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Clustering of AMPA Receptors by the Synaptic PDZ Domain–Containing Protein PICK1

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Summary

Synaptic clustering of neurotransmitter receptors is crucial for efficient signal transduction and integration in neurons. PDZ domain–containing proteins such as PSD-95/SAP90 interact with the intracellular C termini of a variety of receptors and are thought to be important in the targeting and anchoring of receptors to specific synapses. Here, we show that PICK1 (protein interacting with C kinase), a PDZ domain–containing protein, interacts with the C termini of α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA) receptors in vitro and in vivo. In neurons, PICK1 specifically colocalizes with AMPA receptors at excitatory synapses. Furthermore, PICK1 induces clustering of AMPA receptors in heterologous expression systems. These results suggest that PICK1 may play an important role in the modulation of synaptic transmission by regulating the synaptic targeting of AMPA receptors.

Introduction

Postsynaptic membranes in the nervous system are highly organized structures enriched in neurotransmitter receptors. The aggregation and immobilization of neurotransmitter receptors at synapses in the central and peripheral nervous systems appears to be due to the direct interaction of these receptors with intracellular anchoring proteins (Sheng and Kim, 1996; Kornau et al., 1997; O'Brien et al., 1998). Glutamate is the major excitatory neurotransmitter in the central nervous system, and the synaptic clustering of glutamate receptors is critical for efficient excitatory synaptic transmission. Glutamate receptors can be divided into three different subclasses, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA), kainate, and N-methyl-D-aspartate (NMDA) receptors, based on their physiological and pharmacological properties (Hollmann and Heinemann, 1994). AMPA receptors mediate rapid synaptic transmission, while NMDA receptors are important in activity-dependent plasticity and excitotoxicity in the nervous system. These receptors are heteromeric complexes of homologous subunits that differentially combine to form a variety of receptor subtypes.

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Recent studies have revealed that the synaptic protein PSD-95/SAP90 and its family members, SAP102 and PSD-93/Chapsyn-110, physically associate with NMDA receptors and may be involved in the synaptic localization of NMDA receptors (Sheng and Kim, 1996; Kornau et al., 1997; O'Brien et al., 1998). This interaction is mediated by the direct binding of the C termini of the NMDA receptor NR2 subunits to the PDZ (PSD-95, DLG, ZO-1) domains of PSD-95/SAP90 (Kornau et al., 1995). PDZ domains are motifs of ~90 amino acids that have recently been recognized to mediate protein–protein interactions. PSD-95/SAP90 family members colocalize with NMDA receptors at excitatory synapses and induce NMDA receptor clustering when coexpressed in heterologous expression systems (Kim et al., 1996; Kornau et al., 1997; O'Brien et al., 1998). Recent genetic studies in *Drosophila* have shown that the PSD-95-related protein Discs large (DLG) is essential for the synaptic clustering of Shaker-type K^+ channels (Tejedor et al., 1997) and the cell adhesion molecule Fasciclin II (Thomas et al., 1997; Zito et al., 1997) at the neuromuscular junction (NMJ). The interaction of the C termini of proteins with PDZ domains seems to be a generalized mechanism for the subcellular localization of proteins to cell–cell contacts in neurons as well as in many other cell types. For example, proteins like p55, LIN-7, and InaD all contain PDZ domains and associate with the target proteins, glycophorin C, the LET-23 receptor tyrosine kinase, and TRP Ca^{2+} channels, respectively, via their C termini (Saras and Heldin, 1996).

The C termini of the GluR2 and GluR3 subunits of AMPA receptors have recently been shown to interact with a novel PDZ domain–containing protein called GRIP (glutamate receptor–interacting protein) (Dong et al., 1997). GRIP contains seven PDZ domains and appears to serve as an adapter protein to cross-link AMPA receptors and/or to link AMPA receptors to other neuronal proteins. GRIP is selectively expressed in neurons and is colocalized at excitatory synapses with AMPA receptors. However, GRIP does not induce AMPA receptor clustering when they are coexpressed in heterologous expression systems, indicating that other proteins may be required for AMPA receptor synaptic clustering. Recent studies on glutamate receptors in *C. elegans* have indicated that complexes of PDZ-containing proteins may be required for the appropriate synaptic targeting of AMPA receptors (Rongo et al., 1998). Here, we report that the C termini of the GluR2 and GluR3 subunits interact with the PDZ domain–containing protein PICK1 (protein interacting with C kinase). PICK1 was originally isolated due to its interaction with protein kinase C α (PKCα) (Staudinger et al., 1995, 1997). PICK1 is colocalized with AMPA receptors at excitatory synapses and is associated with AMPA receptors in the brain. Moreover, PICK1 induces the clustering of AMPA receptors in heterologous expression systems. These results indicate that PICK1 and GRIP (Dong et al., 1997) as well as other proteins that interact with the C termini of AMPA receptors, such as the N-ethylmaleimide-sensitive factor (NSF) (Nishimune et al., 1998; Osten et al., 1998; Song et al.,

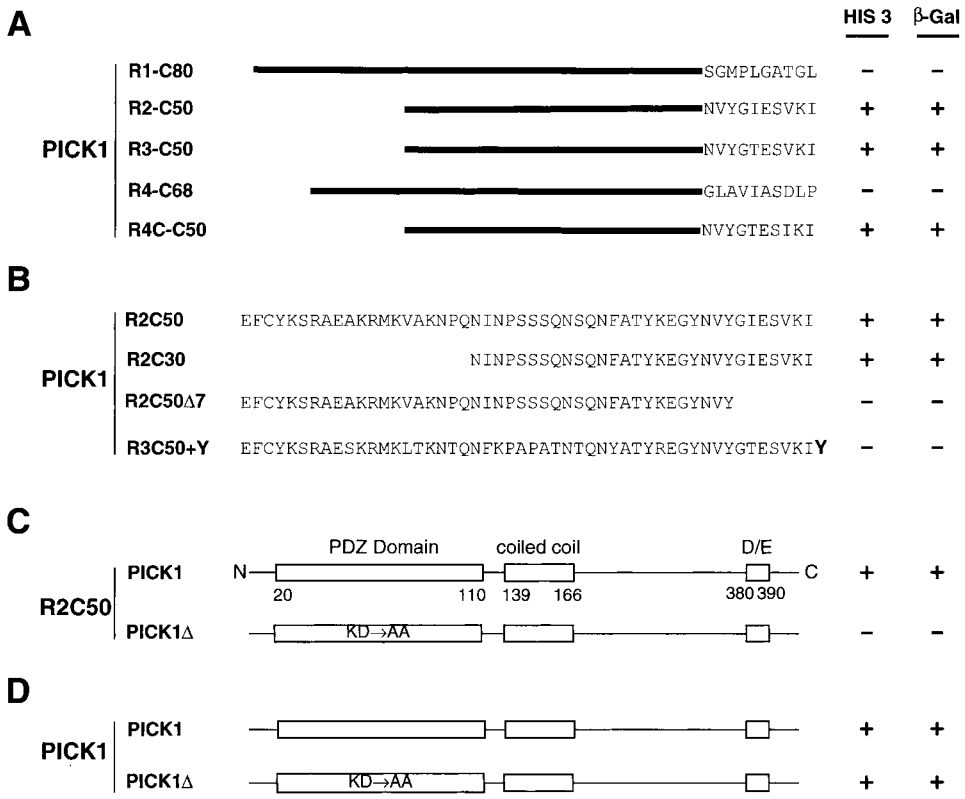

Figure 1. Interaction of AMPA Receptors with PICK1 in the Yeast Two-Hybrid System

(A) PICK1 interacts with the intracellular C-terminal domains of GluR2, GluR3, and GluR4c but not GluR1 and GluR4. The bars represent the approximate length of the intracellular domains, and the sequence of the last ten amino acids is shown. The results from growth assay and β-galactosidase activity are indicated.

(B) Interactions of PICK1 with the AMPA receptor GluR2 and GluR3 C-terminal mutants indicate that the C-terminal amino acids in GluR2 and GluR3 are important.

(C) The PDZ domain of PICK1 is required for the interaction with GluR2, since the GluR2 C-terminal tail does not interact with the PICK1 mutant (*PICK1*Δ) in which K27 and D28, the critical amino acids in the PDZ domain, were mutated to AA.

(D) Both the wild-type PICK1 and the PICK1 (K27D28→AA) PDZ domain mutant are capable of self-association.

1998), may cooperate to regulate the targeting and clustering of AMPA receptors at excitatory synapses.

Results

Interaction of PICK1 with AMPA Receptors in the Yeast Two-Hybrid System

In an effort to search for molecules that may be involved in anchoring and clustering of AMPA receptors at excitatory synapses, we used the C-terminal 50 amino acids of the AMPA receptor GluR2 subunit as bait to screen a rat hippocampal cDNA library using the yeast two-hybrid technique (Fields and Song, 1989; Chevray and Nathans, 1992). Using this method, we have previously demonstrated that GRIP (Dong et al., 1997) and NSF (Song et al., 1998) bind to the C terminus of GluR2. In a similar screen, we isolated two clones that encode nearly the full length of PICK1, a protein that was previously isolated due to its ability to interact with PKCα (Staudinger et al., 1995). Interestingly, PICK1 contains a PDZ domain at the N terminus (Figure 1C; Staudinger et al., 1997). To characterize the specificity of the GluR2–PICK1 binding, we examined the interaction of other AMPA receptor subunits with PICK1 in the yeast two-hybrid system. PICK1 specifically interacted with the C termini of the GluR2, GluR3, and GluR4C subunits but not with the GluR1 and GluR4 subunits (Figure 1A). This result was not surprising, since GluR2, GluR3, and GluR4C have similar C-terminal amino acid sequences and specifically interact with GRIP (Dong et al., 1997). To define the exact site of interaction between GluR2/3 and PICK1, various mutants of the GluR2 and GluR3 subunits were tested for interaction with PICK1. Figure 1B shows that deletions of the N-terminal region of the GluR2 construct had no effect on the interaction. In contrast, the deletion of the last seven amino acids from the GluR2 C termini completely eliminated the interaction with PICK1. Furthermore, the addition of a single amino acid at the C terminus of GluR3 abolished the interaction as well, indicating that the correct amino acid sequence had to be specifically located at the C terminus. The requirement of the intact C termini of GluR2 and GluR3 for the interaction with PICK1 suggested that the PDZ domain of PICK1 is the site of interaction. PDZ domains contain a conserved lysine that is required for interaction with the free carboxyl

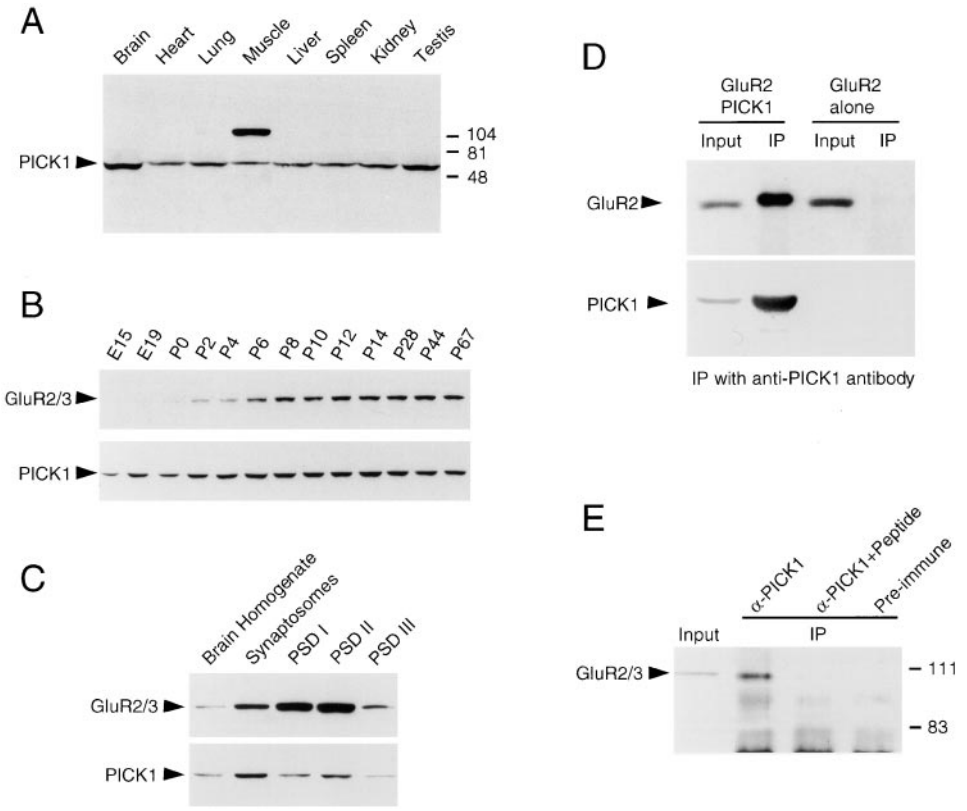

Figure 2. Characterization of PICK1 Expression and Interaction with AMPA Receptors

(A) Tissue distribution of PICK1 protein. Equal amounts of protein were loaded in each lane and probed with an antibody against the last 100 amino acids of PICK1. A 55 kDa band, which corresponded to the predicted size of PICK1, is detected in all tissues, with the highest expression in the brain and the second highest in the testis. In muscle, an additional band appears around 110 kDa.

(B) Development profile of PICK1 and GluR2/3 in the brain. PICK1 appears early in development and gradually increases and reaches a peak at around 2 weeks. In comparison, GluR2/3 is expressed at low levels at embryonic day 19 and postnatal day 0, and then rapidly increases postnatally, reaching a peak at around 2 weeks.

(C) Subcellular distribution of PICK1. PICK1 is enriched in synaptosomes and is present in PSD fractions. GluR2/3, detected by an antibody recognizing both GluR2 and GluR3, is enriched in both synaptosomes and PSD fractions.

(D) Coimmunoprecipitation of PICK1 and GluR2 from transfected HEK 293T cells. Cells were transfected with GluR2 and PICK1 or GluR2 alone and then immunoprecipitated with the anti-PICK1 antibody. The immunoprecipitates were resolved in an SDS–PAGE and probed with anti-PICK1 and anti-GluR2 antibodies. GluR2 was specifically coimmunoprecipitated with PICK1 antibody only in the presence of PICK1.

(E) Coimmunoprecipitation of PICK1 and AMPA receptors from rat brain. Membrane fractions of brain homogenate were solubilized and immunoprecipitated as indicated. The blot shows that the anti-PICK1 antibody specifically pulls down GluR2/3, detected by an antibody recognizing both GluR2 and GluR3. When the antibody was preabsorbed by the immunogen or when the preimmune serum was used, GluR2/3 was not detected.

group on the C terminus of target proteins (Cabral et al., 1996; Doyle et al., 1996; Songyang et al., 1997). To analyze whether the PDZ domain of PICK1 was important in the interaction, the corresponding lysine residue, together with the adjacent aspartic acid residue, were mutated to alanines (K27D28→AA). Mutation of these two residues eliminated the interaction of GluR2 with PICK1, indicating that the PDZ domain is essential for interaction (Figure 1C).

Tissue Distribution, Developmental Expression, and Subcellular Distribution of PICK1 Protein

We compared the expression and distribution of PICK1 with AMPA receptors using an antibody generated against the last 100 amino acids of PICK1. The PICK1 antibody detected a 55 kDa protein, the predicted size of PICK1, in all tissues with the highest expression level in the brain (Figure 2A). This is consistent with Northern analysis (Staudinger et al., 1995). The antibody recognized an additional 110 kDa protein only in muscle, indicating that muscle may contain a specific splice variant of PICK1. This is interesting, since we have recently found that PICK1 interacts with MuSK, a muscle-specific tyrosine kinase involved in the synaptic targeting of the nicotinic acetylcholine receptor (AChR) at the NMJ (J. X. and R. L. H., unpublished data).

The developmental expression of PICK1 and GluR2/3 in the brain is shown in Figure 2B. PICK1 and GluR2 are both highly expressed in the postnatal brain. As has been previously shown, GluR2/3 expression is relatively low early in development and then steadily increases, reaching a peak at postnatal day 14. In contrast, PICK1 protein is detected early in development, suggesting that PICK1 may be important in GluR2-independent processes in early development, and then increases gradually, reaching its peak around postnatal day 14.

We characterized the subcellular distribution of PICK1 in the brain to see if it was colocalized with AMPA receptors in postsynaptic density (PSD) fractions. Synaptosomes were isolated from rat brain homogenates and then extracted with detergents to isolate various PSD fractions (Cho et al., 1992). PICK1, similar to GluR2/3, is highly enriched in the synaptosomal fraction and is present with other synaptic proteins in the detergent-resistant PSDI, PSDII, and PSDIII fractions.

Association of PICK1 with AMPA Receptors In Situ and In Vivo

To further investigate the interaction of PICK1 and AMPA receptors, we tested whether PICK1 and AMPA formed a complex in transfected mammalian cells. HEK 293T cells were transfected with GluR2 in the presence or absence of PICK1, and the cells were solubilized by Triton X-100 and immunoprecipitated by an anti-PICK1 antibody 2 days after transfection. The immunoprecipitates were then resolved by SDS–PAGE and immunoblotted with an affinity-purified anti-GluR2/3 antibody that recognizes both GluR2 and GluR3. As shown in Figure 2D, GluR2 was coimmunoprecipitated with PICK1 from cells cotransfected with GluR2 and PICK1, indicating that PICK1 and GluR2 were associated (left two lanes). In contrast, when cells were transfected with GluR2 alone, GluR2 was not detected in the immunoprecipitates isolated with the PICK1 antibody (right two lanes), indicating the specificity of the coimmunoprecipitation.

To determine whether AMPA receptors and PICK1 interact in vivo, we examined whether GluR2 and PICK1 coimmunoprecipitated from rat brain. Membrane fractions from rat brain homogenate were solubilized by 1% deoxycholate and centrifuged at 100,000 $\times$ g, and the supernatant was immunoprecipitated with an anti-PICK1 polyclonal antibody. The immunoprecipitates were then analyzed by immunoblot techniques with an affinity-purified anti-GluR2/3 antibody that recognizes both GluR2 and GluR3. As shown in Figure 2E, GluR2/3 was coimmunoprecipitated with PICK1, but not when the anti-PICK1 antibody was blocked with antigen or when preimmune serum was used for immunoprecipitation.

PICK1 Is Colocalized with AMPA Receptors at Excitatory Synapses

The subcellular distribution of PICK1 in neurons was further analyzed by immunofluorescence techniques. Primary cultures of rat hippocampal low-density neurons were double labeled with affinity-purified rabbit anti-PICK1 antibody and mouse anti-GluR2 antibody. As shown in Figure 3A, PICK1 is highly enriched at synapses and colocalizes with GluR2 (arrows). Interestingly, PICK1 appears to be present at spiny synapses but not at shaft synapses (data not shown). To test whether PICK1 is exclusively localized at excitatory synapses, we double stained hippocampal neurons with the rabbit anti-PICK1 antibody and an anti-glutamic acid decarboxylase (GAD) mouse antibody that specifically recognizes GABAergic inhibitory synapses. As shown in Figure 3B, PICK1 (arrows) did not colocalize with GAD (arrowheads), indicating that PICK1 is not found at inhibitory synapses.

PKCα Colocalizes with PICK1 and AMPA Receptors at Excitatory Synapses

PICK1 was originally identified due to its interaction with PKCα. To investigate the relationship of PKCα with PICK1 and AMPA receptors, we examined the distribution of PKC in cultured neurons in culture. Neurons were double labeled with antibodies against PKCα and PICK1 or PKCα plus AMPA receptors and then analyzed by immunofluorescence techniques. In Figure 3C, neurons were stained with a monoclonal antibody against PKCα and an affinity-purified rabbit anti-GluR2/3 antibody that recognizes both GluR2 and GluR3. Interestingly, PKCα is highly enriched in excitatory synapses and colocalizes with GluR2/3 (arrows). The interactions of PKCα with PICK1 and PICK1 with AMPA receptors, and the colocalization of PKCα, PICK1, and AMPA receptors at excitatory synapses, indicate that these three proteins may be in a complex at excitatory synapses. However, we have been unable to demonstrate a complex containing PKCα with PICK1 and AMPA receptors in coimmunoprecipitation experiments. This might be due to a low-affinity interaction of PKCα with PICK1.

PICK1 Induces AMPA Receptor Clustering in Heterologous Systems

PDZ domain–containing proteins like PSD-95/SAP90 and PSD-93/Chapsyn-110 have been shown to cluster NMDA receptors in heterologous expression systems (Kim et al., 1996). To examine if PICK1 could induce AMPA receptor clustering, we transfected PICK1 and GluR2 into HEK 293T cells and analyzed GluR2 and PICK1 distribution by immunofluorescence staining techniques. As shown in Figure 4A, when GluR2 was transfected into HEK 293T cells alone, GluR2 staining was diffuse in the cell. Similarly, when PICK1 was transfected into HEK 293T cells, PICK1 was diffusely distributed (Figure 4B), with the exception of some cells that had a few small PICK1 clusters (data not shown). In contrast, when GluR2 was cotransfected with PICK1, GluR2 formed many clusters that colocalized with PICK1 (Figure 4C). To rule out the possibility that the clusters of PICK1 and GluR2 are aggregates inside the cells, due to protein misfolding or misprocessing, we examined whether PICK1 induced GluR2 to cluster on the surface plasma membrane. HEK 293T cells cotransfected with GluR2 and PICK1 were stained live with a mouse anti-N-terminal GluR2 antibody to specifically label GluR2 on the surface. The rabbit anti-PICK1 antibody was then added after fixation and permeabilization. Indeed, many of the clusters of GluR2 induced by PICK1 were on the plasma membrane (Figure 4D).

To examine the specificity of this clustering, a GluR2 construct without the last seven amino acids (*GluR2*Δ*7*) required for interaction with PICK1 was cotransfected with PICK1. Deletion of the last seven amino acids eliminated the PICK1-induced clustering of GluR2 (Figure 5B). In addition, the role of the PDZ domain of PICK1 in GluR2 clustering was examined by cotransfecting the PICK1 PDZ domain mutant (K27D28→AA) with wild-type

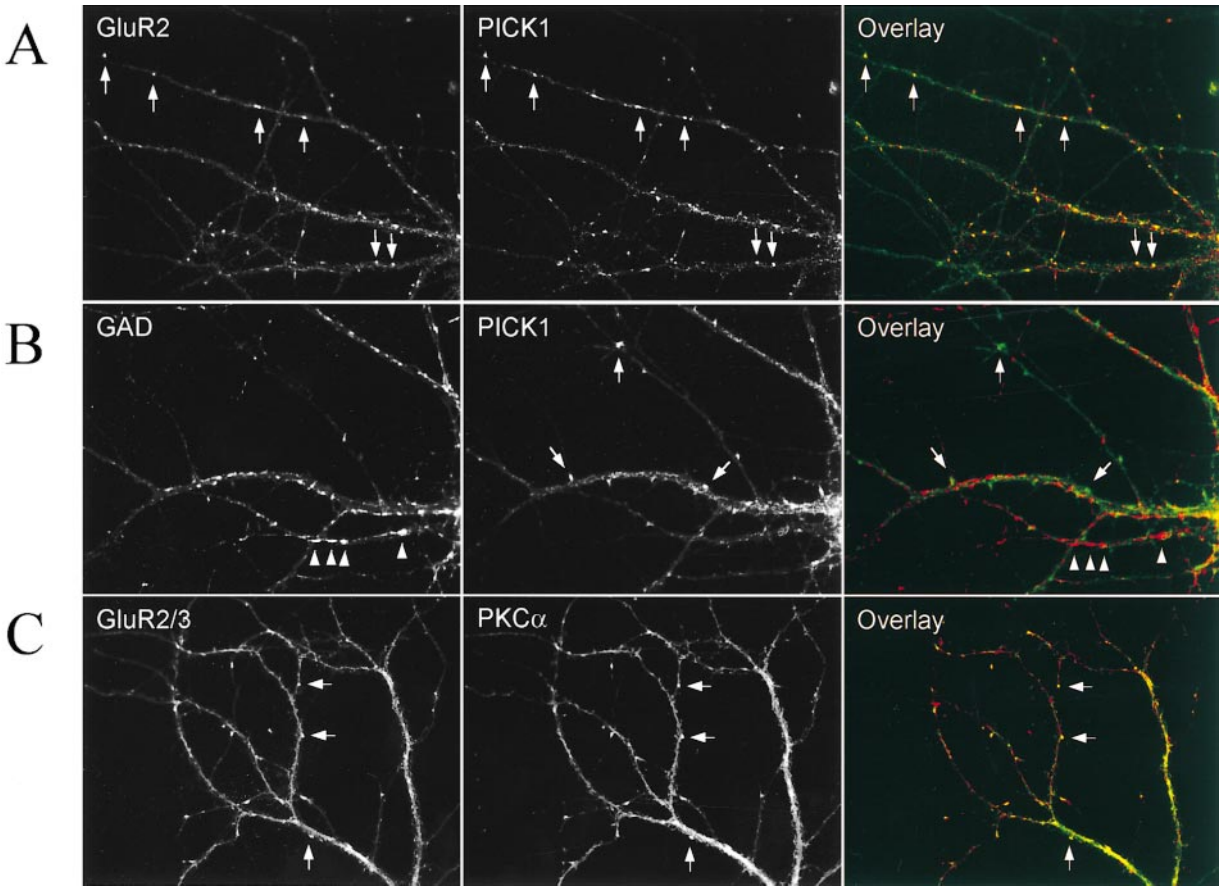

Figure 3. Colocalization of PICK1, AMPA Receptors, and PKCα in Cultured Hippocampal Low-Density Neurons

(A) Neurons were double labeled with the rabbit anti-PICK1 and the mouse anti-GluR2 antibodies. PICK1 (green) colocalizes with GluR2 (red) at excitatory synapses as shown in the overlay panel (arrows).

(B) Neurons were double labeled with the rabbit anti-PICK1 antibody and a mouse anti-GAD antibody that recognizes glutamic acid decarboxylase, a marker for inhibitory synapses. PICK1 (green) does not colocalize with GAD (red) at inhibitory synapses as indicated by arrows (PICK1) and arrowheads (GAD).

(C) Neurons were double labeled with the rabbit anti-GluR2/3 antibody and a mouse anti-PKCα. PKCα (green), which interacts with PICK1, is highly enriched at excitatory synapses and colocalizes with GluR2/3 (red).

GluR2. As shown in Figure 5C, mutation of the PDZ domain also abolished the clustering of the GluR2 subunit. These results indicate that PICK1 can induce GluR2 clustering through the interaction of its PDZ domain with the C terminus of GluR2. However, the molecular mechanism underlying this clustering is not clear, since PICK1, unlike PSD-95 and GRIP, has only one PDZ domain and cannot cross-link AMPA receptors to form a multivalent complex. Analysis of the PICK1 amino acid sequence revealed that, in addition to the PDZ domain, PICK1 contains a coiled-coil domain that could potentially be involved in the dimerization of PICK1. Previous studies have shown that both PDZ and coiled-coil domains mediate self-association of proteins (Adamson et al., 1993; Blake et al., 1995; Brenman et al., 1996). We therefore examined whether PICK1 could interact with itself in the yeast two-hybrid system. Indeed, PICK1 robustly interacts with itself (Figure 1D; Staudinger et al., 1997), and mutation of the PICK1 PDZ domain, which blocks its association with GluR2, does not affect PICK1 self-interaction (Figures 1C and 1D). This result suggested that PICK1 self-association may be independent of its interaction with GluR2 and may allow for the multimeric assembly of PICK1 and GluR2 required for clustering.

Discussion

Recent studies have suggested that the regulation of the expression, transport, and synaptic targeting of neurotransmitter receptors may play important roles in the regulation of synaptic transmission (O'Brien et al., 1998). For example, the insertion of AMPA receptors into the postsynaptic membrane has been proposed to be an essential component of synaptic plasticity during long-term potentiation (LTP) and neuronal development (Isaac et al., 1995, 1997; Liao et al., 1995; Durand et al., 1996; Wu et al., 1996). In the last few years, significant advances have been made in our understanding of the molecular mechanisms underlying the synaptic targeting of neurotransmitter receptors. Recent studies have demonstrated the central role of PDZ domain–containing proteins, such as PSD-95 and GRIP, in the subcellular targeting of neurotransmitter receptors and

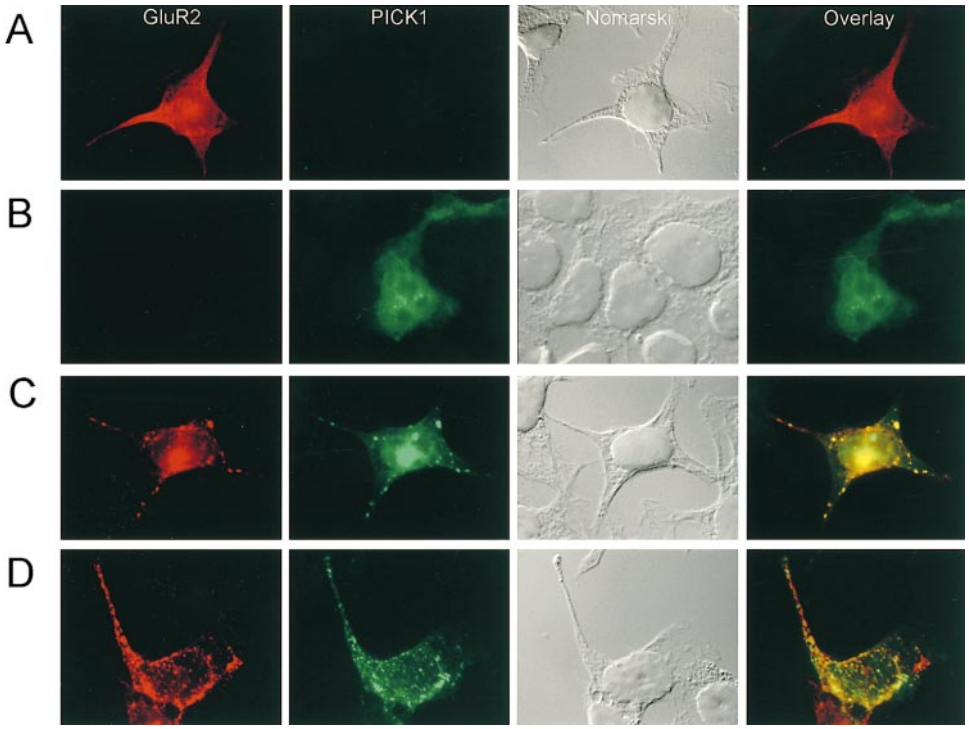

Figure 4. PICK1 Induces GluR2 Clustering in HEK 293T Cells

(A) HEK 293T cells were transfected with GluR2 alone. GluR2 immunostaining was diffuse in the cell.
(B) Cells were transfected with PICK1 alone. PICK1 was diffuse in the cell as well.
(C) Cells were cotransfected with GluR2 and PICK1. GluR2 and PICK1 form many coclusters in the cell.
(D) Surface GluR2 is clustered by PICK1 in cotransfected cells. The anti-N-terminal GluR2 antibody was added to live cells to label surface GluR2, while the PICK1 antibody was added after fixation and permeabilization.

ion channels (Sheng and Kim, 1996; Kornau et al., 1997; O'Brien et al., 1998). PDZ domains are now known to be ubiquitous protein–protein interaction motifs present in hundreds of proteins that are essential for the proper subcellular targeting of proteins (Saras and Heldin, 1996). Here, we report that PICK1, a PDZ domain–

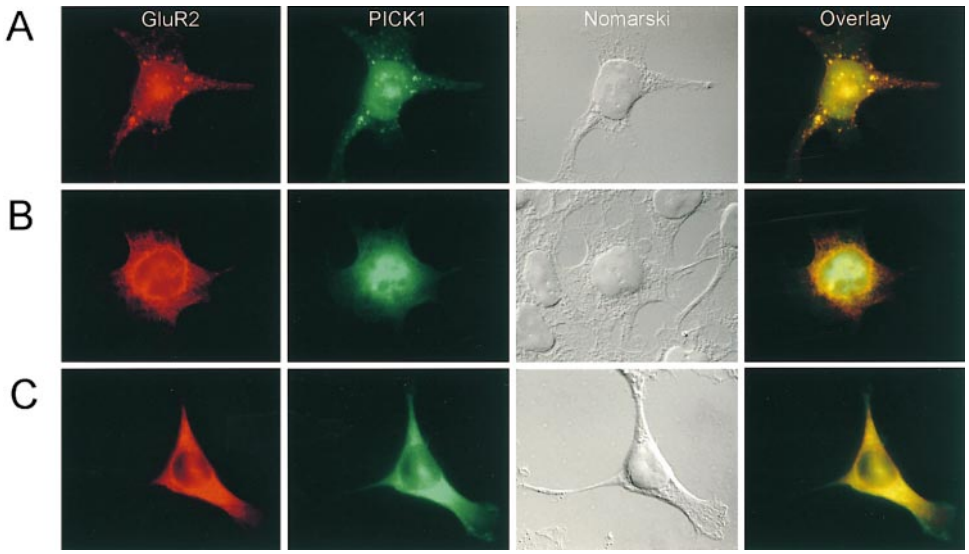

Figure 5. Mutation of GluR2 C Terminus and PICK1 PDZ Domain Abolishes Clustering

(A) Cells cotransfected with wild-type GluR2 and PICK1 show coclusters of GluR2 and PICK1.
(B) Cells transfected with PICK1 and a GluR2 mutant lacking the last seven amino acids (*GluR2*Δ7) failed to form clusters.
(C) Cells transfected with GluR2 and *PICK1*Δ, the K27D28→AA PDZ domain mutant, also did not form clusters.

containing protein, specifically associates with the C termini of the GluR2, GluR3, and GluR4c AMPA receptor subunits in vitro and in vivo. PICK1 is colocalized with AMPA receptors at excitatory synapses, and, moreover, PICK1 clusters AMPA receptors in heterologous expression systems. Overexpression of the C-terminal region of GluR2 that interacts with the PDZ domains of PICK1 and GRIP has been found to disrupt the synaptic clustering of AMPA receptors in neurons (Dong et al., 1997). These data strongly suggest that proteins that interact with the C-terminal tail of GluR2, such as PICK1 and GRIP, are critical for clustering AMPA receptors at excitatory synapses. While both PICK1 and GRIP interact with the C-terminal tail of GluR2, the current evidence suggests that PICK1 may be more directly involved in the clustering of AMPA receptors at excitatory synapses. PICK1 specifically colocalizes with GluR2 at excitatory synapses, while recent data have demonstrated that GRIP is localized at both excitatory and inhibitory synapses (H. Dong and R. L. H., unpublished data). In addition, coexpression of PICK1 with GluR2 in mammalian cells induces receptor clustering. In contrast, we have been unable to induce AMPA receptor clustering with GRIP overexpression (H. Dong and R. L. H., unpublished data). Recent data have also shown that GRIP is localized in post-Golgi vesicles as well as at synapses, suggesting that GRIP may be involved in transporting AMPA receptors to dendrites (H. Dong and R. L. H., unpublished data). In addition, genetic studies in *C. elegans* have recently identified multiple PDZ domain–containing proteins that are essential for targeting of the AMPA-like GLR-1 receptors (Bredt, 1998; Rongo et al., 1998). The results in this paper suggest that GRIP and PICK1, as well as other synaptic proteins, may cooperate in the transport and clustering of AMPA receptors at synapses.

PICK1 was originally isolated, due to its interaction with PKC (Staudinger et al., 1995). Previous studies have demonstrated that PKC directly phosphorylates and modulates AMPA receptors and also plays critical roles in synaptic plasticity (Roche et al., 1994). For example, inhibition of PKC activity blocks the induction of LTP (Bear and Malenka, 1994; Nicoll and Malenka, 1995). The precise role of PKC in LTP and other forms of synaptic plasticity, however, is not clear. The data presented in this paper show that PICK1 interacts with both PKCα and AMPA receptors. In addition, PKCα is highly enriched and colocalizes with PICK1 and AMPA receptors at excitatory synapses. These results suggest that PICK1 could potentially link PKCα and AMPA receptors and therefore modulate AMPA receptor phosphorylation and its function. It would be interesting to examine the potential role of PICK1 in LTP or other forms of synaptic plasticity.

Interestingly, PICK1 and GRIP have also recently been found to interact with the Eph receptor tyrosine kinase at excitatory synapses (Torres et al., 1998). Tyrosine phosphorylation has been shown to be critical for the regulation of the synaptic targeting of AChR at the NMJ (Colledge and Froehner, 1998). At the NMJ, the activation of MuSK leads to the activation of rapsyn, a synaptic protein that is thought to directly interact and cluster the AChR (Colledge and Froehner, 1998). It is interesting to postulate that receptor tyrosine kinases may also play a similar role in the targeting and clustering of glutamate receptors at central synapses.

The large complex signal transduction network that contains AMPA receptors, PICK1, GRIP, Eph receptor tyrosine kinases, and PKC may play an important role in the regulation of AMPA receptor function. However, this large complex may play additional roles in the downstream signaling of AMPA receptor activation. Although the binding of glutamate to AMPA receptors directly gates ion channel gating, it is possible that ligand-induced conformational changes may also transmit novel signals through AMPA receptor–associated protein complexes. Further investigation of members of this complex and the regulation of the protein–protein interactions within this complex will help to elucidate molecular mechanisms involved in synaptic plasticity.

Experimental Procedures

Yeast Two-Hybrid Screening

Yeast two-hybrid screening (Fields and Song, 1989) was performed using a random-primed cDNA library from rat hippocampus subcloned into the SalI/NotI sites of the pPC86 vector (Chevray and Nathans, 1992), which contains the GAL4 activation domain. The bait was the final 50 amino acids of GluR2 subcloned in frame into the SalI/BglII sites of the pPC97 vector, which contains the GAL4 DNA binding domain. The plasmids were transformed into PJ69 yeast cells (James et al., 1996), and positive clones were selected on plates lacking leucine, tryptophan, and adenine. After the growth selection, the clones were further selected in quadruple minus plates (Leu−, Trp−, Ade−, His−) containing X-gal to assay for β-galactosidase activity. Positive clones were cotransformed with either the bait vector or the original pPC97 vector (backbone) into PJ69 and Y190 yeast strains to confirm the interaction. All of the constructs were from PCR products subcloned in frame into pPC97 or pPC86 vectors and were confirmed by sequencing. PICK1 K27D28→AA mutant was generated as described previously (Staudinger et al., 1997).

Antibodies

The anti-PICK1 affinity-purified rabbit polyclonal antibody used in immunostaining and coimmunoprecipitation was generated against a peptide corresponding to residues 2–31 (FADLDYDIEEDKLGIPTV PGKVTLQKDAQN) of mPICK1. The anti-PICK1 rabbit polyclonal antibody used in immunoblots was generated against a fusion protein containing the last 100 amino acids of mPICK1. The anti-GluR2/3 rabbit polyclonal antibody was raised against the C-terminal 20 amino acids of GluR2, and it recognizes both GluR2 and GluR3 AMPA subunits (Blackstone et al., 1992). The anti-N-terminal GluR2 antibody was purchased from Chemicon. The anti-FLAG mouse monoclonal antibody was obtained from Kodak. Horseradish peroxidase–labeled secondary antibody was purchased from Amersham. Rhodamine and FITC-labeled secondary antibodies were from Jackson Laboratory.

Subcellular Fractionation

Synaptosomes and PSD fractions were prepared from rat brains as previously described (Cho et al., 1992), with modifications. The synaptosome fraction isolated by discontinuous sucrose gradient centrifugation was solubilized in ice-cold 0.5% Triton X-100 for 15 min and centrifuged at 32,000 $\times$ g for 20 min to obtain the PSDI pellet. This pellet was either resuspended and solubilized in 0.5% Triton X-100 and then centrifuged at 200,000 $\times$ g for 1 hr to obtain the PSDII pellet, or it was resuspended and solubilized in ice-cold 3% Sarcosyl for 10 min and then centrifuged to obtain the PSDIII pellet. All pellets were resuspended in 40 mM Tris-HCl (pH 8.0). Protein concentrations were measured by a bicinchoninic acid assay (Pierce).

Coimmunoprecipitation and Immunoblotting

Membrane preparations (P2) and solubilization were carried out according to the procedures described by Luo et al. (1997), with modifications. For coimmunoprecipitation, P2 (400 μg per immunoprecipitation) was solubilized by 1% sodium deoxycholate followed by 0.1% Triton X-100, and the preparation was centrifuged for 10 min at 100,000 × g. The supernatant was then used for coimmunoprecipitation. About 5–10 μg of the affinity-purified anti-PICK1 antibody was preincubated with 40 μl of a 1:1 slurry of protein A-Sepharose for 1–2 hr at 4°C. The protein A-antibody complex was spun down at 2,000 rpm for 2 min. The clarified supernatant of solubilized P2 fraction was then added to the Sepharose beads, and the mixture was incubated for 2–3 hr at 4°C. The mixture was washed once with 1% Triton X-100 in Tris-buffered saline (TBS), twice with 1% Triton X-100 in TBS plus 300 mM NaCl, and three times with TBS. The immunoprecipitates were then resolved by SDS–PAGE gel and transferred to a polyvinylidine difluoride (PVDF, Millipore) membrane, and the membrane was blocked and immunoblotted with antibodies. For control, the PICK1 antibody was blocked by preincubating with the immunogenic peptide at a concentration of 100 μg/ml.

Neuronal Staining

Cultured low-density hippocampal neurons were fixed and stained as described (Craig et al., 1994). Affinity-purified rabbit anti-PICK1 antibody and mouse anti-GluR2 antibody were incubated overnight in 3% normal goat serum followed by rhodamine-labeled anti-mouse and FITC-labeled anti-rabbit secondary antibody. The coverslip with neurons was then mounted in Permafluor (Immunon) with 20 mg/ml Dabco and viewed at 63× or 100×. Pictures were taken with a digital camera (MicroMax from Princeton Instruments and Metamorph from Universal Imaging) and colorized when necessary.

HEK 293T Cell Transfection, Coimmunoprecipitation, and Staining

GluR2 cDNA was subcloned into the pRK5 vector. PICK1 (amino acids 1–386) cDNA was subcloned into the pRK5 vector with a Myc-epitope tag of 16 amino acids at the N terminus or the pECE vector with a FLAG-epitope of 9 amino acids at the N terminus. cDNAs were transfected into HEK 293T cells by calcium phosphate coprecipitation. After 48 hr of transfection, cells were collected and solubilized by 2% Triton X-100 and spun down by a microcentrifuge at top speed. Four hundred microliters of clear supernatant was used for each immunoprecipitation. For immunostaining, HEK 293T cells were grown on coverslips coated with 0.2% gelatin. The cells were fixed and double stained with rabbit anti-GluR2/3 and mouse anti-myc or anti-FLAG antibodies. For surface staining of GluR2, the mouse anti-N-terminal GluR2 antibody was added to live cells or fixed cells before permeabilization with 0.2% Triton X-100, and the rabbit anti-myc or rabbit anti-PICK1 antibody was added after permeabilization.

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