Ephrin B proteins function as ligands for B class Eph receptor tyrosine kinases and are postulated to possess an intrinsic signaling function. The sequence at the carboxyl terminus of B-type ephrins contains a putative PDZ binding site, providing a possible mechanism through which transmembrane ephrins might interact with cytoplasmic proteins. To test this notion, a day 10.5 mouse embryonic expression library was screened with a biotinylated peptide corresponding to the carboxyl terminus of ephrin B3. Three of the positive cDNAs encoded polypeptides with multiple PDZ domains, representing fragments of the molecule GRIP, the protein syntenin, and PHIP, a novel PDZ domain-containing protein related to Caenorhabditis elegans PAR-3. In addition, the binding specificities of PDZ domains previously predicted by an oriented library approach (Songyang, Z., Fanning, A. S., Fu, C., Xu, J., Marfatia, S. M., Chishti, A. H., Crompton, A., Chan, A. C., Anderson, J. M., and Cantley, L. C. (1997) Science 275, 73–77) identified the tyrosine phosphatase FAP-1 as a potential binding partner for B ephrins. In vitro studies demonstrated that the fifth PDZ domain of FAP-1 and full-length syntenin bound ephrin B1 via the carboxyl-terminal motif. Lastly, syntenin and ephrin B1 could be co-immunoprecipitated from transfected COS-1 cells, suggesting that PDZ domain binding of B ephrins can occur in cells. These results indicate that the carboxyl-terminal motif of B ephrins provides a binding site for specific PDZ domain-containing proteins, which might localize the transmembrane ligands for interactions with Eph receptors or participate in signaling within ephrin B-expressing cells.

Among the large number of receptor tyrosine kinases identified in metazoan organisms, the members of the Eph family are unusual in several respects. Although only one Eph receptor tyrosine kinase is known to be encoded by the Caenorhabditis elegans genome (the \(\text{cav-1}\) gene product (2)), vertebrates typically possess up to 14 genes for Eph receptors, suggesting that these tyrosine kinases may be important in controlling complex cellular interactions (3, 4). Consistent with this possibility, C. elegans VAB-1 regulates morphogenetic cell movements during ventral closure in the embryo (2) while vertebrate Eph receptors have been implicated in controlling axon guidance and fasciculation, in specifying topographic map formation within the central nervous system, in organizing the movements of neural crest cells during development, in direct fusion of epithelial sheets in closure of the palate, and in angiogenesis (5–15).

Early work on the expression patterns of EphB2 (formerly Nuk) suggested that this receptor is clustered at sites of cell-cell junctions in the developing mouse midbrain and raised the possibility that Eph receptors might mediate signals initiated by direct cell-cell interactions (5). Several lines of evidence support the notion that Eph receptors are normally activated by ligands that are physically associated with the surface of an adjacent cell. All known ligands for the Eph receptors (termed ephrins) are related in sequence but can be divided into two groups based on their carboxyl-terminal motifs. The ephrin A class of ligands become modified by a carboxyl-terminal glycosylphosphatidylinositol moiety, through which the ligand is anchored to the surface of the ligand-expressing cell (7, 9, 16). In contrast, B-type ephrins possess a transmembrane element and a highly conserved cytoplasmic tail comprised of 82–88 carboxyl-terminal residues (17–22). The Eph receptors can, in turn, be divided into A and B subgroups based on their sequence similarity and their propensity to bind soluble forms of either A or B type ephrins, respectively (4, 23, 24). However, although soluble ephrins bind tightly to the relevant receptors, consistent activation of Eph tyrosine kinase activity requires either that the ligands be artificially clustered into oligomers or that receptor-expressing cells be co-cultured with cells expressing membrane-associated ephrins (18). These data suggest that the ability of ephrins to aggregate and thereby activate Eph receptors depends on their attachment to the cell surface, consistent with the view that Eph receptor signaling involves cell-cell interactions. During embryonic development in the mouse, Eph receptors and their ligands are expressed in dynamic but complementary patterns, indicating that Eph receptors are likely activated at boundaries where Eph and ephrin-expressing cells are directly juxtaposed to one another (23, 25).

Genetic analysis of Eph receptor function in C. elegans and the mouse has indicated that Eph receptors have both kinase-dependent and kinase-independent modes of signaling and raised the possibility that B-type Eph receptors and ephrins might mediate bidirectional cell-to-cell signaling (2, 6). Of interest, the binding of Eph receptors to transmembrane ephrin B1 or ephrin B2, as well as treatment of ephrin B-expressing cells with platelet-derived growth factor, leads to the phosphorylation of the ephrins on tyrosine residues within their highly conserved cytoplasmic tails (26, 27). Furthermore, expression of the cytoplasmic tail of a Xenopus ephrin B molecule leads to
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Fig. 1. Amino acid sequence of the cytoplasmic domains of the human B ephrins. Conserved residues among the three B ephrins are highlighted. Asterisks mark conserved tyrosines that are potential sites of phosphorylation. The potential PDZ domain binding site is underlined.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—The B ephrin carboxyl-terminal peptide probe of sequence biotin-Aca-GPPQSPNIPYYKV, related peptides NiPyYPKV, NiPYYKV, NiYPYKV, and DHQPyYNKD were synthesized as described previously (29).

Isolation of PDZ Domain-encoding cDNA Clones—A AElox 10.5 day mouse embryo expression library (Novagen) was plated at an initial density of 10,000 plaque-forming units/15-cm Petri plate. Library screening was performed using a biotinylated peptide probe conjugated to streptavidin-alkaline phosphatase following a procedure similar to that described by Sparkes et al. (30). To isolate more coding sequence for PHIP, an EcoRI/PstI fragment of PHIP cDNA (encoding amino acid residues 462–602) was radiolabeled with [α-32P]dCTP and used to screen the AElox 10.5 day mouse embryo library. The DNA sequencing of positive clones was carried out using the ALF automated DNA sequencer (Amersham Pharmacia Biotech).

Antibodies, Constructs, and Mutagenesis—Anti-ligand antibodies (Santa Cruz) were raised against residues 329–346 of ephrin B1. Anti-FLAG M2 monoclonal antibodies were purchased from Eastman Kodak Co. The expression construct of ephrin B1 cDNA in vector pJFE14 has been described (18). Full-length syntenin cDNA was subcloned in frame into the mammalian expression vector pFLAG CMV2 (Kodak) using standard cloning procedures. For GST fusion constructs, cDNA sequences of syntenin (full-length: residues 1–299; PDZ 1 + 2: residues 101–299; PDZ1: residues 101–211; PDZ2: residues 172–299) were cloned into pGEX4T2 (Amersham Pharmacia Biotech). FAP-1 (Fas-associated phosphatase) PDZ3 and FAP-1 PDZ5 constructs have been described (1). The ephrin B1 Val deletion mutation was constructed by the removal of nucleotides coding for the carboxyl-terminal Val–346 using a polymerase chain reaction-mediated protocol. The PspMI/EcoRI polymerase chain reaction fragment carrying the mutated region was subcloned into the full-length ephrin B1 cDNA in pJFE14. This mutation and all fusion constructs were confirmed by sequencing of both strands of the affected region.

Immunoprecipitation and Western Blot Analysis—Cos-1 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Transient transfections were performed using Lipofectin reagent and Opti-MEM medium (Life Technologies, Inc.) as outlined by the manufacturer. To reduce phosphorylation of ephrin B1 by binding to endogenously expressed EphB receptors or by stimulation with serum growth factors, transfected cells were transferred from 10-cm to 15-cm plates 24 h after transfection and serum-starved in Dulbecco’s modified Eagle’s medium, 0.5% fetal bovine serum 12 h prior to cell lysis. Transfected cells were rinsed once in phosphate-buffered saline A and lysed in phospholipase C lysis buffer (5) with 10 μg/ml leupeptin, 1 mM sodium vanadate, and 1 mM phenylmethylsulfonyl fluoride added. Immunoprecipitations were performed for 1 h at 4 °C using 1 μg of anti-ephrin B1 antibody or 1 μg of anti-interleukin 3 receptor α antibody with protein A-Sepharose. GST mixing experiments were carried out by 1-h incubation at 4 °C of lysate with 5–10 μg of fusion protein immobilized on glutathione-Sepharose. For the peptide competition experiments, peptides were included in the incubation with the GST fusion proteins at a final concentration of 100 μM. Beads for both immunoprecipitations and GST mixing experiments were washed 2–3 times in HNTG buffer (20 mM Hepes, pH 7.5, 10% glycerol, 0.1% Triton X-100, 150 mM NaCl) (5). Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis, transferred to Immobilon-P membrane (Millipore), and immunoblotted with the appropriate antibody. Blots were developed by Enhanced Chemiluminescence (Pierce).

Fluorescence Polarization Analysis—Binding constant determination and peptide competition studies were carried out using fluorescence polarization on a Beacon 2000 Fluorescence Polarization System (Pan Vera, WI) equipped with a 100-μl sample chamber. Fluorescein-labeled probes were prepared through reaction of B ephrin carboxyl-terminal peptides with 5-(and-6)-carboxyfluorescein, succinimidyl ester (Molecular Probes) and purified by reverse-phase high performance liquid chromatography. The authenticity of the fluorescein-labeled peptides was confirmed by mass spectroscopy. In the binding studies, the fluorescein-labeled peptide probe was dissolved in 20 mM Hepes, pH 7.0, 100 mM NaCl, and 2 mM dithiothreitol to a concentration of 25 nM and a known quantity of GST fusion protein was added. The reaction mixtures were allowed to stand for 10 min at room temperature prior to each measurement. All fluorescence polarization measurements were conducted at 22 °C.

RESULTS

Identification of Potential Binding Partners for the Putative PDZ Binding Site of B Ephrins—As one approach toward identifying proteins that interact with the cytoplasmic tails of B-type ephrins, we initially examined the carboxyl-terminal regions of the transmembrane ephrins for conserved peptide motifs that might bind modular domains of intracellular signaling proteins. The extreme carboxyl terminus of the three known B ephrins has a conserved sequence reminiscent of known or predicted binding sites for PDZ domains (Fig. 1). We employed two strategies to identify PDZ domain-containing proteins with the potential to recognize the B ephrins. Firstly, based on the known binding specificities of PDZ domains, we screened through the use of an oriented peptide library technique, revealed the fifth PDZ domain of the cytoplasmic tyrosine phosphate FAP-1 as a possible ephrin B binding partner (Fig. 2A). FAP-1 (also known as PTP-has and PTP-L1) has at least six PDZ domains, an element related to the Band 4.1 cytoskeletal polypeptide, and a carboxyl-terminal tyrosine phosphorylation site that is conserved among ephrin B1-family receptors.

\(^1\) The abbreviations used are: PDZ, PSD95/Dlg, ZO-1; GST, glutathione S-transferase; FAP, Fas-associated phosphatase.
phosphatase domain (31–33). The fifth PDZ domain binds in vitro to peptides with the consensus E(I/Y/V)Y(Y/K)(V/K/I), which closely matches the conserved carboxyl terminus of B-type ephrins (YYKV) (1).

A more direct approach to isolate ephrin B-binding proteins was undertaken by screening a cDNA expression library from a day 10.5 mouse embryo with a peptide probe based on the putative PDZ domain binding site of ephrin B3. The probe was a biotinylated peptide, biotin-Aca-GPPQ3PPNIpYYKV, conjugated to streptavidin-alkaline phosphatase. Although this peptide contained a phosphotyrosine residue at the –3 position relative to the carboxyl-terminal valine, we anticipated that the alkaline phosphatase used in the screen would at least partially dephosphorylate the probe, allowing detection of both tyrosine phosphorylation-dependent and independent binding. The screening of approximately 500,000 cDNA clones yielded four distinct cDNA products that bound to the ephrin B3 carboxyl-terminal peptide, of which 3 were subsequently found to contain PDZ domains upon sequence analysis (Fig. 2, B and C). One of these cDNAs encodes a portion of the adaptor protein GRIP, from the sixth PDZ domain to the carboxyl terminus (amino acid residues 642–1112). GRIP is an –180-kDa protein composed of seven PDZ domains, originally identified by its ability to bind the carboxyl terminus of AMPA receptors through PDZ domains 4 and 5 (34). A second cDNA isolated by this approach contained the entire coding sequence for the PDZ domain-containing protein syntenin. Syntenin was first reported as a transcript down-regulated during melanoma differentiation (termed Mda-9) and subsequently shown to interact via its two PDZ domains with the carboxyl terminus of the transmembrane syndecan proteins (35, 36). A third clone identified in this screen was a partial cDNA encoding the carboxyl-terminal fragment of a novel PDZ domain-containing protein (termed PHIP for ephrin interacting protein). Analysis of the sequence of the PHIP cDNA fragment revealed the presence of two adjacent PDZ domains followed by a 50-amino acid carboxyl-terminal stretch. The PHIP cDNA fragment was subsequently used as a probe to isolate a cDNA from a day 10.5 mouse embryo library. The predicted sequence of PHIP indicates that it encodes a total of three PDZ domains and is closely related to PAR-3, a C. elegans protein involved in regulating polarity of the early embryo (Fig. 2D) (37). Of these candidates, FAP-1 PDZ5 and syntenin were further investigated for their binding to B ephrins.

Syntenin and FAP-1 PDZ5 Bind Ephrin B1 in Vitro—To determine whether either syntenin or FAP-1 could interact with ephrin B1 in vitro, GST fusions containing the fifth PDZ domain of FAP-1 or full-length syntenin were incubated with lysates of ephrin B1-transfected COS-1 cells. Recovery of these immobilized GST fusion proteins and immunoblotting of associated proteins with anti-ephrin B1 antibody revealed that both FAP-1 PDZ5 and full-length syntenin were able to bind intact ephrin B1 (Fig. 3, A and C). The region of syntenin required for binding to ephrin B1 was mapped using GST fusions containing defined fragments of the syntenin protein. The minimal sequence necessary for a strong interaction included both PDZ domains of syntenin but not the amino-terminal third of the protein (Fig. 3D). Interestingly, both PDZ domains of syntenin are also required for binding to the carboxyl-terminal sequence of syndecans, suggesting that the involvement of two PDZ domains in the binding of a single target site may be a common feature of syntenin interactions (36). While the syntenin PDZ1 domain alone was unable to associate with ephrin B1, the second PDZ domain of syntenin alone exhibited a very weak interaction.

In these experiments, neither GST alone nor a GST fusion with the third FAP-1 PDZ domain showed detectable binding to ephrin B1. The identity of the –50-kDa band recognized by GST-FAP-1 PDZ3 is not known but its apparent size does not...
correlate with any of the three known B ephrins. Consistent with this finding, the binding specificity of FAP-1 PDZ3, as previously determined using an oriented peptide library, is significantly different from that of FAP-1 PDZ5 with a preference toward target sequences such as the QSLV-COOH motif in the Fas antigen (1, 33). The inability of the FAP-1 PDZ3 domain to bind ephrin B1 indicates a degree of specificity in recognition of ephrin B1 by PDZ domains.

A hallmark of many PDZ domain binding sites is a requirement for a carboxyl-terminal hydrophobic residue that contacts the PDZ domain through its side chain and carboxyl-terminal carboxylate group (1, 38, 39). The involvement of the carboxyl-terminal Val of ephrin B1 in specific binding to syntenin and FAP-1 PDZ5 was initially evaluated by expressing a deletion mutant of ephrin B1 lacking the terminal Val residue in COS-1 cells. Removal of the carboxyl-terminal Val from full-length ephrin B1 abrogated its binding to both syntenin and FAP-1 PDZ5 GST fusion proteins (Fig. 3, B and C).

As an alternative approach toward investigating the specificity of ephrin B1 interactions with PDZ domain proteins, we employed a specific peptide modeled on the carboxyl terminus of B-type ephrins in competition experiments. For this purpose, lysates of ephrin B1-transfected cells were incubated with either GST-syntenin or GST-FAP-1 PDZ5 in the presence of either GST-syntenin or GST-FAP-1 PDZ5.
absence of a peptide corresponding in sequence to the carboxyl-terminal six residues of B ephrins. The peptide successfully blocked syntenin and FAP-1 PDZ5 binding at a peptide concentration of 100 μM (Fig. 4, A and B). The addition of the unrelated peptide, DHQpYpYND, did not decrease binding, indicating the specificity of the peptide competition (Fig. 4 and data not shown).

**FAP-1 PDZ5 and Syntenin Display Differential Binding to Phosphopeptides**—Binding of B ephrins to their cognate Eph B receptors, expression of an activated Src tyrosine kinase, or treatment of ligand-expressing cells with platelet-derived growth factor results in tyrosine phosphorylation of residues in the ephrin cytoplasmic domain (26, 27). Preliminary evidence based on specific substitutions of the Tyr residues in the ephrin B1 tail suggests that the two tyrosines at the –2 and –3 positions within the PDZ domain binding site are among the phosphorylation sites. To investigate whether tyrosine phosphorylation of these residues might affect PDZ domain binding, the carboxyl-terminal peptide used for the peptide competition described above was also synthesized such that either one or both of the –2 and –3 tyrosine residues were phosphorylated.

The phosphorylated and unphosphorylated peptides were labeled with fluorescein and employed in fluorescence polarization experiments to obtain quantitative measurements of their affinities for FAP-1 and syntenin PDZ domains.

The GST-FAP-1 PDZ5 bound to a fluorescein-labeled NIYYKV peptide with an affinity of 9.9 ± 1.0 μM, while GST-FAP-1 PDZ3 binding was much weaker (65.0 ± 9.6 μM) (Fig. 5A). This is consistent with the GST mixing experiments that indicated FAP-1 PDZ3 does not interact stably with ephrin B1. Similar results were obtained when binding to the three different phosphorylated peptides was investigated, indicating that alternative tyrosine phosphorylation states of the B ephrin carboxyl-terminal sequence had little effect on binding to GST-FAP-1 PDZ5. Similar binding affinity values of 6.8 ± 0.8 μM, 15.4 ± 3.4 μM, and 8.4 ± 2.5 μM were obtained for the NIpYYKV, NIYpYKV, and NIpYpYKV peptides, respectively.

Fluorescence polarization experiments measuring GST-syntenin fusion protein binding to fluorescein-labeled NIYYKV and NIpYYKV peptides yielded nearly identical binding curves (Fig. 5B). Affinity values of 17.7 ± 1.2 μM and 15.4 ± 0.5 μM were obtained, indicating that phosphorylation at the –3 position tyrosine does not significantly affect the PDZ-domain interaction. However, the GST-syntenin fusion protein bound the pYpYKV peptide with a much lower affinity of 151.0 ± 20.9 μM, indicating that phosphorylation at the –2 Tyr can have a detrimental effect on binding to syntenin. A similar low affinity interaction was observed for the YpYKV peptide (data not shown).

**Ephrin B1 and Syntenin Can Associate in Cells**—We have pursued the possibility that B-type ephrins may interact with PDZ domain proteins in vivo by assaying whether ephrin B1 and syntenin associate when co-expressed in COS-1 cells. In cells co-transfected with ephrin B1 and syntenin (tagged at its N terminus with a FLAG epitope) immunoprecipitation of ephrin B1 specifically co-precipitated syntenin (Fig. 6). Precipitation with protein A-Sepharose alone or with an arbitrarily chosen antibody did not yield detectable syntenin, indicating that the interaction is specific. Further, co-immunoprecipitation experiments with the ephrin B1 Val deletion mutant, which fails to interact with PDZ domains in vitro, showed that ephrin B1 lacking the carboxyl-terminal Val did not detectably

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2 G. Mbamalu, S. Holland, and T. Pawson, unpublished results.
associate with syntenin (Fig. 6). While the truncated protein could be successfully immunoprecipitated by antibodies against ephrin B1, syntenin could not be co-immunoprecipitated with the mutant protein. These results demonstrate that ephrin B1 and syntenin can associate in cells and show that an intact PDZ domain binding site in ephrin B1 is necessary for its interaction with syntenin in vivo.

**DISCUSSION**

In an effort to identify components of the cytoplasmic domain that may contribute to ephrin B function, we have demonstrated that the carboxyl-terminal residues of B ephrins constitute a binding site for PDZ domains, a class of protein module known to mediate specific protein-protein interactions. Several lines of evidence indicate that the carboxyl-terminal YYKV sequence, conserved among all 3 known B ephrins, represents a PDZ domain binding site. Firstly, a biotinylated peptide probe with a sequence corresponding to the carboxyl-terminal residues of ephrin B3 identified cDNAs coding for the known PDZ domain-containing proteins syntenin and GRIP, as well as a cDNA for PHIP, a novel PDZ domain-containing protein. In addition, a fourth PDZ-containing protein, FAP-1, was identified as a binding candidate based initially on the predicted binding specificity of its fifth PDZ domain.

Secondly, in vitro studies with syntenin and FAP-1 have demonstrated specific interactions of the PDZ domains of these proteins with the carboxyl terminus of ephrin B1. The finding that the carboxyl-terminal Val residue of ephrin B1 is absolutely required for these interactions indicates that binding occurs in a manner characteristic of other PDZ domain interactions with carboxyl-terminal target sequences. Similar results were also obtained from in vitro binding experiments with ephrin B2 (data not shown), suggesting that PDZ domain interactions may be common to all B ephrins. In vitro experiments were also performed with separate GST fusions of GRIP PDZ6 and GRIP PDZ7. Interactions with ephrin B1 or with the fluorescent GNIYYKV peptide were not detected in GST mixing and fluorescence polarization experiments (data not shown). It is possible that binding to ephrin B1 may require both PDZ6 and PDZ7 of GRIP in a fashion reminiscent of the requirement of both syntenin PDZ domains for binding. We are currently investigating this possibility. Lastly, we have demonstrated that B ephrin-PDZ domain interactions can occur in vivo.

**FIG. 4.** FAP-1 PDZ5 and syntenin binding to ephrin B1 can be blocked by addition of peptides corresponding to the carboxyl-terminal sequence of B ephrins. Peptides of the indicated sequence were included at a concentration of 100 μM in incubations of GST fusion proteins with lysates of COS-1 cells transfected with ephrin B1. Associated proteins were separated on a 10% polyacrylamide-SDS gel and analyzed by immunoblotting with antibodies against ephrin B1. A, competition of FAP-1 PDZ5 binding to ephrin B1 using the indicated peptides. A peptide of sequence DHQpYpYND was added at a concentration of 100 μM as a negative control. Immunoprecipitation of ephrin B1 was included as a positive control. B, peptide competition of the binding of full-length syntenin to ephrin B1.

**FIG. 5.** Fluorescence polarization analysis of GST-FAP-1 PDZ3, GST-FAP-1 PDZ5, and GST-syntenin binding to fluorescein-labeled peptides corresponding to the carboxyl terminus of ephrin B1. A, solutions containing the indicated final concentration of GST-FAP-1 PDZ3 (○) or GST-FAP-1 PDZ5 (●) fusion protein in mixtures containing 25 nM fluorescein-labeled NIYYKV peptide probe, 20 mM phosphate, pH 7.0, 100 mM NaCl, and 2 mM dithiothreitol were monitored for fluorescence polarization at 22 °C. The GST-FAP-1 PDZ5 fusion protein was also measured for binding to the phosphorylated peptides, NIpYYKV (▲), NIYpYKV (▲), and NIpYpYKV (■). The fluorescence polarization values obtained for the peptide in absence of added GST fusion protein has been subtracted from the polarization values displayed. B, a binding of a GST fusion of full-length syntenin to the NIYYKV (○), NIpYYKV (▲), and NIpYpYKV (■) peptides as measured by fluorescence polarization.
Co-immunoprecipitation of syntenin-FLAG with ephrin B1. COS-1 cells were co-transfected with either ephrin B1 and syntenin-FLAG or with the ephrin B1 Val deletion and syntenin-FLAG as indicated. Cell lysates were immunoprecipitated with antibodies against ephrin B1 or interleukin 3 receptor a or were treated with protein A-Sepharose only. Immunocomplexes were subjected to SDS-polyacrylamide gel electrophoresis (10%) and blotted with anti-FLAG antibodies.

**FIG. 6.** Co-immunoprecipitation of syntenin-FLAG with ephrin B1. OS-1 cells were co-transfected with either ephrin B1 and syntenin-FLAG or with the ephrin B1 Val deletion and syntenin-FLAG as indicated. Cell lysates were immunoprecipitated with antibodies against ephrin B1 or interleukin 3 receptor a or were treated with protein A-Sepharose only. Immunocomplexes were subjected to SDS-polyacrylamide gel electrophoresis (10%) and blotted with anti-FLAG antibodies.

in vivo, since syntenin can be successfully co-immunoprecipitated with full-length ephrin B1 but not with ephrin B1 truncated in its PDZ domain target site. Thus far, we have not detected consistent complexes of ephrin B1 with GRIP or FAP-1 in vivo, but it remains possible that these are also physiological binding partners for B-type ephrins.

The effect of the phosphorylation state of two adjacent tyrosines at positions −2 and −3 relative to the carboxyl-terminal Val of the PDZ domain target site was examined using a fluorescence polarization assay. Structural studies of PDZ domains have suggested that interactions between PDZ domains and residues at the −2 and −3 positions of the carboxyl-terminal target site confer binding specificity (38–40). In one case, modification of residues at these positions by serine phosphorylation has been reported to regulate PDZ domain binding. The specific association between the second PDZ domain of PSD-95 and the inward rectifier potassium (K+) channel Kir2.3 is disrupted by protein kinase A-mediated phosphorylation of a key serine residue at the −2 position from the carboxyl terminus of Kir2.3 (41). Our results with B class ephrins and the PDZ domain proteins FAP-1 and syntenin suggest that the phosphorylation of residues within the PDZ domain binding site has different effects on different PDZ domains. The results with FAP-1 PDZ5 suggest that the PDZ domain residues, which contact the tyrosines in the binding site of B ephrins are able to accommodate the addition of two phosphate groups. This is consistent with observations that the single PDZ domain of AF-6 binds an unphosphorylated peptide with the consensus target sequence AYYV and a corresponding peptide phosphorylated at the −2 Tyr with approximately equal affinity.3 In contrast, GST-syntenin exhibited significantly decreased binding to peptides phosphorylated at the −2 residue of the PDZ domain binding site. It will be of interest to determine whether the lower affinity values seen in our in vitro studies are significant in vivo. These data suggest one mechanism through which tyrosine phosphorylation of ephrin B1 may regulate interactions with modular cytoplasmic proteins.

While we have identified a PDZ domain binding site in B-type ephrins, the issue of how PDZ domain interactions relate to ephrin B function remains to be determined. Possible roles for PDZ domain-ephrin B associations, however, can be proposed based on known functions of PDZ domains. Several examples have highlighted the importance of PDZ domain inter-
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Rosene-dependent signaling pathway may be assembled around a PDZ domain-containing protein in a manner similar to the InaD complex. Furthermore, the PDZ domain-containing protein PDS-95, which associates with glutamate receptors and K⁺ channels, also interacts through its PDZ domains with neuronal nitric-oxide synthase and a Ras GTPase activating protein (p135 SynGAP) (53, 54). PDZ domain-containing proteins may thereby serve as adaptors to directly activate signaling pathways. In this context, it is of interest that phosphorylation of the Tyr residues in the carboxyl-terminal ephrin B1 motif may regulate interactions with PDZ domains, as suggested by our results with syntenin.

Further work is required to distinguish among these possible roles for PDZ domain-ephrin B interactions. Cell culture experiments using the Val deletion mutant of ephrin B1 that is unable to bind the PDZ domains of syntenin and FAP-1 are currently in progress to determine the effect of uncoupling PDZ domain interactions from B ephrins. These studies, along with further characterization of the binding candidates identified in this report, will help to determine the physiological relevance of PDZ domain interactions in the function of B ephrins.

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Note Added in Proof—A recently published mammalian PAR-3 homolog, ASIP, is closely related to PHIP (Izumi, Y., Hirose, T., Tamai, Y., and Ohno, S. (1998) Genes Dev. 12, 95–106). Orioli, D., Henkemeyer, M., Lemke, G., Klein, R., and Pawson, T. (1996) EMBO J. 15, 755–766.

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