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PDZ Proteins Bind, Cluster, and Synaptically Colocalize with Eph Receptors and Their Ephrin Ligands

Richard Torres,* Bonnie L. Firestein,† Hualing Dong,‡ Jeff Staudinger,§ Eric N. Olson,‖ Richard L. Huganir,‡ David S. Bredt,† Nicholas W. Gale,* and George D. Yancopoulos*#

*Regeneron Pharmaceuticals
Tarrytown, New York 10591-6707
†Department of Physiology
University of California
San Francisco, California 94143
‡Howard Hughes Medical Institute
Department of Neuroscience
The Johns Hopkins University
School of Medicine
Baltimore, Maryland 21205
§Department of Molecular Endocrinology
GlaxoWellcome
Research Triangle Park, North Carolina 27709
‖Department of Molecular
Biology and Oncology
The University of Texas
Southwestern Medical Center
Dallas, Texas 75235

Summary

Localizing cell surface receptors to specific subcellular positions can be critical for their proper functioning, as most notably demonstrated at neuronal synapses. PDZ proteins apparently play critical roles in such protein localizations. Receptor tyrosine kinases have not been previously shown to interact with PDZ proteins in vertebrates. We report that Eph receptors and their membrane-linked ligands all contain PDZ recognition motifs and can bind and be clustered by PDZ proteins. In addition, we find that Eph receptors and ligands colocalize with PDZ proteins at synapses. Thus, PDZ proteins may play critical roles in localizing vertebrate receptor tyrosine kinases and/or their ligands and may be particularly important for Eph function in guidance or patterning or at the synapse.

Introduction

The localization and clustering of cell surface receptors and channels to specific subcellular positions can be critical for their proper functioning. The best studied examples of such localizations occur at synapses, where neuronal processes interconnect with each other or couple to their nonneuronal targets; membrane proteins critical in sending and receiving synaptic signals are often highly concentrated at pre- and postsynaptic sites. It has recently been appreciated that synaptic membrane proteins are physically linked to an array of cytoskeletal components and intracellular signaling molecules, forming a large synaptic macromolecular complex (reviewed by Kennedy, 1997; Ziff, 1997; Craven and Bredt, 1998). Critical links in this complex have been found to be proteins containing PDZ domains, thus dubbed for the first three proteins for which this domain was defined: the PSD-95 postsynaptic density protein (Cho et al., 1992), the DlgA *Drosophila* discs-large imaginal disc protein (Woods and Bryant, 1991), and the ZO-1 tight junction protein (Itoh et al., 1993). PDZ proteins are also involved in clustering and localizing cell surface receptors outside of the synapse (reviewed by Kim, 1995; Craven and Bredt, 1998).

PDZ proteins are modular proteins that can act as adaptors, attaching via certain of their PDZ domains to the C termini of membrane receptors and channels, while also binding to cytoskeletal or signaling proteins via other PDZ domains or other modules (reviewed by Craven and Bredt, 1998). The initial consensus C-terminal peptide sequence shown to be required for interacting with PDZ domains was (T/S)XV (Kim et al., 1995; Kornau et al., 1995), though there appear to be additional determinants of binding, because different PDZ domains appear to be able to distinguish between binding partners containing the same consensus motif; related C-terminal motifs such as VKI and YYV have also been shown to bind to PDZ domains (Hata et al., 1996; Dong et al., 1997).

Receptor tyrosine kinases remain a receptor class that has not been shown to interact with PDZ-containing proteins in vertebrates. However, such a link has been made in the pathway that controls vulval induction in *C. elegans*. Several genetic mutants in this pathway have been characterized, including ones in PDZ-containing proteins known as Lin-2A, Lin-7, and Lin-10 (Hoskins et al., 1996; Simske et al., 1996; Kaech et al., 1998) as well as in a receptor tyrosine kinase known as Let-23 (Aroian et al., 1990). Current evidence suggests that a complex involving Lin-2A, Lin-7, Lin-10, and Let-23 ensures that the receptor is localized to the basolateral cell surface so as to have access to its ligand and accordingly mediate its signaling role; mutation of Lin-2A, Lin-7, or Lin-10 mislocalizes the Let-23 receptor and results in a signaling defect (Hoskins et al., 1996; Simske et al., 1996; Kaech et al., 1998). The localization of growth factor receptors to the basolateral or the apical membrane of polarized epithelial cells has led to the suggestion that similar requirements for receptor localization, mediated by PDZ-containing proteins, may be occurring in vertebrates (Simske et al., 1996; Bredt, 1998).

The Eph family of receptor tyrosine kinases, as well as their membrane-linked ligands, seem like ideal candidates for localization by PDZ-containing proteins. The Eph family is the largest known family of receptor tyrosine kinases, with at least 14 members, and eight "ephrin" ligands for this family have been described to date (reviewed by Gale and Yancopoulos, 1997; Flanagan and Vanderhaeghen, 1998; Holland et al., 1998). The Eph receptors and their ligands can broadly be divided into two subclasses based on structural homologies and binding specificities, with a great deal of redundancy within a subclass in terms of receptor/ligand binding

#To whom correspondence should be addressed (e-mail: gdy@regpha.com).

specificities. The ligands in one subclass (referred to as the ephrin-A subclass) are linked to the membrane via a glycosylphosphatidylinositol (GPI) linkage, while the members of the other subclass (referred to as the ephrin-B subclass) are transmembrane proteins; ephrin-A ligands preferentially bind to receptors of the EphA subclass, while ephrin-B ligands preferentially bind to receptors of the EphB subclass (Brambilla et al., 1995; Gale et al., 1996a, 1996b). Unlike most other ligands for receptor tyrosine kinases, the ephrins cannot act as soluble mediators. The ephrins are cell surface–bound proteins that require membrane linkage to somehow cluster them and thus to allow them to activate Eph receptors on adjacent cells; soluble versions of the ligands are inactive unless artificially clustered (Davis et al., 1994). Thus, it was proposed that the ephrins might provide key cues in a cell-to-cell contact–dependent fashion and that specific mechanisms are involved in clustering the ligands and therefore allowing them to activate their receptors (Davis et al., 1994); recent evidence suggests that such clustering might be inducibly regulated by various growth factors and the protein kinase C (PKC) pathway (Bruckner et al., 1997; Stein et al., 1998). Remarkably, there appears to be reciprocal signaling between ligands and receptors in the B subclass in that ephrin-B ligands not only activate their respective receptors but are in turn activated upon engaging their receptors, as judged by tyrosine phosphorylation of the ephrin-B cytoplasmic domains (Holland et al., 1996; Bruckner et al., 1997); once again, clustering of receptors seems important for this ligand activation. Consistent with important roles for receptor and ligand clustering, Eph receptors have been visualized on developing neuritic processes by electron microscopy, revealing clustered receptors at points of process contact (Henkemeyer et al., 1994). It thus seems likely that regulated processes involved in localizing and clustering Eph family receptors and/or ligands are crucial to ensure the signaling processes required for the biological processes in which the Eph family is involved, such as in repulsive interactions between neuritic processes during axon guidance or during the establishment of boundaries between neighboring cell compartments in the nervous system or the vasculature (Gale and Yancopoulos, 1997; Flanagan and Vanderhaeghen, 1998; Holland et al., 1998; Wang et al., 1998; Yancopoulos et al., 1998). It seems possible that PDZ proteins are involved in such regulated clustering of Eph receptors and their ligands.

Here, we report that many of the Eph receptors and their ephrin-B ligands contain consensus C-terminal motifs reminiscent of those binding PDZ domains. We further identify PDZ-containing proteins that can specifically bind and cluster both Eph receptors and their ligands in heterologous cells and that are tyrosine phosphorylated upon association with the Eph receptors. We also show that such interactions can be detected in vivo, suggesting that PDZ-mediated clustering may be important for previously defined developmental roles of the Eph family in neural guidance and tissue patterning. Interestingly, we find that Eph receptors and ligands can be colocalized with PDZ-containing proteins at neuronal synapses, suggesting a new role for these developmentally important molecules during synaptic plasticity. Thus, the role of PDZ-containing proteins can be extended to the clustering of receptor tyrosine kinases and their ligands, perhaps recruiting them to macromolecular complexes that include additional membrane components, such as neurotransmitters or ion channels. Our findings provide for a new modality of regulated signaling in such complexes and also suggest that Eph receptor tyrosine kinases may play as important a role at central synapses as the muscle-specific kinase (MuSK) receptor tyrosine kinase has recently been shown to do at the neuromuscular junction (NMJ) (Valenzuela et al., 1995b; DeChiara et al., 1996; Glass et al., 1996).

Results

Isolation of PDZ-Containing Proteins that Interact with the C Termini of Eph Family Receptors and Ligands in a Yeast Two-Hybrid System

To determine if the Eph receptors and their ligands could potentially interact with PDZ-containing proteins, we examined their C-terminal sequences (Figure 1). Remarkably, we found that almost all of these sequences ended in a valine, as do most known consensus sequences for PDZ domain binding (Songyang et al., 1997). However, while many of the Eph receptors (of both the A and B subclasses) ended in the consensus sequence VXV (or a variation in which the valine at the −3 position was replaced by an alternate hydrophobic residue) (Figures 1A and 1C), the ephrin-B ligands ended in the consensus YKV (Figure 1B). The VXV sequence was most similar to the VKI C termini of the GluR2 and GluR3 subunits of the AMPA receptor, which are known to interact with the synaptic protein GRIP, which contains seven PDZ domains (Dong et al., 1997), while the YKV consensus was most similar to the YYV C termini of the neurexins, also known to be PDZ binders (Hata et al., 1996). Examination of the cytoplasmic tails of other receptor tyrosine kinases revealed that ending in a VXV consensus was the exception rather than the rule (Figures 1C and 1D). Remarkably, the MuSK receptor tyrosine kinase, which is localized to the NMJ and is critical for its formation (Valenzuela et al., 1995b; DeChiara et al., 1996; Glass et al., 1996), has a similar C-terminal motif that is conserved from man to torpedo fish (Figure 1C). Interestingly, the C terminus of ErbB2 also conforms to this consensus (Figure 1D), and this receptor is known to localize to the muscle side of the NMJ (Zhu et al., 1995), as MuSK does.

We next focused on the VXV tails of the Eph receptors to see if they could indeed bind to PDZ domains by screening for such binding using the yeast two-hybrid system. Baits were generated that encoded the C termini of mouse EphB2 (the last 96 residues) (Henkemeyer et al., 1994) or rat EphA7 (the last 98 residues) (Valenzuela et al., 1995a), fused downstream to the LexA DNA–binding domain; corresponding "control baits" were generated in which the last 3 residues (either VEV or IQV; see Figure 1A) were deleted. The first two baits were then introduced into the L40 double reporter (yeast *HIS3* and bacterial *lacZ* genes) yeast strain and utilized in two-hybrid screens with a mouse embryonic day 11 (E11) cDNA library that was fused in-frame downstream

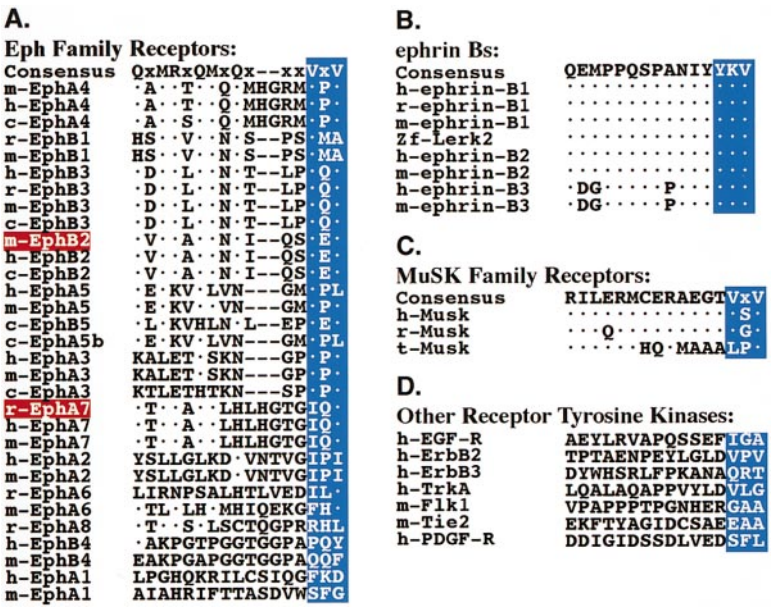

Figure 1. C Termini Alignment of Various Receptor Tyrosine Kinases and Ephrins Reveals Potential PDZ Binding Motifs

Examination of the C termini of Eph family receptors (A), Ephrin-B ligands (B), MuSK orthologs (C), and other receptor tyrosine kinases (D) for potential PDZ-binding motifs. Letters in front of each sequence signify species (c, chicken; h, human; m, mouse; r, rat; t, torpedofish; and Zf, zebrafish). Within the alignments, dots define amino acid residues with identity with the consensus sequence at the top of each panel, dashes denote gaps introduced to maximize the alignment, and "x" denotes any amino acid. The two Eph receptors used below as baits, EphB2 and EphA7, are highlighted in red, and the VXV consensus sequence is highlighted in blue.

of the GAL4 transcription activation domain (GAL4-TAD). cDNA clones that resulted in a HIS3+/LacZ+ phenotype were then screened for interaction with the control baits containing the C-terminal deletions to ensure the isolation of clones that specifically interacted with the desired VEV or IQV C termini. From the approximately 6 $\times$ 10^6 transformants that were screened with the EphB2 bait, two distinct cDNA clones were isolated that interacted in a VEV-dependent manner. One of the two clones contained the entire open reading frame of mouse PICK1, a single PDZ domain–containing protein that was previously identified as a PKC-α-interacting protein (Staudinger et al., 1995, 1997) (Figure 2A). The other clone encoded a protein fragment that displayed >99% amino acid identity to the portion of rat GRIP (Dong et al., 1997) containing the last four PDZ domains and was thus assumed to represent the mouse GRIP counterpart (Figure 2A). Approximately 9 $\times$ 10^6 transformants were screened with the EphA7 bait, and from the HIS3+/LacZ+ clones that were analyzed, only one distinct cDNA clone was obtained that required the IQV for interaction. This clone encoded the entire open reading frame of what appeared to be the mouse counterpart of the recently described human Syndecan-interacting protein (89.6% amino acid identity), designated syntenin (Grootjans et al., 1997), which contains two PDZ domains (Figure 2A).

To characterize the above PDZ domain–containing proteins further in terms of their binding specificity and to determine if they could bind ephrin-B ligands as well as Eph receptors, we directly compared the binding of all three to the above baits as well as to a bait containing the C terminus of ephrin-B1 (Davis et al., 1994) and to a number of control baits (Figure 2B). Under these conditions, the EphA7 and ephrin-B1 C termini were found to bind to all three PDZ-containing proteins, while EphB2 bound only to PICK1 and GRIP PDZs 4–7; in all cases tested, this binding absolutely depended upon the last three residues of the baits, and binding was not noted to control baits such as LexA alone or LexA fusions to Lamin or non-C-terminal regions of receptor tyrosine kinases (Figure 2B and data not shown).

Since GRIP has multiple PDZ domains, further two-hybrid assays were performed with smaller GRIP fusion proteins (Figure 2C), which revealed that binding to both Eph receptors and ephrin-B ligands depended upon PDZ domains 6 and 7 of GRIP; interestingly, the AMPA receptor binds to PDZ domains 4 and 5 of GRIP (Dong et al., 1997), leaving open the possibility of simultaneous binding of GRIP to multiple partners.

Confirmation that Interactions between PDZ-Containing Proteins and Either Eph Receptors or Ephrins Occur in Mammalian Cells

The above interactions were next explored via "pull-down" assays in which glutathione S-transferase (GST) fusion proteins bearing the above identified PDZ domains were used to pull down various full length receptor tyrosine kinases expressed in mammalian cells, verifying the same specificity of interactions for GRIP and syntenin as noted in yeast (Figure 2D). As expected, in these assays, we did not observe binding to a control EphA5 receptor in which the C-terminal PDZ recognition motif was destroyed by addition of a C-terminal "myc" tag. Next, we examined the interactions in mammalian cells by cotransfecting expression constructs for both the PDZ-containing proteins as well as their potential partners into the same COS7 cells. When PICK1 was coexpressed with EphB2 (flag tagged at its N terminus), ephrin-B1, and EphA7, coimmunoprecipitation of PICK1 and each of its potential partners was obvious, indicating complex formation (Figures 3A, lanes 1–3; 3B; and 3C). Coexpression using deleted versions of EphB2 or ephrin-B1 lacking their 3 C-terminal residues prevented complex formation (Figure 3A, lane 4 and data not shown), indicating that the noted interactions depended upon the C-terminal residues of Eph family members.

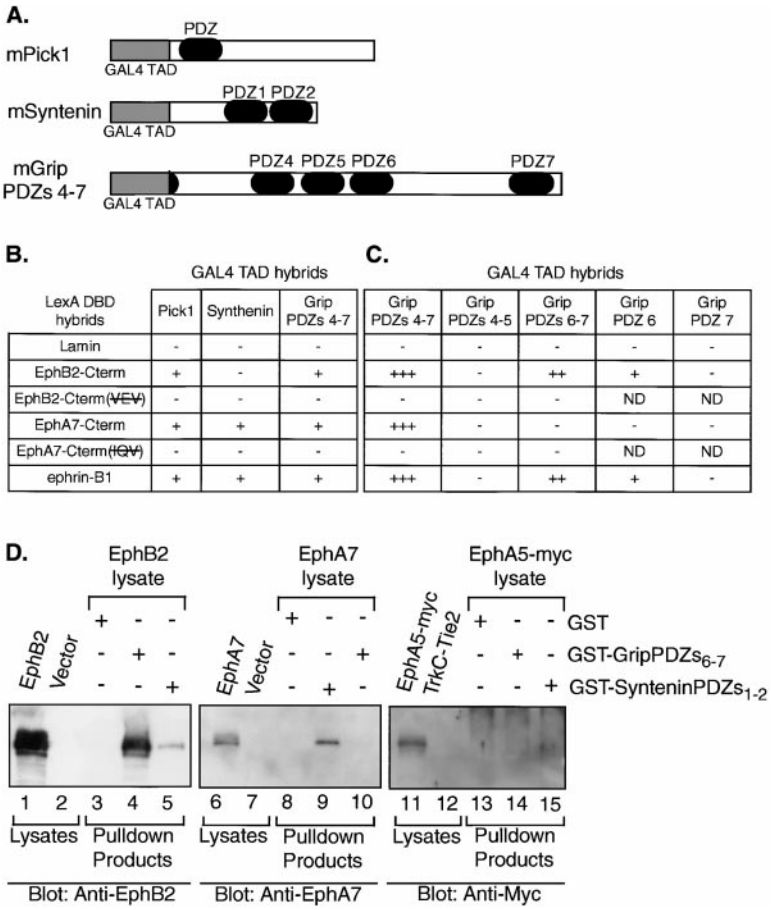

Figure 2. Identification and Characterization of PICK1, Syntenin, and GRIP Interactions with the C Termini of EphB2, EphA7, and Ephrin-B1 in Yeast Two-Hybrid Screens

(A) Schematic representation of the regions of PICK1, syntenin, and GRIP that were isolated as fusion proteins (to the GAL4 transcription activation domain GAL4-TAD) in yeast two-hybrid screens for interaction with the C termini of Eph receptors.

(B) The specificity of interaction of these fusion proteins for various baits (fused to the LexA DNA binding domain LexA-DBD), as indicated.

(C) Determination of which PDZ domain(s) of GRIP is required for interaction with the various Eph family members using different GRIP deletion mutants as indicated. The number of plus signs indicates qualitative evaluation of strength of interaction and ND indicates interaction not determined.

(D) GST pulldown assays showing that GST fusion proteins to GRIP (PDZ domains 6 and 7) or syntenin (PDZ domains 1 and 2) can coprecipitate EphB2 and EphA7 with the same specificity as exhibited in yeast two-hybrid screens. A mutant form of the EphA5 receptor, in which the C-terminal PDZ recognition motif is destroyed by appending a C-terminal myc tag, is used as a negative control. Lysates from COS cells transfected with each of the indicated receptors was mixed with the GST fusion protein as indicated, precipitations were performed, and immunoblotting was done with the indicated antibodies to detect pulldown products. Immunoblotting of 10% of total lysates utilized in binding assays is shown.

Similarly, coexpression of GRIP with its potential partners in COS7 cells demonstrated coprecipitation, and thus complex formation, with the EphB2 and EphA7 receptors as well as the ephrin-B ligands but not with a variety of control receptors (Figure 3D and data not shown). Confirmation of specific interaction between syntenin and EphA7 and ephrin-B1 was also obtained (data not shown).

PICK1 Gets Tyrosine Phosphorylated When Complexed with EphB2

The demonstration that PDZ-containing proteins directly interact with the Eph receptors defines a new class of proteins that can associate with vertebrate receptor tyrosine kinases. Many of the previously identified interactors with receptor tyrosine kinases have been shown to be substrates of the kinase, and their tyrosine phosphorylation often regulates their activity in some way (reviewed by van der Geer et al., 1994). To examine whether PDZ-containing proteins could be substrates for the Eph receptors, we determined whether PICK1 was tyrosine phosphorylated upon its association with the EphB2 receptor. Indeed, levels of PICK1 tyrosine phosphorylation precisely correlated not with the levels of its expression but only with whether it was associated with EphB2 (Figure 3A, lane 2; note that levels of PICK1 tyrosine phosphorylation correlate not with PICK1 protein levels but with association with EphB2). Comparable experiments did not reveal that GRIP was tyrosine phosphorylated when in complex with EphB2 nor that PICK1 was tyrosine phosphorylated when complexed with the ephrin ligands. Thus, at least some PDZ-containing proteins become tyrosine phosphorylated when complexed to receptor tyrosine kinases.

PICK1 Induces Clustering of EphB2 and Ephrin-B Ligands

Our efforts to identify PDZ interactions for Eph family members was inspired by observations that Eph receptors and ligands were found in clusters in vivo and that clustering was apparently required for their ability to activate their partners (Davis et al., 1994; Henkemeyer et al., 1994). We thus were motivated to explore whether PDZ-containing proteins could actually cause clustering of Eph family members. COS7 cells have been utilized in the past to demonstrate that PSD-95 family members form coclusters when coexpressed with either NMDA receptors or Shaker-type K^+ channels, as dramatically demonstrated by double immunofluorescence microscopy (for example, Kim et al., 1995, 1996). We thus performed similar experiments using Eph receptors, ephrins, and PDZ-containing proteins. When PICK1, EphB2, and ephrin-B1 are individually expressed in COS7 cells, a diffuse staining pattern is detected for all three proteins after visualization with TR or FITC-coupled secondary antibodies (Figures 4A, 4B, and 4C). When PICK1 is coexpressed with either EphB2 or ephrin-B1, dramatic coclustering is observed (Figures 4D and 4F), though in

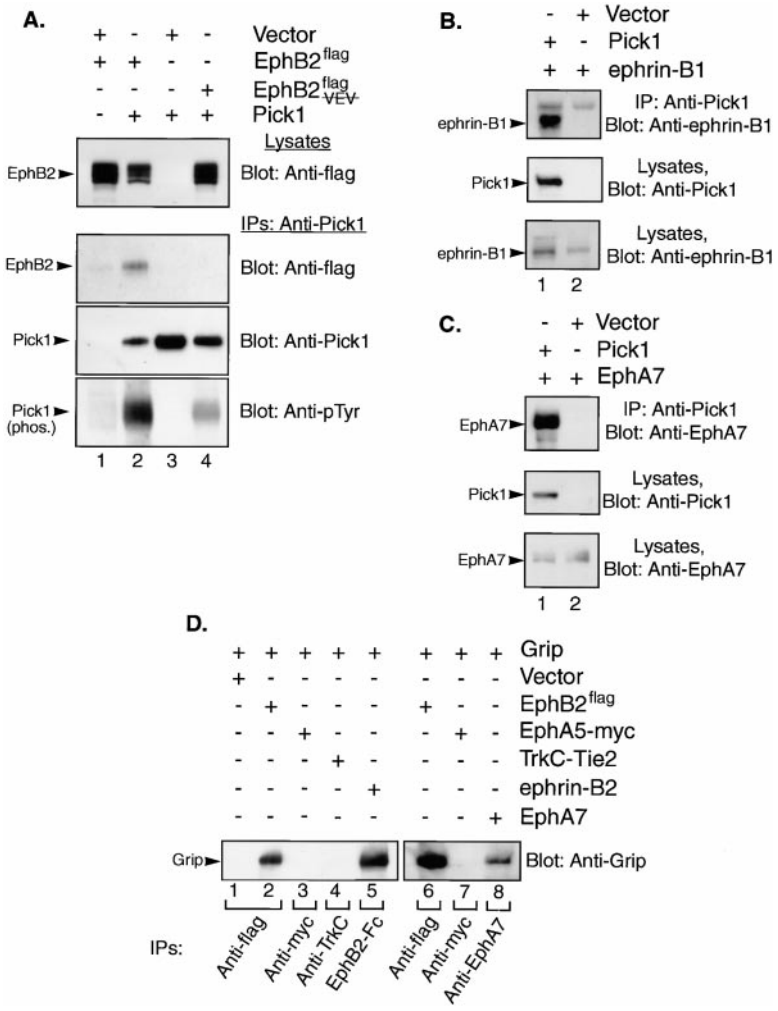

Figure 3. Interactions of Eph Family Members and PDZ Proteins in Mammalian Cells and Tyrosine Phosphorylation of PICK1 When Complexed with EphB2

Eph family receptors and ligands interact with PICK1 (A–C) and GRIP (D) in transfected mammalian cells. Various combinations of expression constructs, as indicated above each panel, were transfected into COS7 cells. The cells were subsequently lysed and immunoprecipiated with the indicated antibodies and then immunoblotted with indicated antibodies to test for coprecipitation of associated proteins; in some cases, lysates from the cells were directly immunoblotted to control for protein expression levels as indicated.

(A) PICK1 specifically associates with the C terminus of EphB2 in a VEV-dependent fashion and is tyrosine phosphorylated when associated with EphB2.

(B) PICK1 specifically associates with ephrin-B1.

(C) PICK1 specifically associates with EphA7.

(D) GRIP specifically associates with EphB2, ephrin-B2, and EphA7.

multiple experiments, coclustering with ephrin-B1 seemed more efficient than with EphB2; since only cell surface receptors or ligands were visualized in the above assays, clusters were not due to aggregates forming intracellularly. Coclustering depends on the C-terminal VEV residues of EphB2, since coclusters were not observed when using $EphB2_{VEV}^{flag}$ (Figure 4E). PICK1 was also able to cluster ephrin-B2 and ephrin-B3 ligands but not ephrin-A members (data not shown). However, we were not able to induce coclusters of Eph members with two membrane-associated guanylate kinase (MAGUK) proteins, PSD-95 and hCask, or with GRIP or syntenin (data not shown).

Eph Family Members Associate with PDZ Proteins in the Brain and Colocalize with PDZ Proteins at Synapses

Having demonstrated that associations between Eph family members and PDZ-containing proteins can occur when these proteins are expressed in heterologous cells, we next decided to explore whether these associations are physiologically relevant by examining whether they normally occur in vivo. We thus performed anti-PICK1 immunoprecipitations from protein extracts of adult mouse forebrain and whole mouse embryos (E12.5). Consistent with the presence of in vivo complexes between PICK1 and EphB2, EphB2 could be detected by immunoblotting in the PICK1 immunoprecipitates from adult brain but not in nonimmune serum precipitates from the same sample (Figure 5A, top panel; compare lanes 1 and 2). No EphB2 could be detected in the PICK1 immunoprecipitates from the E12.5 brain sample (Figure 5A, top panel, lane 3), but immunoblotting of either the immunoprecipitates or total lysates revealed much lower PICK1 levels at this earlier developmental stage (Figure 5A, lower panel; compare lanes 1 and 3 and lanes 4 and 5), presumably making detection of any complexes very difficult. Interaction between PICK1 and EphB2 was also detected in P3 mouse hindbrain protein extracts (data not shown).

Coprecipitation of ephrins and PICK1 could not be detected (data not shown), though similar difficulties in detecting coprecipitation have been noted for other partners of PDZ proteins (Hata et al., 1996; Niethammer et al., 1996; Dong et al., 1997), perhaps because detergents may disrupt weak PICK1–ephrin complexes or because only a small proportion of ephrins may be associated with any one PDZ-containing protein. We thus attempted to obtain other types of evidence that PDZ-mediated clustering of ephrins, as well as of Eph receptors, might be physiologically relevant. Since partners

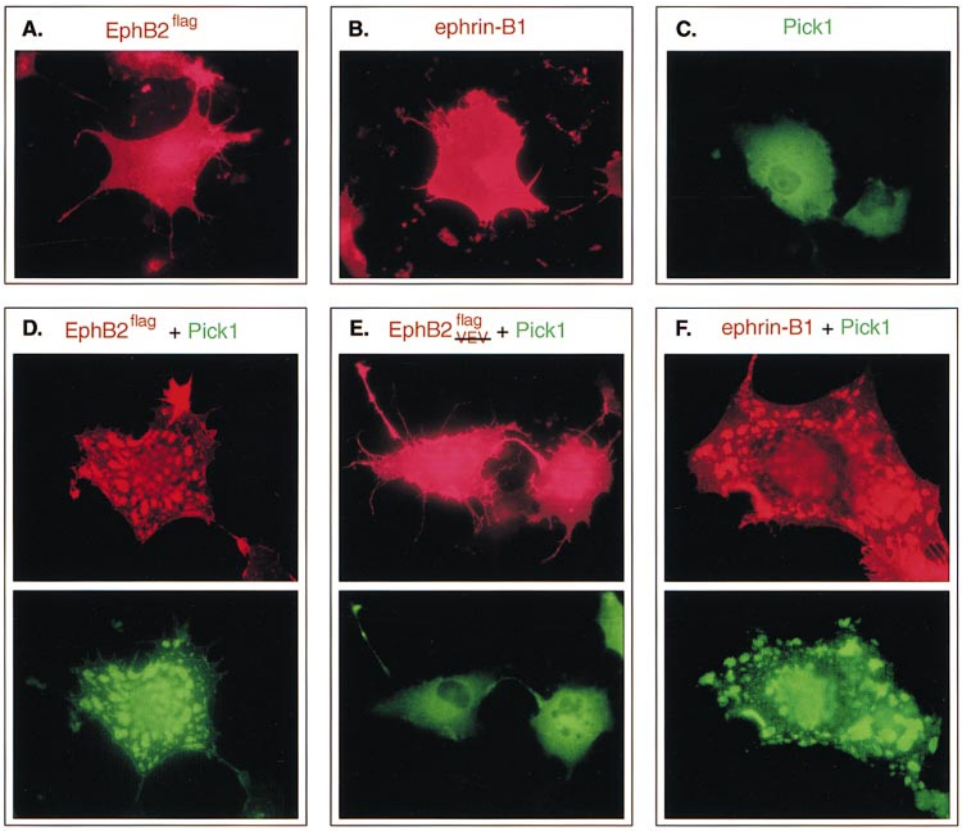

Figure 4. PICK1 Induces Clustering of Eph Receptors and Ligands in Heterologous Cells

COS7 cells were transfected with the various expression constructs listed on top of the panels, and 48 hr posttransfection immunofluorescence was performed to determine the cellular distributions of EphB2^{flag}, ephrin-B1, and PICK1. When expressed individually, EphB2^{flag} (A) and ephrin-B1 (B) were diffusely expressed on the cell surface, while PICK1 was diffusely noted in the cytoplasm (C). Coclustering was observed when PICK1 was expressed with EphB2^{flag} (D) or ephrin-B1 (F) but not when PICK1 was expressed with a version of EphB2 lacking VEV at its C terminus (E). The two colors seen to originate from the secondary antibodies utililized (red stain originates from TR and green stain originates from FITC).

of PDZ proteins are often localized to synapses, we decided to determine whether either Eph receptors or their ligands were synaptically localized. Thus, subcellular fractionations on adult rat cortex were performed using a differential centrifugation procedure (Li et al., 1996; Wang et al., 1997). PSD-95, a protein highly concentrated in the postsynaptic density, is highly enriched in crude synaptosomes and is further enriched in more purified pre- and postsynaptic membranes, while it is not found in the cytosolic fraction of synaptosomes or in the synaptic vesicle fraction enriched in synaptophysin (Figure 5B; Li et al., 1996; Wang et al., 1997). The GluR2/3 subunits of the AMPA receptor are also enriched in synaptic membranes, though not as highly as PSD-95 (Figure 5B). Ephrin-B ligands, EphB2 receptors, and GRIP were also enriched in crude synaptosomes and in the pre- and postsynaptic membrane to an extent similar to GluR2/3, indicating their localization to synaptic membranes (Figure 5B). Interestingly, PICK1 was present in synaptic membranes, though not as enriched as ephrin-B, EphB2, and GRIP (Figure 5B). PICK1 was, however, substantially more enriched in synaptic membrane fractions than Neurofilament H, an axonal protein present but not enriched at synapses (Figure 5B). These data are consistent with the notion that PICK1 is present not only at synapses but elsewhere in the adult rat cortex as well. Similar studies were not performed with syntenin, as immunoblotting antibodies for syntenin are not currently available.

To localize the Eph family members and PDZ proteins at a higher resolution, double-labeling studies were performed using confocal microscopy on low-density embryonic hippocampal neuronal cultures. Consistent with the above subcellular fractionation experiments, immunohistochemical analysis of cultured embryonic hippocampal neurons shows that ephrin-B ligands and EphB2 as well as PICK1 are concentrated and clustered at synapses along the neuronal processes in these cultures, as judged by colocalization with the synaptic marker synaptophysin in overlays (Figure 6); previous studies with GRIP have similarly localized it to synapses in embryonic hippocampal neuronal cultures (Dong et al., 1997). Thus, Eph receptors and ligands seem to colocalize with PDZ proteins in adult rat cortex as well as in embryonic hippocampal neuronal cultures.

Discussion

Receptor tyrosine kinases represented a receptor class that had not previously been shown to interact with

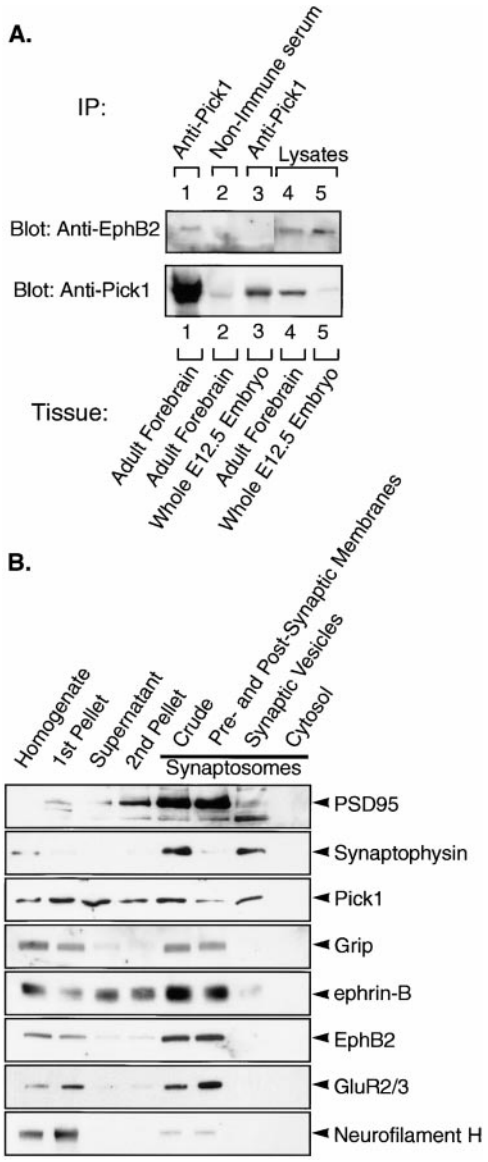

Figure 5. Coassociation of EphB2 and PICK1 in Brain Extracts and Localization of EphB2, Ephrin-B Ligands, GRIP, and PICK1 in Subcellular Fractions Enriched for Synaptic Membranes

(A) Protein extracts (3 mg) derived from the indicated tissue sources were immunoprecipitated with EphB2-specific antibodies and immunoblotted for PICK1, revealing coprecipitation of PICK1 with EphB2 in adult forebrain extracts; 50 μg of lysate are depicted as controls.

(B) Subcellular fractionations of adult rat cortex were performed as described in Experimental Procedures with progressive enrichment for synaptic membranes as verified by immunoblotting these fractions for control proteins, such as PSD-95 (a synaptic membrane marker), synaptophysin (a synaptic vesicle localized marker for the synaptic vesicle fraction), and GluR2/3 (AMPA receptor subunits localized to synaptic membranes). Immunoblotting revealed that EphB2, ephrin-B, and GRIP were enriched in the synaptic membrane fraction to a similar extent as GluR2/3. PICK1 was present but not as highly enriched in the synaptic membrane fraction (though it was present there and more highly enriched than Neurofilament H, an axonal protein present but not enriched at synapses) and was also enriched in synaptic vesicles; thus, PICK1 is most likely present at multiple locations within, as well as outside of, synapses in the adult rat cortex.

PDZ-containing proteins in vertebrates. However, such a link had been made in the pathway that controls vulval induction in *C. elegans* (Hoskins et al., 1996; Simske et al., 1996). The Eph family and its membrane-linked ephrin-B ligands seemed like ideal candidates for localization by PDZ-containing proteins. Clustering of Eph family receptors and their ligands had been suggested to be important for the function of this family in neuronal guidance or during the establishment of boundaries between neighboring cell compartments in the nervous system or the vasculature (Gale and Yancopoulos, 1997; Flanagan and Vanderhaeghen, 1998; Holland et al., 1998; Wang et al., 1998; Yancopoulos et al., 1998). We have found that many of the Eph receptors, as well as their ephrin-B ligands, contain consensus C-terminal motifs reminiscent of those binding PDZ domains. Among other receptor tyrosine kinases examined, it is notable that MuSK and ErbB2, both of which are clustered at the NMJ, have consensus PDZ recognition motifs at their C termini. Pursuing the possibility that motifs at the C terminus of particular receptor tyrosine kinases may interact with PDZ domains, we have shown that PDZ-containing proteins specifically bind and cluster both Eph receptors and their ligands in heterologous cells, and we provide evidence that these interactions are physiologically relevant. Included among this evidence are our findings that Eph receptors and ligands can be colocalized with PDZ proteins at neuronal synapses, suggesting fascinating new roles for the Eph family.

Since ephrins must be clustered in order to activate their receptors on an apposing cell, and since such clustering may be regulated by various growth factor treatments and the PKC pathway (Bruckner et al., 1997; Stein et al., 1998), exploring the role of PDZ proteins in such processes becomes a high priority. Remarkably, PICK1 was first discovered as a protein interacting with PKC (Staudinger et al., 1995, 1997), suggesting that it is a good candidate for playing a role in PKC regulation of clustering. In addition to the requirement for clustering of both Eph receptors and ligands during engagement and activation of their partners, subcellular localization via PDZ interactions may be critical for other aspects of Eph function, even outside of the synapse. Thus, Eph family members may need to be localized to the tip of growing axons during neuronal guidance or to the apposing cell surfaces of two adjacent cells during boundary formation or vascular patterning.

Contemplating the multiplicity of potential combinations of Eph, ephrin-B, and PDZ partners is quite overwhelming but could certainly allow for many variations on a theme. Our findings have probably only begun to pair potential partners to each other. Coupling a particular Eph receptor to one PDZ protein may bring it into a very different macromolecular complex than when it is coupled via another PDZ protein; the signaling and functional sequelae of these different couplings for the same receptor could be profound. It will be fascinating to determine whether particular biological processes involve different partners. For example, one could imagine that Eph receptors and/or ligands are coupled via one set of PDZ proteins when they mediate repulsive interactions but via a completely different set when involved in boundary establishment. Strikingly, the ability of

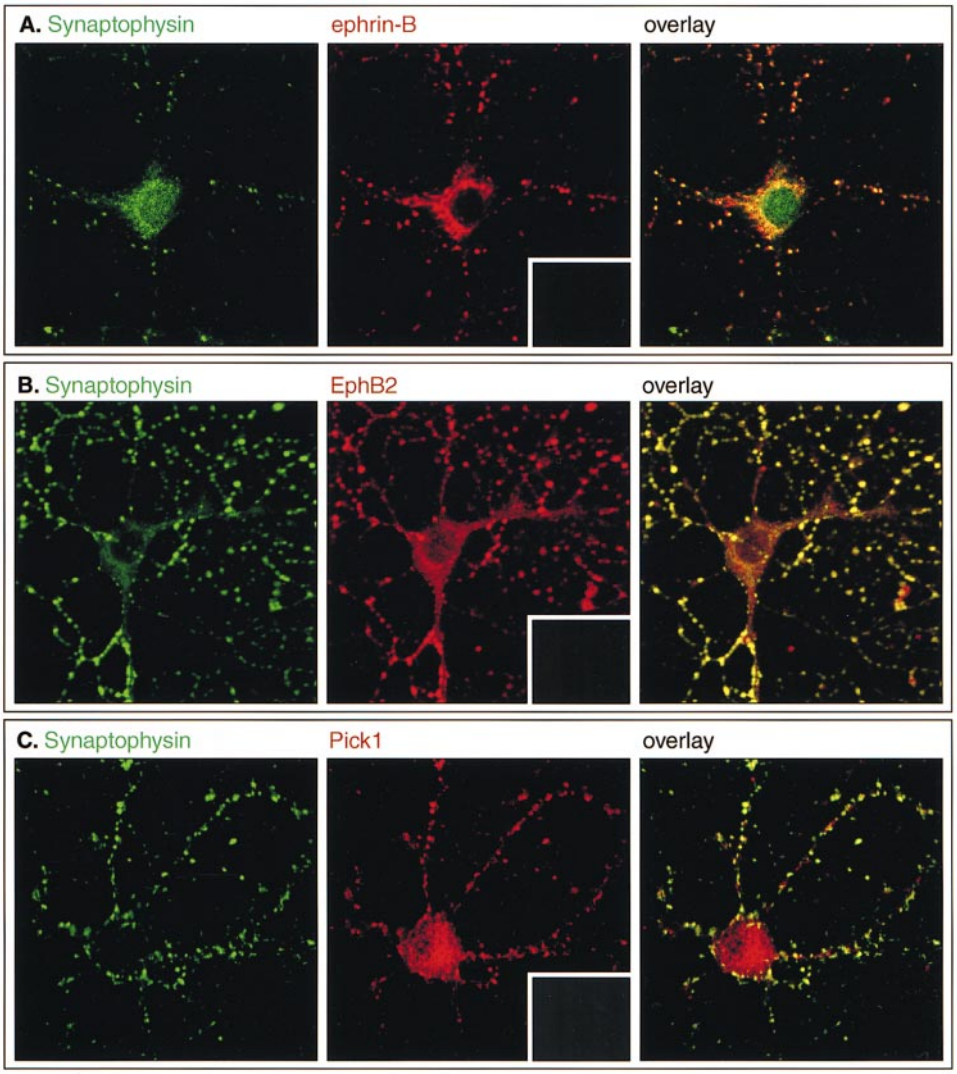

Figure 6. EphB2, Ephrin-B, and PICK1 Are Synaptically Localized in Cultured Rat Hippocampal Neurons

Hippocampal neurons were isolated from rats at E17, cultured for 2 weeks, and then double labeled with antibodies for synaptophysin (left panels) as well as antibodies specific for ephrin-B, EphB2, and PICK1 (middle panels), which were visualized with either Cy2 (green color for synaptophysin) or Cy3 (red color for center panels). Colocalization was demonstrated in overlays (right panels) in which yellow color indicates simultaneous labeling with both Cy2 and Cy3. Insets in the middle panels show specificity controls in which the immunostaining was done in the presence of competition with corresponding antigen used to generate each antibody.

PICK1 to cluster Eph family members represents the first example of clustering by a PDZ protein that is not in the PSD-95 subfamily. It will be interesting to see whether other classes of PDZ proteins can also cluster Eph family members and whether this leads to different functional sequelae. In addition to coupling Eph receptors to other classes of cell surface receptors or to intracellular proteins, PDZ proteins may also mediate coclustering of different Eph receptors with each other or coclustering of Eph receptors with ephrin ligands on the same cell surface.

Localization of Eph receptors and ligands to the synapse suggests previously unexpected roles for this family. Thus, the same molecular players involved in guiding and shaping initial neuronal contacts may continue to be involved as these contacts develop into functional synapses; such roles should perhaps have been anticipated based on the high levels of Eph receptors in synapse-rich structures in the adult, such as the hippocampus (for example, Maisonpierre et al., 1993; Valenzuela et al., 1995a). One could imagine that the Eph family plays a stabilization or adhesive role at the synapse. Alternatively, the Eph family may be involved in functions more analogous to its developmental roles in repulsion and thus contribute to synaptic plasticity by destabilizing synapses. Furthermore, the reciprocal signaling ability of Eph receptors and the ephrin-B ligands (Holland et al., 1996; Bruckner et al., 1997) suggests that they may be involved in bidirectional signaling at the synapse. Since PICK1 is known to interact with PKC, it may provide for functional coupling of Eph receptors and ligands to intracellular kinase cascades. It will certainly

be of interest to determine the list of other molecules that are linked to the Eph receptors and ligands at the synapse and to learn whether the Eph family provides a signaling capability that can modify the activity of associated synaptic components, such as neurotransmitter receptors, ion channels, adhesion molecules, and intracellular signaling molecules. The presence of distinct receptor types within the same complexes is certainly suggested by the recent observation that PICK1—like GRIP—can bind and cluster AMPA receptors (Xia et al., submitted) as well as Eph family members. In addition, Eph family members in large complexes may otherwise impact intracellular signaling. Recent studies have identified a synaptic ras GTPase–activating protein (synGAP) that associates with the PDZ domains of the PSD-95 protein family (Chen et al., 1998; Kim et al., 1998), and it has been reported that a rasGAP also associates with EphB2 (Holland et al., 1997). Proteins (such as extracellular signal–related protein kinase 2– [ERK2-] type mitogen-activated protein kinase [Suzuki et al., 1995], α calcium/calmodulin-dependent kinase II [αCaMKII] [Goldenring et al., 1984; Kelly et al., 1984], and CRIPT [Niethammer et al., 1998]) that regulate phosphorylation of cytoskeletal proteins or the interaction of proteins with the cytoskeleton are enriched in synapses. Thus, the clustering of ephrins and their receptors may reflect their role in synapse remodeling via regulation of the synaptic cytoskeleton. It is also interesting to consider the possibility that by inducing tyrosine phosphorylation of associated PDZ proteins, as we have shown can be the case with PICK1, Eph receptors may modify the coupling functions of these proteins and thus alter the assembled macromolecular complex itself.

There are many analogies between synapses in the central nervous system and the most studied individual synapse of all, i.e., the NMJ. Recent studies have demonstrated crucial roles for receptor tyrosine kinases at the NMJ. In particular, agrin activation of the MuSK receptor tyrosine kinase is responsible for initiating all aspects of NMJ formation (DeChiara et al., 1996; Glass et al., 1996), and MuSK remains highly localized to the postsynaptic muscle membrane in the adult (Valenzuela et al., 1995b). In addition, ErbB receptors are localized to the postsynaptic side of the NMJ (Zhu et al., 1995), where they play key roles in regulating levels of acetylcholine receptor expression in response to nerve-derived neuregulin. It does not seem too surprising that we have found that MuSK and ErbB receptors have PDZ-binding motifs at their C termini; indeed, we have recently found that PDZ proteins, including PICK1, associate with the C terminus of MuSK (R. T. et al., unpublished data). However, it does seem quite surprising that, despite the importance of receptor tyrosine kinases at the NMJ, not much attention has been paid to the role of such receptors in central synapses. We now provide the first demonstration of a family of receptor tyrosine kinases whose members are localized to central synapses within PDZ-containing complexes. Since this is the largest family of receptor tyrosine kinases, it may have the diversity required to differentially act at different types of synapses, particularly when coupled to the diversity of choice possible with PDZ-containing partners. The roles of the Eph family at central synapses may ultimately prove to be as important as those served by other receptor tyrosine kinases at the NMJ or those played by the Eph family during development.

Experimental Procedures

Yeast Two-Hybrid Screens

To identify mammalian proteins that interact with the C termini of Eph family members, a mouse E11 Matchmaker cDNA library (Clontech) was transformed into the *Saccharomyces cerevisiae* L40 reporter strain bearing either the LexA–EphB2–C term or LexA–EphA7–C term fusion plasmids; cDNA clones that resulted in a HIS3+/LacZ+ phenotype were then screened for interaction with several additional baits, including the LexA-EphB2(-VEV) and LexA-EphA7(-QV) baits and the LexA-ephrin-B1 bait, as well as control baits such as LexA-lamin. Standard yeast manipulations, yeast two-hybrid screens, and the *HIS3* and *lacZ* reporter assays were performed as previously described (Vojtek et al., 1993; Schreiber-Agus et al., 1995).

GST Pulldown Assays

Five micrograms of GST or GST fusion proteins were incubated with 500 μg of COS7 cell lysates expressing the indicated receptors, and binding asssays were performed as indicated in Hannon et al. (1993). Following separation on SDS-PAGE gels and transfer to nylon membranes, immunoblotting was performed with the indicated antibodies.

Antibodies

The affinity-purified rabbit polyclonal PICK1 antibody utilized for the immunoprecipitations with COS7 lysates (Figure 3) and for the clustering assay in COS7 cells (Figure 4) was generated with a peptide (ADLDYDIEEDKLGIP) corresponding to the N-terminal region of mPICK1. The anti-PICK1 affinity-purified rabbit polyclonal utilized in Figure 5 was generated against a longer peptide containing residues 2–31 of mPICK1. The anti-GRIP antibody is an affinity-purified rabbit polyclonal antibody raised against a peptide to the C terminus of GRIP. The anti-EphA7 affinity-purified rabbit polyclonal antibody was raised against the KADQEGDEELYFHFKFPGTK TYID peptide from the juxtamembrane region of EphA7. The anti-myc 9E10 mouse monoclonal antibody was obtained from Calbiochem. The mouse monoclonal anti-phosphotyrosine antibody (4G10) was obtained from UBI. The anti-EphB2 rabbit polyclonal antibody was kindly provided by Sacha Holland and Tony Pawson. The anti-ephrin-B(C-18) antibody was obtained from Santa Cruz. The mouse monoclonal anti-PSD-95 antibody was obtained from ABR. The mouse monoclonal anti-synaptophysin antibody was obtained from Sigma.

COS7 Expression Studies, Immunoprecipitations, and Immunoblotting

The PICK1 expression construct was generated by subcloning the full length mouse pick1 cDNA into the pMT21 mammalian expression vector. The GRIP expression construct was generated by subcloning the full length rat GRIP cDNA into the pBK mammalian expression vector. $EphB2^{flag}$ was constructed in pMT21 with a flag tag at the N terminus, and $EphB2_{\Delta VEV}^{flag}$ was constructed by introducing a stop codon at the −3 amino acid position of the C terminus, therefore deleting the last 3 residues (-VEV). The EphA7 construct was generated by subcloning the rat ephA7 cDNA into pMT21. EphA5-myc was constructed by introducing a triple myc tag at the C terminus of the rat ephA5. COS7 cell transfections, cell lysis, tissue lysis, immunoprecipitations, and immunoblotting were performed as previously described (Henkemeyer et al., 1994; Holland et al., 1997).

Immunohistochemistry

Clustering Assay in COS7 Cells

COS7 cells plated on coverslips in 35 mM wells were transfected with each of the indicated expression constructs. Forty-eight hours posttransfection, cells were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 15 min. The anti-flag mouse monoclonal M2 antibody (to detect $EphB2^{flag}$ or $EphB2_{S}^{flag}$)

(Kodak Scientific) and EphB2-Fc (to detect ephrin-B1) were then utilized followed by permeabilization of the cells and incubation with the anti-PICK1 antibody; this protocol allowed one to detect only cell surface receptors and ligands. Goat anti-mouse conjugated to TR (to detect M2), goat anti-human (to detect EphB2-Fc), or goat anti-rabbit conjugated to FITC (to detect PICK1) were utilized as secondary antibodies to visualize the proteins. All secondary antibodies were obtained from Jackson ImmunoResearch Laboratories. Photos were taken under fluorescence microscopy at 40× objective.

Rat Hippocampal Cultures

E17 rat hippocampal cultures were prepared as previously described (Banker and Cowan, 1977; Goslin and Banker, 1991) and plated on coverslips. After 10–14 days in vitro, cells were washed with PBS twice and fixed with methanol at −20°C for 15 min. Cells were washed three times with PBS + 0.1% triton (PBST) and incubated with the various affinity-purified primary antibodies (anti-PICK1, anti-EphB2, anti-ephrin-B, and mouse anti-synaptophysin) for 2 hr at 25°C. Cells were washed with PBST three times and incubated with secondary antibodies (donkey anti-mouse Cy2 and goat anti-rabbit Cy3) for 2 hr at 25°C to visualize the proteins. Cells were washed three times with PBST and mounted by using Fluoromount-G. Secondary antibodies conjugated to Cy2 or Cy3 were purchased from Jackson Laboratories. Fluoromount-G was purchased from Southern Biotechnology Associates (Birmingham, AL). Photos were taken with confocal microscopy, model MRC1024 from Biorad, at the 60× objective.

Synaptosomal Fractionation

Synaptosomes were prepared as described by Li et al. (1996) with modification. Four adult rat cortices were homogenized in 36 ml homogenization buffer (HB; 320 mM sucrose, 4 mM HEPES [pH 7.4], 1 mM EGTA, and 1 mM PMSF) with ten strokes at 900 rpm of a loose-fitting glass Teflon homogenizer (Kontes, #22). The homogenate was centrifuged at 1000 g for 10 min. The supernatant was collected and centrifuged at 12,000 g for 15 min, and the second pellet was resuspended in 24 ml HB and centrifuged at 13,000 g for 15 min. The resulting pellet represented a crude synaptosomal fraction. This crude fraction was lysed by osmotic shock and homogenized by three strokes in a glass Teflon homogenizer at 2000 rpm, and the homogenate was spun at 33,000 g for 20 min to yield a supernatant and pellet (pre- and postsynaptic membranes). The supernatant was spun at 251,000 g for 2 hr. The resulting supernatant (cytosol) contained soluble proteins, and the pellet (synaptic vesicles) contained synaptic vesicle proteins. Ten micrograms of each fraction was separated on SDS-PAGE gels, and Western blot analyses were performed with the indicated antibodies.

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Note Added in Proof

Following submission of this manuscript, a report by Hock et al. revealed that the Eph family of RTKs is capable of interacting with the PDZ-containing protein AF6: Hock, B., Bohme, B., Karn, T., Yamamoto, T., Kaibuchi, K., Holtrich, U., Holland, S., Pawson, T., Rubsamen-Waigmann, H., and Strebhardt, K. PDZ-domain-mediated interaction of the Eph-related receptor tyrosine kinase EphB3 and the ras-binding protein AF6 depends on the kinase activity of the receptor. Proc. Natl. Acad. Sci. USA *95*, 9779–9784.