HeLa cells and did not colocalize with Δ121, as expected since Δ121 lacks the PDZ domain (Figure 4A, upper panel; Perez et al., 2001). However, in cells expressing GRIP, GluR2, and Δ121, all three peptides colocalized in a perinuclear pattern that resembled the pattern seen when Δ121 was expressed on its own (Figure 4A, middle panel). The clusters of GRIP, GluR2, and Δ121 were unlikely to be nonspecific aggregates, because a mutant of GluR2, GluR2-AVKI, which does not interact with GRIP (Osten et al., 2000), did not colocalize with GRIP and Δ121 when they were triply transfected into cells (Figure 4A, lower panel).

We further confirmed a PICK1, GRIP, and GluR2 triple complex using a GST pulldown assay. As shown previously, GRIP strongly bound to the GluR2 C terminus GST fusion (R2C) (Osten et al., 2000), and Δ121 did not bind to GST-R2C (Figure 4B; Perez et al., 2001). However, in the presence of GRIP, Δ121 strongly bound to GST-R2C, but not GST-GluR2C-SVKE, a mutant of GluR2 that does not interact with GRIP (Figure 4B). These data demonstrate that Δ121-GRIP-GluR2 can form a triple complex both in vitro and in vivo.

Disruption of the PICK1 Intramolecular Interaction Facilitates the Intermolecular PICK1-GRIP Association

If the PICK1 PDZ-BAR domain interaction masks the BAR domain, disrupting the PDZ-BAR interaction would be expected to enhance the PICK1-ABP/GRIP association. To study this question, we first compared the distributions of GRIP when coexpressed with PICK1 or Δ121 in HeLa cells. As shown before (Figure 2A), GRIP and Δ121 colocalized extensively in perinuclear clusters (Figure 4C, upper panel). In contrast, only a fraction of GRIP puncta colocalized with PICK1 (Figure 4C, lower panel). This indicates that the exposure of the PICK1 BAR domain, such as takes place upon the deletion of the PICK1 PDZ domain, can promote the PICK1-GRIP interaction. We next asked if a peptide that binds to the PICK1 PDZ domain and that disrupts the PDZ-BAR interaction could enhance the PICK1-ABP/GRIP association. Lysates from 293T cells expressing PICK1-Flag were incubated with GST-Br, the sequence in ABP/

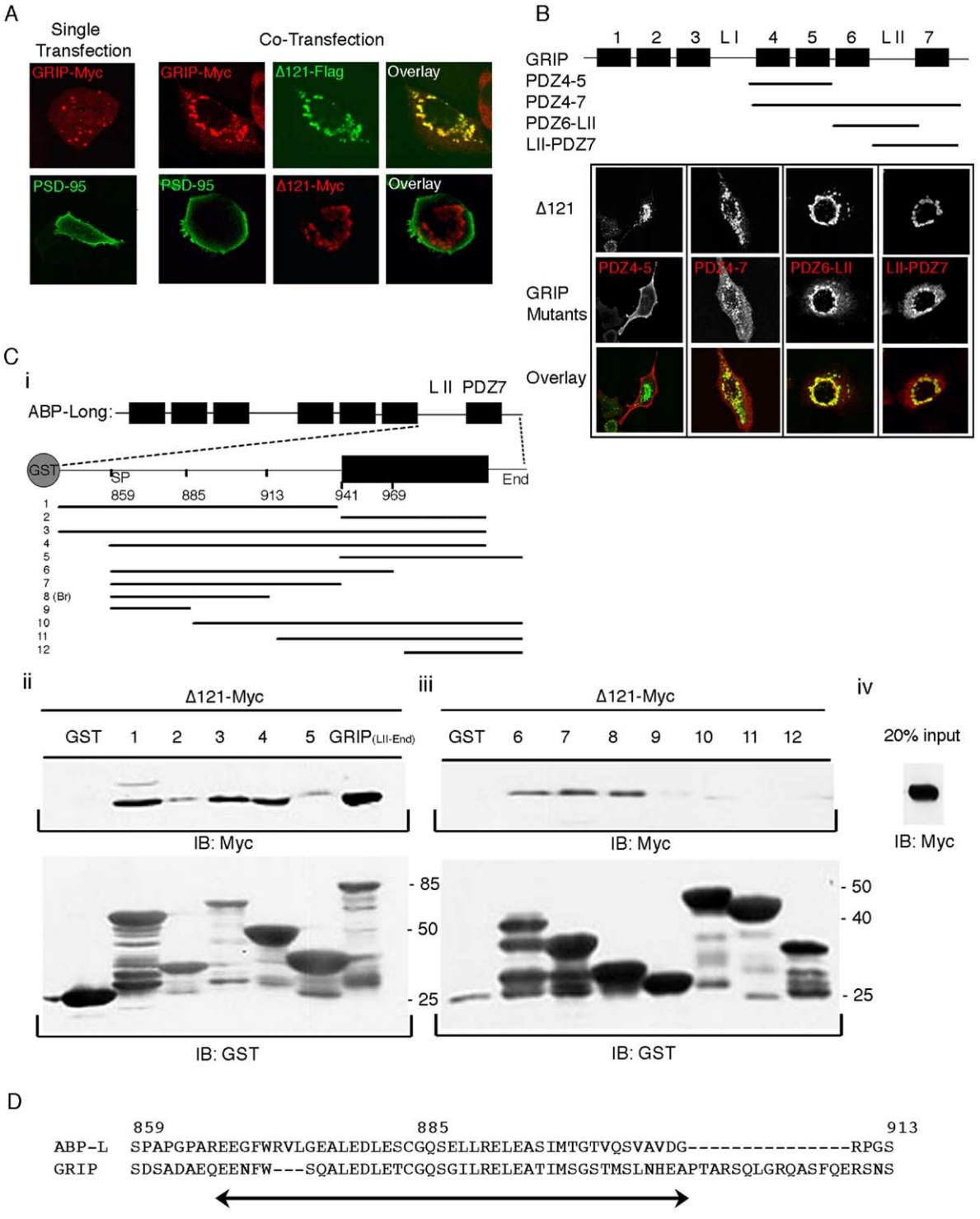


Figure 2. PICK1 Colocalizes with GRIP/ABP and Binds to GRIP/ABP In Vitro

(A) GRIP but not PSD-95 colocalizes with Δ121 in HeLa cells. GRIP and PSD-95 were either expressed alone (left) or coexpressed (right) with Δ121. When coexpressed with Δ121, GRIP, but not PSD-95, colocalized with Δ121 in large perinuclear clusters.

(B) Immunostaining of HeLa cells cotransfected with different GRIP mutants and Δ121.

(C) A 55 aa sequence in ABP LII mediates the interaction with Δ121. (Ci) Schematics of GST-ABP mutants. "SP" refers to the alternative "splicing point". (Gii and Giii) Lysates from 293T cells expressing Δ121 were divided equally to bind to GST alone, GST-ABP mutants, or a GST-GRIP mutant containing LII ([Cii], right). Bound proteins were detected by IB. 10% input of Δ121 was also shown (Civ).

(D) The 55 aa Br of ABP were aligned with the same region of GRIP. There is an 82% similarity between ABP and GRIP in the marked region.

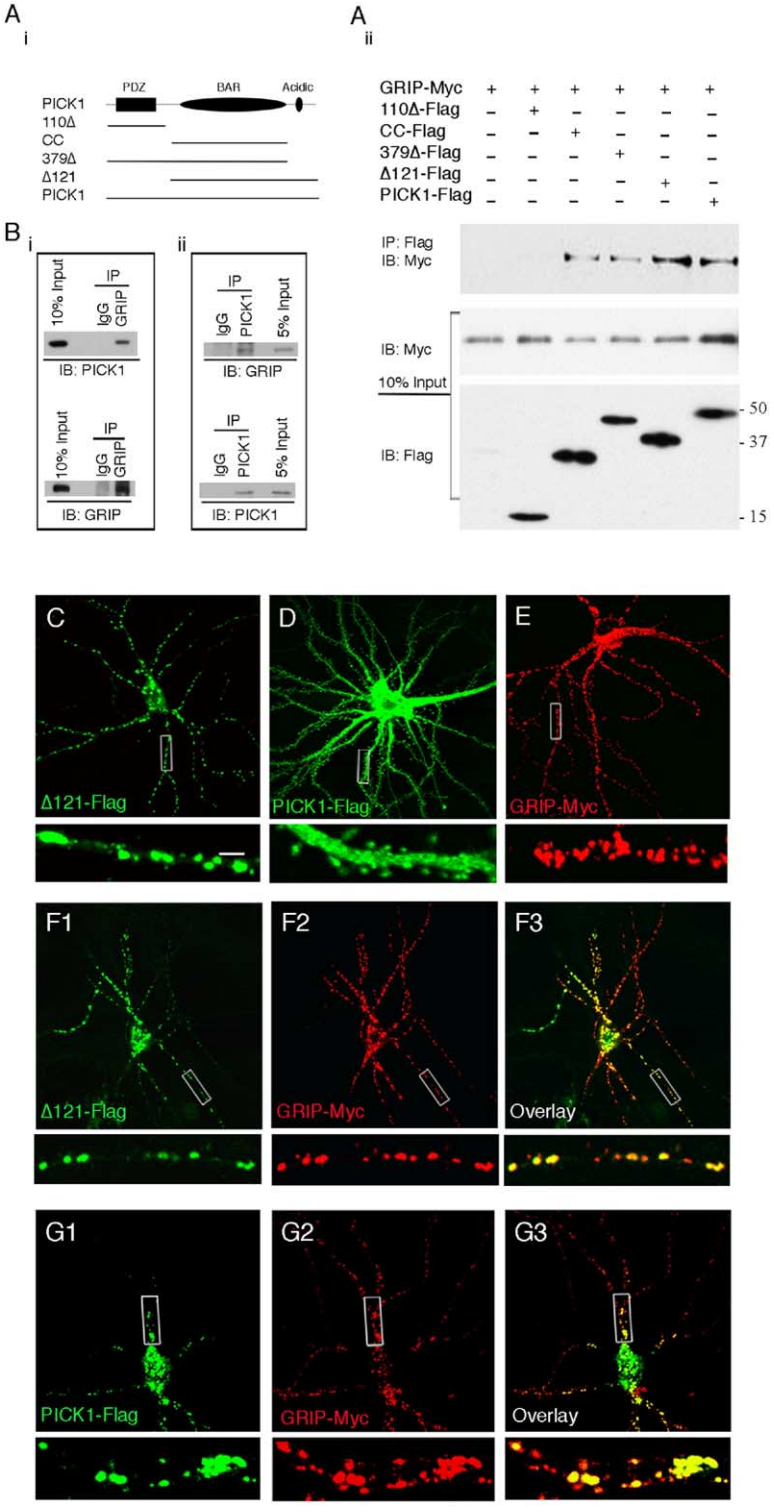


Figure 3. PICK1 Interacts with GRIP In Vivo and Colocalizes with GRIP in Cultured Hippocampal Neurons

(A) PICK1 interacts with GRIP in 293T cells. (Ai) Schematics of PICK1 and PICK1 mutants. (Aii) GRIP was expressed alone or together with PICK1 and PICK1 mutants. Cell lysates were subjected to IP and IB assays with indicated antibodies.

(B) PICK1 associates with GRIP in rat brain tissue. Rat brain cortex homogenates were precipitated with a control IgG or an anti-GRIP antibody (Bi) and a control IgG or an anti-PICK1 rabbit antibody (Bii). Bound proteins were detected by IB.

(C–G) Immunostaining of neurons expressing Δ121(C), PICK1 (D), or GRIP (E) or coexpressing GRIP and Δ121 (F1–F3) and GRIP and PICK1 (G1–G3). Scale bar, 20 μm. The GRIP construct used in this experiment is the nonpalmitoylated isoform. White boxes in panels (C)–(G) define enlargements shown in lower panels.

GRIP that binds to PICK1 (see Figure 2C). Figure 4D shows that the PKCα C-terminal peptide significantly enhanced the interaction between GST-Br and PICK1. This shows that disruption of the PICK1 PDZ-BAR domain interaction facilitates the association of PICK1 with the Br region of ABP/GRIP.

Functions of the PICK1-ABP/GRIP Interaction in GluR2 Trafficking

These data suggest a model in which PICK1 targets activated PKCα to the ABP/GRIP-GluR2 complex and enables PKCα to phosphorylate GluR2 at S880 and thus facilitates AMPAR trafficking. This model predicts

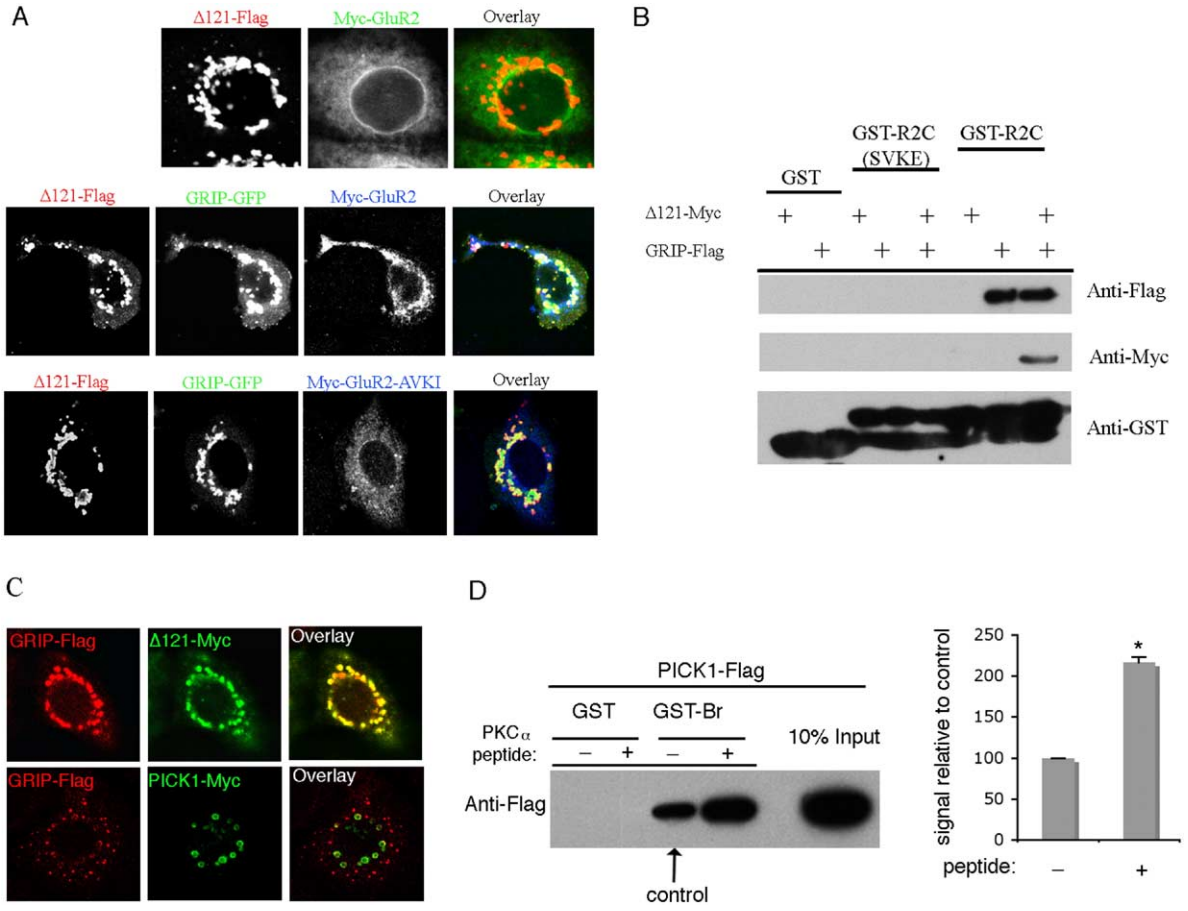


Figure 4. Δ121, GRIP, and GluR2 Colocalize in HeLa Cells and Form a Complex In Vitro

(A) When cells were cotransfected with GluR2 and Δ121 (top panel), GluR2 did not colocalize with Δ121. When cells were triple transfected with GRIP, GluR2, and Δ121 (middle panel), three proteins formed perinuclear clusters. When cells were triple transfected with GRIP, GluR2-AVKI, and Δ121 (bottom panel), GluR2-AVKI did not colocalize with the coclusters of GRIP and Δ121.

(B) Δ121, GRIP, and GluR2 form a complex in vitro. Lysates from 293T cells expressing GRIP or Δ121 or both GRIP and Δ121 were divided equally to bind to GST alone or GST-GluR2-SVKE or GST-GluR2C as indicated. Bound proteins were detected by IB.

(C) Colocalization of Δ121 or PICK1 with GRIP in HeLa cells. Δ121 (top panel) or PICK1 (bottom panel) was coexpressed with GRIP. Compared with Δ121, PICK1 only partially colocalized with GRIP.

(D) Peptide that binds to the PICK1 PDZ domain enhances the PICK1-ABP/GRIP association. Lysates from 293T cells expressing PICK1 were divided equally to incubate with GST or GST-Br in the absence or presence of the peptide as indicated. Bound proteins were probed with an anti-Flag antibody. Data from three experiments were quantitated by scanning densitometry (n = 3, t test, *p < 0.001). Bar graph shows mean ± SD.

several consequences of disrupting the PICK1-ABP/GRIP interaction. First, interference with the PICK1-ABP/GRIP interaction should impair phosphorylation of GluR2 at S880; second, disruption of the PICK1-ABP/GRIP interaction should impair GluR2 trafficking; third, the PICK1-ABP/GRIP interaction may function in recycling of GluR2; and finally, the PICK1-ABP/GRIP interaction should contribute to the mechanism of LTD. We next tested each of these predictions.

The PICK1-ABP/GRIP Interaction Mediates GluR2 S880 Phosphorylation by PKC

The PICK1-ABP/GRIP interaction may facilitate the GluR2 phosphorylation by PKC by targeting activated PKC to the ABP/GRIP-GluR2 complexes, leading to S880 phosphorylation and AMPAR trafficking. To test a role for the PICK1-ABP/GRIP interaction in GluR2 phosphorylation by PKC in a physiological context, we expressed ECFP-tagged ABP/GRIP Br (ECFP-Br) in neurons and examined its effect on GluR2 phosphorylation by PKC by immunoblotting using a phosphopeptide antiserum specific for GluR2-S880-PO_4. We hypothesized that ECFP-Br would act in a dominant-negative fashion by binding to PKC-PICK1 complexes, preventing PKC-PICK1 from associating with ABP/GRIP and hindering S880 phosphorylation. In HeLa cells, ECFP-Br colocalized with PICK1 or Δ121 in patterns similar to those seen when wt GRIP was cotransfected with PICK1 or Δ121, respectively (Figure 5A). We conclude that the ECFP-Br is capable of interacting with PICK1 (also see Figures 2C and 4D). ECFP-Br was then expressed from high-titer Sindbis virus in cultured

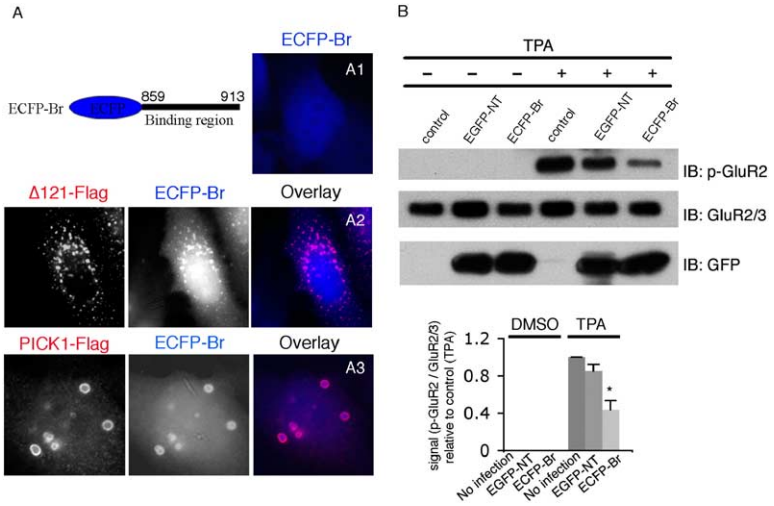


Figure 5. The Br Suppresses S880 Phosphorylation of GluR2

(A) ECFP-Br colocalizes with Δ121 or PICK1 in HeLa cells. When expressed alone, ECFP-Br was diffuse (A1). When coexpressed with Δ121 (A2) or PICK1 (A3), ECFP-Br colocalized with them.

(B) Expression of ECFP-Br suppresses S880 phosphorylation of GluR2 in hippocampal cultures. Neurons expressing ECFP-Br or EGFP-NT or that were uninfected were treated with DMSO or TPA for 15 min. Neurons were then lysed and lysates were subjected to IB. Data from three experiments were quantitated by scanning densitometry (n = 3, t test, *p < 0.001). Bar graph shows mean ± SD.

neurons. In our culture conditions, GluR2-S880-PO_4 was below the detectable level in the absence of treatment with the PKC activator TPA (Figure 5B). Treatment with TPA caused robust phosphorylation of GluR2 S880 (Figure 5B). However, the GluR2-S880-PO_4 level induced by TPA was significantly reduced in neurons expressing ECFP-Br. Expression of a control peptide, a 53 aa sequence from the N terminus of ABP-L that is not required for the ABP interaction with PICK1 (Figure 2), fused to the C terminus of EGFP (EGFP-NT) from high-titer Sindbis virus did not significantly affect the GluR2-S880-PO_4 level induced by TPA. We conclude that the PICK1-ABP/GRIP interaction functions in the GluR2 S880 phosphorylation by PKC.

Interference with the PICK1-GRIP/ABP Interaction Impairs the Surface Expression of GluR2 but Not GluR1

Trafficking of AMPARs to the neuronal plasma membrane is a necessary step to establish chemical communication at glutamatergic synapses. Both PICK1 and ABP/GRIP bind to GluR2 and play roles in AMPAR surface expression (reviewed by Bredt and Nicoll, 2003; Malinow and Malenka, 2002). Therefore, we examined if the PICK1-ABP/GRIP interaction functions in surface expression of AMPAR in cultured hippocampal neurons. To test this, we investigated the effects of expression of two dominant-negative mutants, the Δ121 mutant of PICK1 and the Br of ABP/GRIP, on the AMPAR surface expression in neurons.

We first expressed Δ121-Flag from Sindbis virus in cultured hippocampal neurons. Plasma membrane expression of endogenous GluR1 or GluR2 was quantified by surface labeling of live neurons with antibody recognizing, respectively, the extracellular region of GluR1 or of GluR2. Figure 6A shows three neurons, two expressing Δ121 (green channel) and the other neighboring neuron not expressing this peptide. The surface levels of GluR2 (red channel) were reduced significantly in neurons expressing Δ121 as compared with the level in the neuron that did not express Δ121 (fluorescence of surface GluR2 = 40.7% of that in uninfected cells, n = 45 cells; Figures 6A and 6Ci). Interestingly, expression of Δ121 did not have a significant effect on surface GluR1 levels (fluorescence of surface GluR1 = 98.4% of that in uninfected cells, n = 25 cells; Figures 6B and 6Cii). This indicates that expression of Δ121 in neurons specifically disrupts the surface expression of a population of receptors containing GluR2, but not receptors containing GluR1.

We next used expression of the ABP Br as an alternative approach to interfere with the PICK1-ABP/GRIP interaction. Figure 6D shows two neurons, one expressing ECFP-Br (blue channel) and another neighboring neuron that did not. The surface levels of GluR2 (red channel) were significantly reduced in the neuron expressing ECFP-Br as compared with that in the uninfected neuron (fluorescence of surface GluR2 = 43.1% of that in uninfected cells, n = 59 cells; Figures 6D and 6Fi). We note that expression of ECFP-Br did not have a significant effect on surface GluR2 in a population of infected neurons (11/59, 18.6%). Nevertheless, all cells were included in the quantitation in Figure 6Fi. Similar to Δ121, expression of ECFP-Br failed to have a significant effect on surface GluR1 levels (fluorescence of surface GluR1 = 99% of that in uninfected cells, n = 28 cells; Figures 6E and 6Fii). Neither surface GluR1 nor surface GluR2 expression was affected by the EGFP-NT control peptide (images not shown, data quantitated in Figure 6G), further indicating the specificity of the effect of ECFP-Br. The effects of expression of the two dominant-negative constructs on surface GluR2 expression are unlikely to be due to a global reduction of GluR2 levels, as expression of either Δ121 or ECFP-Br did not significantly decrease expression of total GluR2 (Figures 6H–6N). These data suggest that PICK1 binding to ABP/GRIP via BAR domain-Br interaction is required for surface targeting of a population of receptors containing GluR2, but not for receptors containing GluR1.

The PICK1-ABP/GRIP Interaction Functions in Recycling of GluR2

What is the mechanism underlying the reduced levels of surface GluR2 in neurons expressing the dominant-negative mutants? GluR2 continually undergoes endo-

cytosis, and recycling of internalized GluR2 back to the plasma membrane is important in maintaining the basal level of synaptic transmission (Daw et al., 2000; Ehlers, 2000; Hirbec et al., 2003). Therefore, we asked if interference with the PICK1-ABP/GRIP interaction impaired GluR2 recycling and thus reduced surface GluR2 levels. We adopted a protocol previously employed to study AMPAR recycling in cultured neurons (Passafaro et al., 2001). Live hippocampal cultures were first surface labeled with a GluR2 antibody and then briefly treated with NMDA (20 μM for 4 min) to induce endocytosis of receptor-antibody complexes. Remaining surface antibodies were stripped away, and neurons were then returned to culture media at 37°C for 45 min for receptor recycling. After recycling, neurons were fixed and stained with a secondary antibody (red channel) to detect GluR2-antibody complexes that had recycled back to surface. Neurons were then permeabilized to detect GluR2-antibody complexes that remained in internal compartments (blue channel). As shown in Figures 7A–7D, recycling of GluR2 back to surface was diminished significantly in neurons expressing ECFP-Br as compared to uninfected neurons or neurons expressing the control peptide, EGFP-NT. This finding suggests that the PICK-ABP/GRIP interaction is required for recycling of receptors back to the plasma membrane. This also suggests that the reduced levels of surface GluR2 observed after expression of the dominant-negative mutant (Figures 6A–6G) result from the impaired GluR2 recycling caused by interference with the PICK1-ABP/GRIP interaction.

The PICK1-ABP/GRIP Interaction Is Involved in NMDA-Induced Endocytosis of GluR2

We finally tested the involvement of the PICK1-ABP/GRIP interaction in activity-dependent regulation of AMPAR trafficking. We employed a chemical LTD (chem-LTD) protocol to analyze GluR2 trafficking in neurons expressing the dominant-negative mutant, ECFP-Br, or a control peptide, EGFP-NT. The chem-LTD protocol has been shown to share an expression mechanism with canonic LTD that is induced by low-frequency electrical stimulation (Lee et al., 1998). Live hippocampal cultures were first surface labeled with a GluR2 antibody and then briefly treated with NMDA to induce endocytosis of receptor-antibody complexes. GluR2 antibodies remaining on the cell surface and internalized GluR2 antibodies were then sequentially detected by immunofluorescence. ECFP-Br overexpression diminished significantly the NMDA-induced endocytosis in infected neurons as compared to uninfected neurons and neurons infected with EGFP-NT (Figures 7E–7H). This suggests that the PICK1-ABP/GRIP interaction is involved in activity-dependent modification of receptor abundance at the cell surface during LTD. Therefore, we conclude that the PICK1-GRIP/ABP interaction contributes to the mechanisms of GluR2 S880 phosphorylation by PKC, GluR2 surface expression, recycling, and NMDA-induced endocytosis.

Discussion

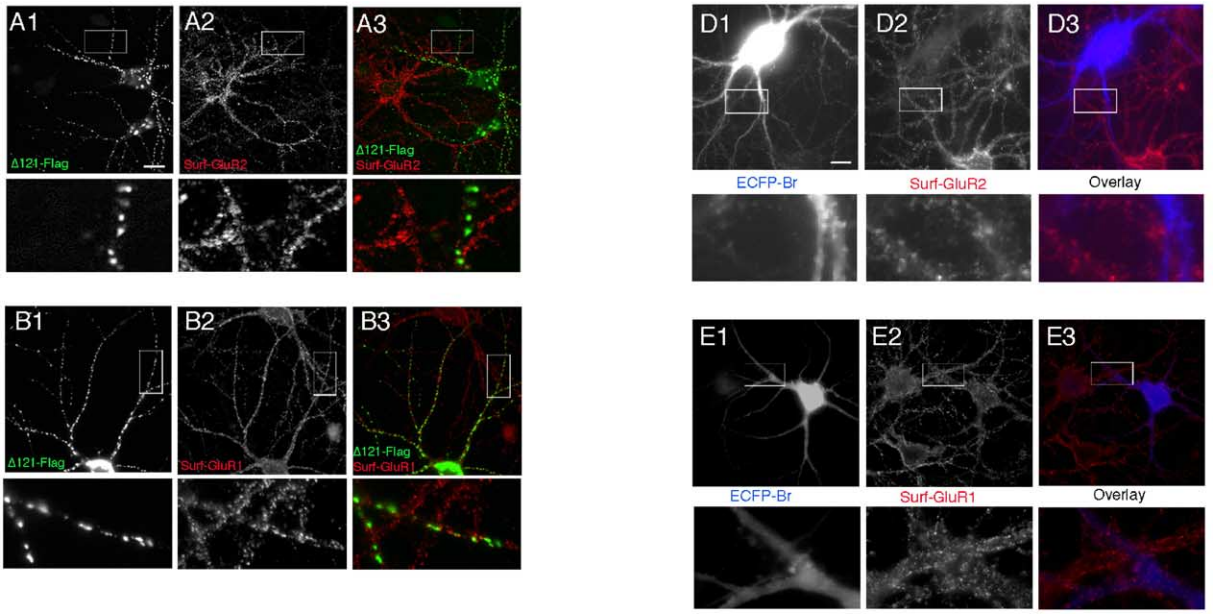
Intramolecular Interaction of PICK1

PICK1 has been suggested to be involved in both AMPAR endocytosis (Chung et al., 2000, 2003; Hanley et al., 2002; Kim et al., 2001; Perez et al., 2001; Seidenman et al., 2003) and exocytosis (Gardner et al., 2005; Greger et al., 2002; Liu and Cull-Candy, 2005; Figures 7A–7D). Although it appears paradoxical that PICK1 could function in both inward and outward trafficking of AMPAR, a general role for PICK1 in trafficking is suggested by the finding that PICK1 dimers contain a BAR domain (Figure 1I), which is a sensor for lipid membrane curvature (Peter et al., 2004). This suggests that PICK1 may direct AMPARs to endocytic or exocytic buds through BAR domain binding to curved bud membranes. In this case, the primary function of PICK1 may be to prime AMPARs for trafficking, rather than to determine the AMPAR trafficking direction. PICK1 may also coordinate AMPAR endocytosis with the activities of proteins that function in bud formation, including dynamin and clathrin, possibly by concentrating AMPAR in regions of curved membranes to enable dynamin and clathrin to execute the formation of an endocytic vesicle (Carroll et al., 1999; Man et al., 2000; Wang and Linden, 2000). During exocytosis, PICK1 may provide an analogous function by accumulating receptors in specific internal membrane domains from which PICK1, together with the small GTPases, the Rab proteins (Park et al., 2004), may direct receptor recycling back to the plasma membrane. In both heterologous cells and cultured neurons, PICK1 that is not bound to a receptor is diffusely distributed in the cytoplasm, but upon binding to its PDZ ligands, such as GluR2 and PKCα, PICK1 is rapidly transported, possibly via interactions with curved membranes, to vesicular clusters (Perez et al., 2001; Xia et al., 1999). It is now evident that this rapid redistribution is coupled to a PICK1 intramolecular rearrangement induced by ligand binding. We show here that the PICK1 PDZ domain directly interacts with its BAR domain, preventing the BAR domain from binding to curved membranes. Binding a ligand to the PDZ domain of PICK1 disrupts this intramolecular interaction and exposes the BAR domain. In addition, disruption of the PICK1 PDZ-BAR domain interaction can facilitate BAR domain interaction with other molecules, such as ABP/GRIP. Thus, the PDZ-BAR interaction serves as an important regulator of PICK1 function by controlling the availability of the PICK1 BAR domain for membranes or interacting proteins.

The PICK1-ABP/GRIP Interaction

ABP/GRIP has been suggested to form a supramolecular complex associated with AMPARs, which anchors receptors to membranes both at synapses and at intracellular locations (Braithwaite et al., 2002; Daw et al., 2000; DeSouza et al., 2002; Dong et al., 1997; Fu et al., 2003; Osten et al., 2000; Srivastava et al., 1998). During receptor trafficking, the PICK1-ABP/GRIP interaction may target PICK1-PKCα complexes to ABP/GRIP-AMPAR complexes, leading to phosphorylation of GluR2 at S880 and receptor trafficking (Figure 8). The PICK1-ABP/GRIP interaction may also function in targeting vesicles containing AMPARs bound to PICK1 to the anchoring proteins, ABP/GRIP, leading to receptor anchorage.

LII of GRIP, which contains the binding site for the PICK1 BAR domain, also interacts with kinesins (isoforms 5A-C) (Setou et al., 2002), which are molecular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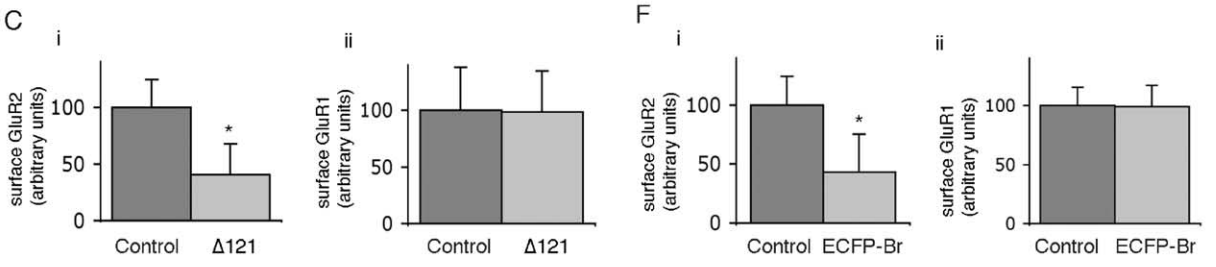
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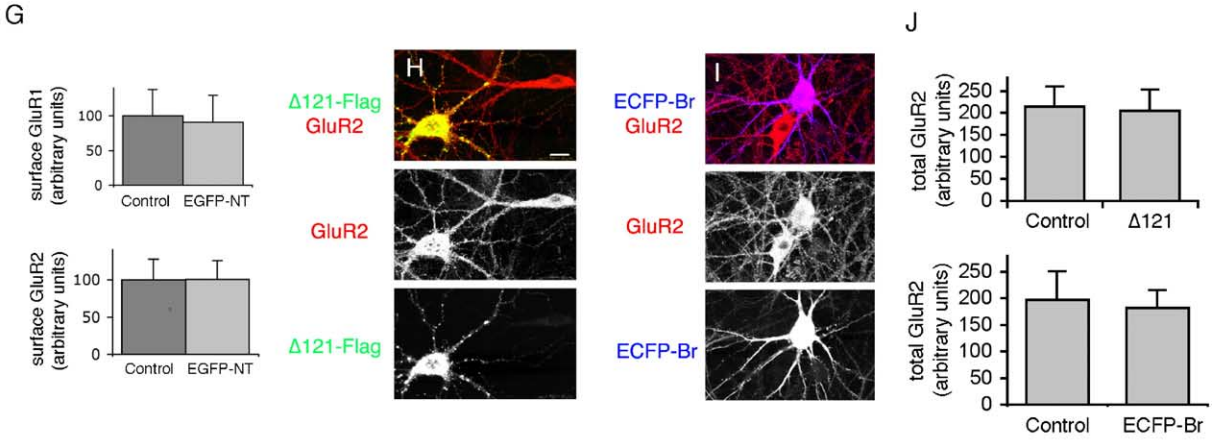
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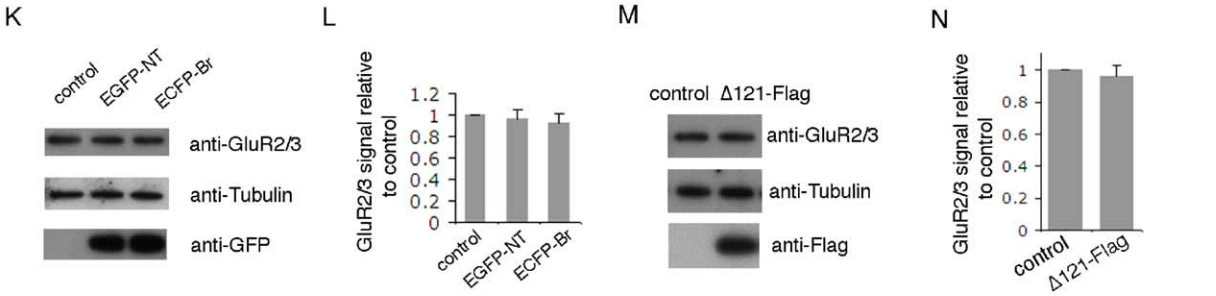
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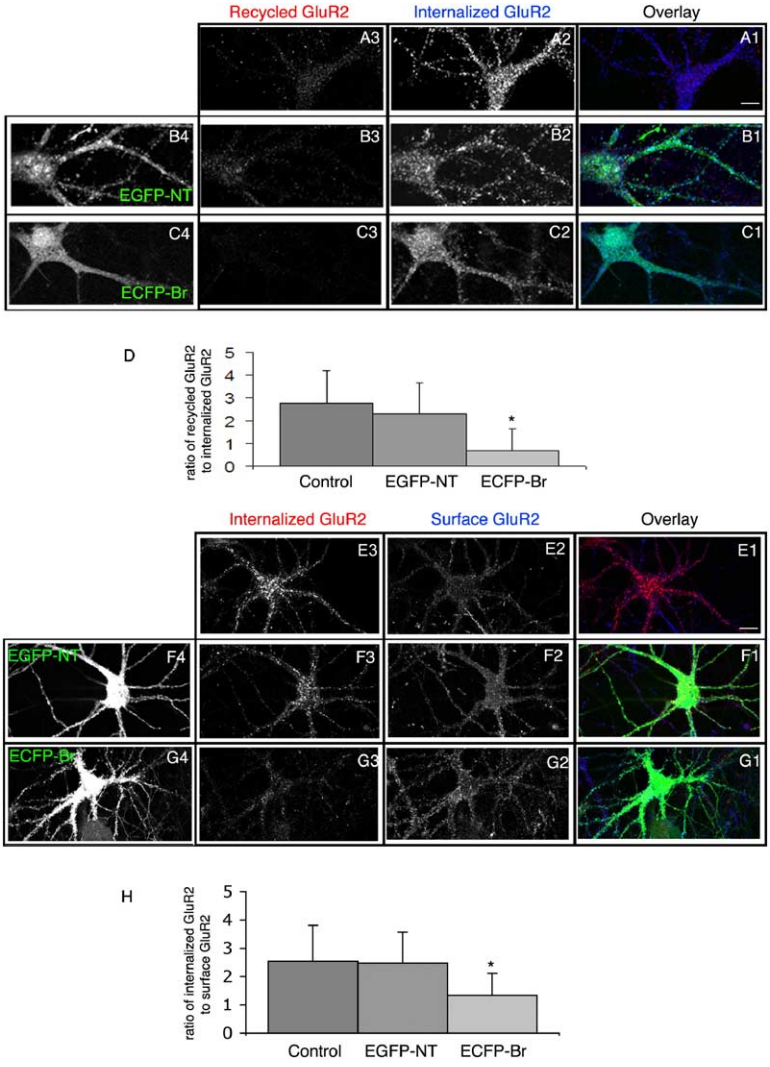

Figure 7. The PICK1-ABP/GRIP Interaction Functions in GluR2 Recycling and the NMDA-Induced Endocytosis of GluR2

(A–D) Expression of ECFP-Br, but not EGFP-NT, impaired GluR2 recycling. Neurons uninfected (A1–A3) or infected with Sindbis virus expressing EGFP-NT (B1–B4) or ECFP-Br (C1–C4) were stained live with an anti-GluR2 antibody, followed by treatment of NMDA. Neurons were then returned back to growth media for 45 min. Recycled and internalized GluR2 were sequentially labeled with Rhodamine (under unpermeabilized condition) and Cy5 (under permeabilized condition) conjugated secondary antibodies, respectively. Scale bar, 20 μm. (D) Quantitation of the ratio of recycled to internalized GluR2 in neurons expressing EGFP-NT (B1–B4) (n = 14, t test, $p > 0.05$) or ECFP-Br (C1–C4) (n = 16, t test, $^*p < 0.001$), respectively.

(E–H) Expression of ECFP-Br, but not EGFP-NT, impaired NMDA-induced endocytosis of GluR2. Neurons uninfected (E1–E3) or infected with Sindbis virus expressing EGFP-NT (F1–F4) or ECFP-Br (G1–G4) were stained live with an anti-GluR2 antibody, followed by treatment of NMDA. Surface and internalized GluR2 were sequentially labeled with Cy5 (under unpermeabilized condition) and Rhodamine (under permeabilized condition) conjugated secondary antibodies, respectively. Scale bar, 20 μm. (H) Quantitation of the ratio of internalized to surface GluR2 in neurons expressing EGFP-NT (F1–F4) (n = 13, t test, $p > 0.05$) or ECFP-Br (G1–G4) (n = 23, t test, $^*p < 0.001$), respectively.

Bar graphs in (D) and (H) show mean ± SD.

motors that govern the dendritic trafficking of the GRIP-AMPAR complex. In our preliminary experiments, a GST fusion to the Br of ABP that binds to PICK1 did not pull down kinesin5A from cultured hippocampal neurons (data not shown). Either another sequence in LII binds to kinesin5A or the Br is not sufficient for kinesin5A binding. We note that the C termini of several membrane proteins including metabotropic glutamate

Figure 6. Interference with the PICK1-ABP/GRIP Interaction Impairs the Surface Expression of Endogenous GluR2 but Not GluR1

(A and B) Neurons expressing Δ121-Flag were stained live with an anti-GluR2 (A) or an anti-GluR1 (B) antibody to detect surface GluR2 or GluR1, respectively. Neurons were then permeabilized to detect Δ121-Flag. Scale bar, 20 μm. White boxes in each triplet of upper panels define enlargements shown in lower panels.

(C) Quantitation of surface levels of GluR2 (Ci) or GluR1 (Cii) on neurons shown in (A) or (B) (n = 45 for GluR2, n = 22 for GluR1, t test, $^*p < 0.001$).

(D and E) Neurons expressing ECFP-Br were stained live with an anti-GluR2 (D1–D3) or an anti-GluR1 (E1–E3) antibody to detect surface GluR2 or GluR1. Scale bar, 20 μm. White boxes in each triplet of upper panels define enlargements shown in lower panels.

(F) Quantitation of surface levels of GluR2 (Fi) or GluR1 (Fii) shown in (D) or (E) (n = 59 for GluR2, n = 27 for GluR1, t test, $^*p < 0.001$).

(G) Quantitation of surface levels of GluR2 or GluR1 in neurons expressing EGFP-NT (images now shown) (n = 27 for GluR1, n = 32 for GluR2, t test).

(H–N) Total GluR2 level was not significantly affected in neurons expressing the dominant-negative constructs. Neurons expressing Δ121 (H) or ECFP-Br (I) were stained with indicated antibodies. Quantitation of total GluR2 in neurons expressing Δ121 (n = 10) or ECFP-Br (n = 10) (J). Scale bar, 20 μm. Neurons uninfected or infected with high-titer Sindbis virus expressing ECFP-Br (K) or EGFP-NT (K) or Δ121 (M) were lysed with 1% Triton X-100 buffer, and lysates were subjected to IB with indicated antibodies. Data from three repeats were quantitated by scanning densitometry, respectively (n = 3, t test, $p > 0.05$) (L and N).

Bar graphs in (C), (F), (G), and (H)–(N) show mean ± SD.

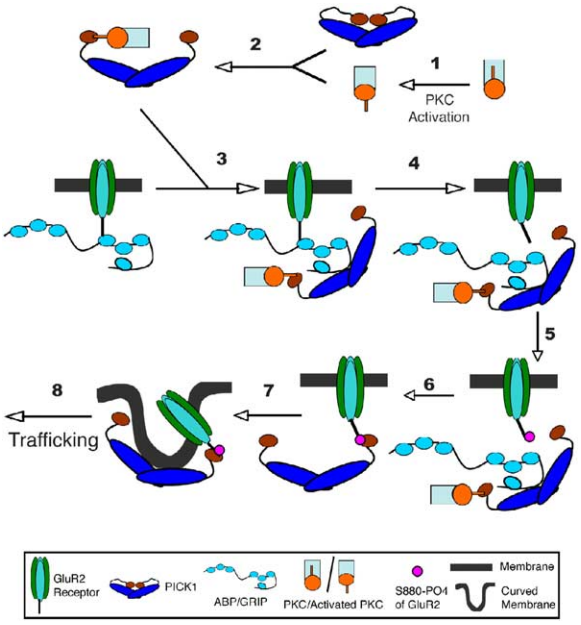

Figure 8. Model for the Role of the PICK1-ABP/GRIP Interaction in AMPAR Trafficking

GluR2 receptors are anchored at synaptic and intracellular membranes by ABP/GRIP but undergo cycling between these membranes in association with PICK1. Trafficking of GluR2 receptors from ABP/GRIP anchorage requires the PICK1-ABP/GRIP interaction. Activation of PKCα (1) causes PKCα to bind to the PICK1 PDZ domain, which disrupts the PICK1 PDZ-BAR domain interaction and leads to the exposure of the PICK1 BAR domain (2). The PICK1-PKCα complex is targeted to the ABP/GRIP-GluR2 complex through the interaction of the exposed BAR domain with the Br sequence of ABP/GRIP (3). PICK1 competes with ABP/GRIP for the GluR2 interaction (4). PKCα phosphorylates S880 of GluR2. GluR2 phosphorylated at S880 cannot bind back to ABP/GRIP (5) but is able to bind to PICK1 (6). The PICK1 BAR domain directs the PICK1-GluR2 complex to curved membranes (7), where GluR2 receptors bud from the plasma membrane and internalize or bud from an internal membrane prior to reinsertion into synapses (8).

receptors (Dev et al., 2000; Hirbec et al., 2002) and Ephrin and Eph receptors (Torres et al., 1998) bind to PDZ domains of both PICK1 and ABP/GRIP. Thus, the regulation of GluR2 trafficking coordinated by the PICK1-ABP/GRIP interaction described here may also apply to these membrane proteins.

ABP has two major alternative splicing isoforms: a short form (ABP-S) and a long form, containing, respectively, six and seven PDZ domains (DeSouza et al., 2002). Although both forms bind to GluR2, only the long form has the PICK1 binding site (Figures 2C and 2D). Thus, differential ABP splicing may regulate the interaction of ABP with PICK1. In rat brain, ABP-S is expressed at low levels prior to postnatal day 9, whereupon its expression increases (Srivastava et al., 1998). Thus, during development, a population of AMPAR may be formed that is anchored by ABP-S but is not sensitive to PICK1 regulation. No short form counterpart of GRIP has yet been described.

Implications for AMPAR Trafficking

How does the PICK1-ABP/GRIP interaction function in AMPAR trafficking? Dynamic regulation of the GluR2 interaction with ABP/GRIP or PICK1 is critical for both constitutive and regulated trafficking of AMPA receptors (reviewed by Bredt and Nicoll, 2003; Malinow and Malenka, 2002). We conclude that the PICK1-ABP/GRIP interaction plays a role in phosphorylation of GluR2 at S880 by PKC, since impairment of the PICK1-ABP/GRIP interaction decreases PKC phosphorylation of GluR2 S880. The PICK1-ABP/GRIP interaction may dock the PICK1-PKC complex at the GluR2-ABP/GRIP complex facilitating GluR2 phosphorylation. Since activation of PKC is involved in both inward and outward trafficking of AMPAR (Chung et al., 2000; Daw et al., 2000; Hirbec et al., 2003; Perez et al., 2001; also see Figure S1 in the Supplemental Data available online), the PICK1-ABP/GRIP interaction may contribute to AMPAR trafficking. Supporting this notion, we find that expression of dominant-negative mutants in hippocampal cultures reduces surface levels of endogenous GluR2. Thus, the PICK1-ABP/GRIP interaction is likely to regulate GluR2 surface expression by facilitating GluR2 phosphorylation at S880 by PKC.

How does impairment of the PICK1-ABP/GRIP interaction reduce surface GluR2? Under basal conditions, GluR2 receptors continually cycle between membranes at synaptic and intracellular sites (reviewed by Bredt and Nicoll, 2003; Malinow and Malenka, 2002). Recycling of receptors back to the plasma membrane is required for maintenance of basal synaptic transmission and de-depression (Daw et al., 2000; Ehlers, 2000; Hirbec et al., 2003; Park et al., 2004). Interference with the PICK1-ABP/GRIP interaction impaired receptor recycling and reduced surface levels of GluR2. This indicates that the PICK1-ABP/GRIP interaction is required for recycling of internalized receptors back to the plasma membrane. In promoting recycling, the PICK1-ABP/GRIP interaction may target PKC to internalized GluR2 in a complex with internal ABP/GRIP, leading to S880 phosphorylation of GluR2 by PKC, which in turn facilitates the dissociation of GluR2 from internal ABP/GRIP anchorage and makes possible subsequent recycling back to the plasma membrane. This interpretation is consistent with the findings by others that PKC activation and dissociation of GluR2 from internal ABP/GRIP anchorage are required for GluR2 recycling back to synapses during de-depression (Daw et al., 2000; Hirbec et al., 2003). Our interpretation is also consistent with a previous report that most basal S880-PO_4 GluR2 is localized in internal pools and is largely excluded from synapses in cultured hippocampal neurons (Chung et al., 2000). Of note, two recent papers also demonstrate the involvement of PICK1 in exocytosis of GluR2-containing receptors into the plasma membrane in cerebellar stellate cells (Gardner et al., 2005; Liu and Cull-Candy, 2005), suggesting a function of PICK1 in receptor exocytosis in both hippocampus and cerebellum (Gardner et al., 2005; Greger et al., 2002; Liu and Cull-Candy, 2005; Figures 7A–7D).

As both PICK1 and ABP/GRIP play roles in LTD induction (Chung et al., 2003; Kim et al., 2001; Matsuda et al., 2000; Seidenman et al., 2003; Xia et al., 2000), it is not surprising that the PICK1-ABP/GRIP interaction is also involved in LTD. We have shown that interference with the PICK1-ABP/GRIP interaction impairs chem-LTD in cultured hippocampal neurons. Thus, interference with the PICK1-ABP/GRIP interaction not

only decreases the surface levels of GluR2 under basal conditions, it also impairs NMDA-induced endocytosis of GluR2 that remains at the plasma membrane. Therefore, the PICK1-ABP/GRIP interaction may function in different pathways under different conditions. In the absence of plasticity-inducing stimuli, the PICK1-ABP/GRIP interaction may primarily facilitate recycling of internalized receptors back to the plasma membrane. This is consistent GluR2-S880-PO_4 presence primarily in internal compartments under basal conditions and absence from synaptic sites (Chung et al., 2000). During hippocampal LTD, phosphorylation of synaptic GluR2 at S880 enhances receptor endocytosis (Kim et al., 2001; Seidenman et al., 2003), which is in agreement with our finding that the PICK1-ABP/GRIP interaction is involved in the induction of LTD (Figures 7E–7H). Following PKCα activation by synaptic activity and the subsequent binding of activated PKCα to PICK1, the PICK1-PKCα complex may be targeted to synaptic ABP/GRIP-GluR2 complexes to enable PKCα to phosphorylate GluR2 at S880, thereby promoting the dissociation of receptors from the anchorage protein, ABP/GRIP.

We have reported that binding of ABP/GRIP to GluR2 suppresses phosphorylation of GluR2 at S880 by PKC (Fu et al., 2003). Thus, GluR2 release from ABP/GRIP may be a prerequisite for efficient GluR2 phosphorylation by PKC (Fu et al., 2003). The mechanism of dissociation of ABP/GRIP from GluR2 is not known. Binding of PICK1 to ABP/GRIP-GluR2 may simply position PICK1 near the GluR2-ABP/GRIP complex, enabling PICK1 to compete efficiently with ABP/GRIP for binding to GluR2. During the transfer of GluR2 from ABP/GRIP to PICK1, GluR2 could be efficiently phosphorylated by PKC. Alternatively, a not yet determined mechanism could actively disassemble the ABP/GRIP-GluR2 complex, exposing the GluR2 C terminus to PKCα that is docked in a complex with PICK1 at GRIP/ABP linker II.

What is the function of the PICK1-ABP/GRIP interaction in the trafficking of the AMPAR GluR3 subunit? GluR3 mainly forms heteromers with GluR2 in hippocampus (Wenthold et al., 1996), and GluR3 is capable of binding to both PICK1 and ABP/GRIP. Thus, the mechanism described here for regulating GluR2 trafficking may also apply to GluR3. Indeed, a GluR2/3 heteromer, in which both subunits are capable of binding to both PICK1 and ABP/GRIP, might represent an "ideal substrate" for the regulation by the PICK1-ABP/GRIP interaction. Interestingly, our dominant-negative mutants lack an effect on surface GluR1 levels. GluR1 trafficking is regulated differently from GluR2 and appears to dominate over GluR2 in determining receptor synaptic insertion (Passafaro et al., 2001; Shi et al., 2001), while GluR2 dominates over GluR1 for endocytosis (Lee et al., 2004). It remains to be determined why impairment of the PICK1-ABP/GRIP interaction does not affect surface levels of GluR1. The exocytotic reinsertion of internalized GluR1/2 heteromers back to the plasma membrane may not require the PICK1-ABP/GRIP interaction, if GluR1 dominates over GluR2 in exocytotic processes. Alternatively, interactions of GluR1 with other proteins, such as SAP97, may dominate in trafficking of GluR1/2 heteromers (for review, see Bredt and Nicoll, 2003).

Blocking the interactions of GluR2 with other binding partners, such as NSF (Noel et al., 1999) or stargazin (Chen et al., 2000), or the GRIP interaction with liprin-α (Wyszynski et al., 2002) impairs surface expression of both GluR1 and GluR2, indicating that these interactions function in AMPAR trafficking without restriction to GluR2. In contrast, interference with the PICK1-ABP/GRIP interaction in the current work impaired surface levels of GluR2, but not GluR1, indicating that the PICK1-GRIP/ABP interaction regulates surface expression of receptors that lack GluR1. Notably, overexpression of PICK1 reduced levels of surface GluR2, but not GluR1, in hippocampal neurons (Terashima et al., 2004). Our study thus suggests a specialized pathway that is dedicated to the regulation of GluR2 trafficking. The PICK1-ABP/GRIP interaction may enable GluR2 receptors to cycle constitutively in hippocampal neurons in a rapid time scale.

Experimental Procedures

Expression Vectors

Plasmids and viral vectors expressing PICK1, GluR2, ABP/GRIP, and GST-BAR and His6-135Δ have been described before (DeSouza et al., 2002; Hanley et al., 2002; Osten et al., 2000; Perez et al., 2001). GST-ABP/GRIP mutants (a gift from S. DeSouza) were cloned by PCR and ligated into pGEX-4T1 (Pharmacia). The Br of ABP (aa 859–913) was cloned by PCR and ligated into pECFP-C1 (Clontech). ECFP-Br was PCR amplified and subcloned into pSinRep5 (Invitrogen). The PICK1 BAR domain point mutant was generated by the QuickChange mutagenesis kit (Stratagene).

Antibodies

The following antibodies were used: anti-Myc, His6, GST, tubulin, and PICK1 N18 antibodies (Santa Cruz); anti-Flag antibodies (Sigma); anti-GRIP and anti-PSD-95 antibodies (Upstate); mouse anti-GluR2 and rabbit anti-GluR2/3 (Chemicon) and rabbit anti-GluR1 (Oncogene) antibodies; anti-S880-PO_4 GluR2 antibody (Perez et al., 2001); anti-PICK1 antibody (a generous gift from Richard L. Huganir) and anti-GFP antibody (Fu et al., 2003).

Cell Culture and Immunocytochemistry

HeLa cell transfection and immunostaining were described before (DeSouza et al., 2002), as were hippocampal primary neuron culture, Sindbis virus preparation, neuronal infection (at DIV 17–22) (Osten et al., 2000), and fixed staining of recombinant proteins (Osten et al., 2000). For live staining, neurons were incubated with an anti-GluR2 N terminus antibody (10 μg/ml) or an anti-GluR1 N terminus antibody (10 μg/ml) for 15 min at 37°C. Neurons were fixed with 4% paraformaldehyde (PFA)/4% sucrose for 10 min at room temperature (RT) and stained with secondary antibody under unpermeabilized condition. For chem-LTD assay, after live staining for surface GluR2, neurons were washed and incubated with NMDA (100 μM) for 8 min at 37°C. 100 μM NMDA was employed to achieve maximal endocytosis of surface GluR2, which facilitates identification of differential effects of our dominant-negative mutant from control on GluR2 trafficking. Surface antibodies were detected under unpermeabilized condition, and neurons were then permeabilized to detect internalized receptor-antibody complexes. The recycling assay protocol was adopted from a previous study with modifications (Passafaro et al., 2001). For recycling assay, after live staining for surface GluR2, neurons were washed and incubated with NMDA (20 μM) for 4 min at 37°C. Surface remaining antibodies were then stripped away by stripping buffer (0.5 M NaCl and 0.2 M acetic acid) on ice for 4 min (Carroll et al., 1999; Passafaro et al., 2001). Neurons were then washed extensively with ice-cold TBS (Tris-buffered saline) and returned back to growth media at 37°C for 45 min for recycling. After recycling, neurons were fixed, and GluR2-antibody complexes recycling back to the surface were detected with secondary antibody. Neurons were then permeabilized, and internal GluR2-antibody complexes were detected with another secondary antibody. In the recycling assay, we employed

a low concentration of NMDA for a shorter time to induce GluR2 endocytosis, which maintained neuron viability during the assay.

Image Analysis and Quantitation

Images were acquired on a Nikon PCM 2000 confocal or Zeiss Axovert 200 fluorescence microscope and analyzed with Simple32 Imaging software. To analyze GluR1 and GluR2 surface fluorescence, 20 μm of dendrites from infected and uninfected cells were traced and surface fluorescence was analyzed with Simple32 Imaging software. To analyze endocytosis of surface GluR2 or recycling of internalized GluR2 after NMDA treatment, the ratio of the internalized to surface GluR2 or the recycled to internalized GluR2 fluorescence from 20 μm of dendrites from infected and uninfected neurons was calculated. Each experiment was performed at least three times. Error bars are standard errors, and significance was determined by the t test.

293T Cell Culture and GST Pulldown

Plasmids were transfected into 293T cells with effectene reagent (Qiagen) following the manufacturer's instructions. At 48 hr after transfection, cells were lysed with 1% Triton X-100 buffer, centrifuged, and the supernatants were used in GST pulldown and IP experiments.

GST-fusions were purified as described (Hanley et al., 2002). Lysate from cells transfected with Δ121-Myc was divided equally to bind to ABP or GRIP GST fusions immobilized on glutathione-agarose beads (GAB) for 2 hr. Bound proteins were eluted and detected by IB. For GST-GluR2C binding assay, GST fusion proteins were immobilized on GAB, and beads were then incubated with lysate of cells transfected with corresponding constructs indicated in Figure 4B. Bound proteins were eluted and detected by IB. For the peptide-enhanced interaction between ABP/GRIP and PICK1, PKCα C terminus was added into lysate of cells expressing PICK1, which was incubated with GST-Br immobilized on GAB for 30 min. Bound proteins were eluted and detected by IB. Films of Western blots from three experiments were analyzed with NIH Image 1.62. t test was carried out to determine the significance.

Immunoprecipitation and Immunoblotting

293T cell lysates were incubated with 1 μg M2 anti-Flag antibody to precipitate Flag-tagged species for 2 hr. Proteins from IPs were eluted and detected by IB. For peptide-blocking assays, peptides were solubilized in DMSO and incubated with lysates of cells expressing Δ121 and 135Δ for 30 min at RT (final concentration of peptide: 10 mM). IP and IB assays were then performed. For IP assay of endogenous proteins, rat brain cortex was lysed in modified RIPA buffer (1% NP-40 plus 0.25% deoxycholate and 0.05% SDS). Extract was clarified, and supernatant (3–4 μg/μl) was used for IP. Antibody was added for 4–6 hr, followed by protein A agarose for 2 hr. Beads were then washed once with lysis buffer, twice with lysis buffer plus 0.5 M NaCl, and twice with lysis buffer. Bound proteins were eluted and detected by IB.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/47/3/407/DC1/.

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