The Eph family of receptor tyrosine kinases has been implicated in many developmental patterning processes, including cell segregation, cell migration, and axon guidance. The cellular components involved in the signaling pathways of the Eph receptors, however, are incompletely characterized. Using a yeast two-hybrid screen, we have identified a novel signaling intermediate, SHEP1 (SH2 domain-containing Eph receptor-binding protein), which is expressed in the embryonic and adult brain. SHEP1 contains an Src homology 2 domain that binds to a conserved tyrosine-phosphorylated motif in the juxtamembrane region of the EphB2 receptor and may itself be a target of EphB2 kinase activity, since it becomes heavily tyrosine-phosphorylated in cells expressing activated EphB2. SHEP1 also contains a domain similar to Ras guanine nucleotide exchange factor domains and binds to the GTPases R-Ras and Rap1A, but not Ha-Ras or RalA. Thus, SHEP1 directly links activated, tyrosine-phosphorylated Eph receptors to small Ras superfamily GTPases.

The Eph receptor tyrosine kinases, together with their ephrin ligands, regulate cell segregation, cell migration, and axon guidance and sprouting in the developing embryo, but unlike other families of receptor tyrosine kinases, they have only modest effects on cell proliferation (1–4). Activation of Eph receptors by their cognate ligands leads to cell repulsion in Eph receptor-expressing non-neuronal cells (5) and to growth cone collapse in receptor-expressing neurons (6–8). Changes in cell adhesion to various substrates have also been documented following Eph receptor activation (9, 10).

Nearly all of the effectors known to interact with activated Eph receptors are well characterized signaling proteins that contain SH2 domains. They include cytoplasmic tyrosine kinases of the Src family (11, 12); the adaptors Grb2, Grb10 (13), and Nck (14); the Ras GTPase-activating protein (15); and the p85 subunit of phosphatidylinositol 3-kinase (16). The Src-like adaptor protein is the only novel protein identified because of its interaction with the cytoplasmic domain of an Eph receptor (17). Src-like adaptor protein consists of an Src homology 2 (SH2) and an SH3 domain but does not have a catalytic domain, and its function is unknown.

To further characterize the signaling pathways activated downstream of Eph receptors, we have searched for proteins that interact with autophosphorylated sequence motifs of Eph receptors. We employed the yeast two-hybrid system to screen a mouse embryo cDNA library using the phosphorylated cytoplasmic domain of EphB2 (18) as the bait (12, 19). Two of the isolated clones encoded a protein fragment that was distantly related to the SH2 domains of known proteins. Using expressed sequence tag (EST) data bases and RT-PCR, we obtained a full-length sequence for this novel protein, which we have designated SHEP1 (for SH2 domain-containing Eph receptor-binding protein). The structural features of SHEP1 suggest that this protein may be an important signaling intermediate downstream of the Eph receptors.

**EXPERIMENTAL PROCEDURES**

**Cloning of SHEP1 cDNA—**Screening of the GenBank tm EST data base using the BLAST algorithm with the partial SHEP1 sequence isolated from the two-hybrid screen retrieved partially overlapping clones containing additional sequences. Further rounds of EST data base searches and reverse transcription polymerase chain reaction amplifications of several murine tissues with the sense primer AGCGCGCGCCCTGTACCATGGACGCATC (containing a NotI site, underlined) and the antisense primer GAGACCTTTCGCAACTTCT yielded the entire coding sequence of SHEP1.

**Yeast Two-hybrid Assay—**The L40 yeast strain was co-transformed with LexA-EphB2 cytoplasmic domain constructs (12) and a VP16-SHEP1 SH2 domain construct. Co-transformants were first selected on growth assay on histidine-deficient medium.

**Cell Culture and Transfection—**The 293T human embryonal kidney cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C. Superfect (Qiagen)-mediated transfections were carried out in accordance with the manufacturer’s instructions. Cells were harvested 24–48 h post-transfection.

**Preparation of Antibodies—**A GST fusion protein of the SHEP1 SH2 domain (aa 167–324) was expressed in bacteria and purified on glutathione-agarose for injection into rabbits. The immune serum was affinity-purified on a GST-SHEP1 SH2 domain column and then absorbed to test for interaction by growth assay on histidine-deficient medium.

**RT-PCR, reverse transcription PCR; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; aa, amino acids; GEF, guanine nucleotide exchange factor; GTPases, guanine 5’-O-(thiotriphosphate); SCR, structurally conserved region.**
The anti-EphB2 antibodies were absorbed on a human Fc column (Cappel).

**Immunoprecipitation and Western Blot Analysis—**Transiently transfected 293T cells and adult mouse spleen were lysed in radioimmune precipitation buffer supplemented with protease inhibitors and sodium orthovanadate. Extracts were precleared for 1 hr at 4 °C with protein G-Sepharose (for immunoprecipitation with monoclonal antibodies) or with staphylococcus A (for immunoprecipitation with polyclonal antibodies) and then immunoprecipitated with anti-Myc 9E10 monoclonal antibody bound to protein G-Sepharose or anti-EphB2 or anti-SHEP1 polyclonal antibodies bound to staphylococcus A. Immunoprecipitates were separated by SDS-PAGE, transferred to PVDF membranes (Millipore Corp.), and probed with peroxidase-conjugated PY20 anti-phosphotyrosine antibody (Transduction Laboratories), anti-Myc 9E10 antibody, or polyclonal antibodies followed by peroxidase-conjugated protein A. Detection was with an enhanced chemiluminescence system (Amer sham Pharmacia Biotech).

**GST Fusion Protein Binding Assays—**GST fusion proteins of the SHEP1 SH2 domain (aa 167–324), the SHEP1 guanine nucleotide exchange factor (GEF) domain (aa 474–854), or GST alone were expressed in bacteria and purified on glutathione-agarose beads. Transiently transfected 293T cells and adult mouse spleen were lysed in radioimmune precipitation buffer supplemented with protease inhibitors and sodium orthovanadate. Extracts were precleared for 1 hr at 4 °C with protein G-Sepharose or anti-EphB2 or anti-SHEP1 polyclonal antibodies bound to staphylococcus A. Immunoprecipitates were separated by SDS-PAGE, transferred to PVDF membranes (Millipore Corp.), and probed with peroxidase-conjugated PY20 anti-phosphotyrosine antibody (Transduction Laboratories), anti-Myc 9E10 antibody, or polyclonal antibodies followed by peroxidase-conjugated protein A. Detection was with an enhanced chemiluminescence system (Amer sham Pharmacia Biotech).

**RESULTS**

cDNA Cloning of SHEP1—The cytoplasmic domain of the EphB2 receptor tyrosine kinase expressed as a LexA fusion protein was used to screen a VP16 yeast two-hybrid library. The kinase domain of EphB2 is highly active in yeast, resulting in tyrosine autophosphorylation on several residues (12). Accordingly, a large proportion of the clones isolated from the screen encoded the SH2 domains of known signaling molecules. Two identical clones corresponding to a partial sequence of a novel SH2 domain were also isolated (schematically shown in Fig. 1B). We designated the novel protein SHEP1.

Screening of EST data bases with the novel SHEP1 sequence revealed a number of mouse (Fig. 1B) and human (not shown) SHEP1 sequences as well as sequences corresponding to a second closely related protein, which we designated SHEP2 (not shown). The SHEP1 cDNA encodes an 854-amino acid protein (Fig. 1A). A stop codon in frame with the initial methionine is present in the 5′-untranslated sequence (not shown), indicating that the entire coding sequence was identified. A phylogenetic tree (Fig. 2A) illustrates the relationships between the SHEP1 SH2 domain and other previously known SH2 domains.

The program ProfileScan (23) also indicated the presence of a weak but significant homology between the carboxyl-terminal portion of SHEP1 and CDC25-like Ras GEF domains (24). The SHEP1 sequences corresponding to the three structurally conserved regions (SCRs) characteristic of GEF domains are highlighted in Fig. 1A. Additional sequence similarities are present throughout the GEF domain. The relationship between the GEF domain of SHEP1 and other previously known GEF domains is illustrated in the phylogenetic tree shown in Fig. 2B. The SHEP1 GEF domain is somewhat divergent from those previously identified, as indicated by the longer branch connecting SHEP1 to the tree.

A notable feature of SHEP1 in the region between the SH2 domain and the GEF domain is the high proportion of prolines (13% of the amino acids) and serines (21% of the amino acids) (Fig. 1A). The proline residues form five PXXP motifs, which conform to the consensus sequence of SH3 domain binding sites (25). This proline-serine-rich region also contains many potential sites of phosphorylation by proline-directed kinases, such as mitogen-activated protein kinases (26). Finally, five ty-
Rosines are present in the serine/proline-rich region of SHEP1, and four are in the amino-terminal segment that precedes the SH2 domain (Fig. 1A). If phosphorylated, these tyrosines may create binding sites for SH2 or phosphotyrosine-binding domains (27).

When SHEP1 was identified, its sequence was not closely related to that of any other known protein. While this work was in progress, sequences closely related to SHEP1 were submitted to GenBank™, which showed that SHEP1 is part of a family of proteins. The SHEP1 family includes HrSH2 (GenBank™ accession number AB010891), a protein of unknown function isolated from the tunicate Halocynthia roretzi, and BCAR3 (breast cancer anti-estrogen resistance gene 3; GenBank™ accession number U92715), a protein isolated based on its ability to confer tamoxifen resistance to estrogen-dependent breast cancer cells (28). BCAR3 is the same protein that we had designated SHEP2.

Expression of SHEP1 in Fetal and Adult Murine Tissues—The SHEP1 EST clones (Fig. 1B) were obtained from a wide variety of embryonic and adult tissues, and by RT-PCR we found that SHEP1 transcripts are present in adult brain, spleen, and thymus as well as embryonic brain (Fig. 3A). Hence, SHEP1 mRNA has widespread expression, suggesting that SHEP1 functions in a number of embryonic and adult tissues. To study the SHEP1 protein, we generated a polyclonal antibody to amino acids 167–324 of SHEP1. With this antibody, we immunoprecipitated a single 130-kDa protein from adult mouse spleen and from 293T cells transfected with full-length SHEP1 cDNA (Fig. 3B). Notably, the apparent size of the SHEP1 protein determined by SDS-PAGE is substantially larger than the size of 94 kDa calculated based on the amino acid sequence, suggesting that the mature protein may be post-translationally modified.

Association of SHEP1 with Eph Receptors—To further characterize the binding of SHEP1 to Eph receptors, we incubated an immobilized GST fusion protein of the SH2 domain of SHEP1 with extracts of 293T cells expressing tyrosine-phosphorylated EphB2 or EphA4 (12). Immunoblotting showed that the Eph receptors bind the SH2 domain of SHEP1 (Fig. 4A), suggesting that SHEP1 interacts with full-length Eph receptors belonging to both the A and the B subclass (29). Two-hybrid analysis with LexA-EphB2 fusion proteins showed that the SHEP1 SH2 domain interacts with wild-type, phosphorylated EphB2 but not with a kinase-inactive, unphosphorylated mutant (K662R) (12). Mutation of tyrosine 605 in the juxtamembrane domain of EphB2 to glutamic acid (Y605E) or phenylalanine (Y605F) also abrogated binding. This result indicates that this tyrosine is required for binding of the SHEP1 SH2 domain. Mutation of tyrosine 611 to phenylalanine (Y611F), a mutation that severely impairs kinase activity and autophosphorylation (12), also abrogated binding of the SHEP1 SH2 domain. Binding, however, was preserved when tyrosine 611 was mutated to glutamic acid (Y611E), a mutation that does not impair kinase activity and autophosphorylation, indicating that juxtamembrane tyrosine 611 is not required for binding of the SHEP1 SH2 domain. Finally, no binding of SHEP1 to EphB2 was observed when both tyrosines 605 and 611 were mutated to glutamic acid.

\[^3\] Zisch, A. H., Pazzagli, C., Freeman, A. L., Schneller, M., Hadman, M., Smith, J. W., Ruoslahti, E., and Pasquale, E. B., Oncogene, in press.
The association of SHEP1 with EphB2 was further verified by co-immunoprecipitation. EphB2 and a Myc-tagged SHEP1 construct containing the SH2 domain were co-transfected in 293T cells, and cell extracts were immunoprecipitated with either anti-Myc antibodies or anti-EphB2 antibodies. Probing by immunoblotting with anti-phosphotyrosine antibodies revealed the presence of EphB2 in the SHEP1 immunoprecipitates (Fig. 5). Conversely, tyrosine-phosphorylated SHEP1 was detected in the EphB2 immunoprecipitates. Probing with anti-Myc antibodies revealed that SHEP1 expressed in the absence of EphB2 had a lower apparent molecular weight than SHEP1 co-expressed with EphB2. These results suggest that EphB2 causes SHEP1 phosphorylation, probably on multiple residues.

 Association of SHEP1 with Ras Family GTPases—The presence of both an SH2 and a GEF domain in SHEP1 suggests a novel signaling pathway linked to small Ras superfamily GTPases. GEF domains typically bind and activate small GTPases, and the GDP is then replaced by GTP, which is abundant in the cytoplasm (24). In in vitro exchange assays, the GST fusion protein containing the GEF domain of SHEP1 did not detectably promote the release of 3H-labeled GDP from Ras family GTPases, and the GDP is then replaced by GTP, which is abundant in the cytoplasm (24). In in vitro exchange assays, the GST fusion protein containing the GEF domain of SHEP1 did not detectably promote the release of 3H-labeled GDP from GST fusions of Ha-Ras and R-Ras (Fig. 6C). As shown before (32), GST-CDC25 efficiently promoted 3H-labeled GDP release from Ha-Ras. GST-CDC25 also promoted a slow release of the labeled nucleotide from R-Ras, showing that the GST-R-Ras fusion protein was competent to bind and release GDP. Exchange assays using RalA and Rap1A yielded results similar to those obtained with R-Ras (not shown). Therefore, the GEF domain of SHEP1 binds R-Ras and Rap1A without promoting GDP/GTP exchange, at least under the conditions of our in vitro experiments.

**DISCUSSION**

We report here the isolation of a novel protein, designated SHEP1, that contains an SH2 domain in its N-terminal region, a proline/serine-rich central region, and a C-terminal guanine nucleotide exchange factor domain. SHEP1 associates with Eph receptors via its SH2 domain in a phosphorylation-dependent manner. Co-expression of activated EphB2 receptor and SHEP1 results in tyrosine phosphorylation of the latter, although it is not known if SHEP1 is a direct or indirect target of EphB2. The C-terminal portion of SHEP1, which contains the GEF domain, preferentially binds to the small Ras family GTPases R-Ras and Rap1A, although no exchange activity was observed.

Proteins containing SH2 domains and proteins containing GEF domains are two major classes of proteins that regulate normal cellular signals and can transform cells (34). However, typically SH2 and GEF domains do not occur in the same polypeptide (24, 25, 30). The only exception, in addition to SHEP family proteins, are the proto-oncogenes Vav and Vav2, which are related to each other (35). However, the role of Vav in signaling is likely to be distinct from that of SHEP1. The SH2 domain of Vav, which is located in the carboxy-terminal part of the protein, is only distantly related to that of SHEP1 (Fig. 2A). The GEF domain of Vav is a Dbl homology domain rather than a CDC25-like domain. In addition, Vav contains an array of other protein and lipid interaction domains (35). Nevertheless, the characterization of Vav as being required in early embryogenesis (36, 37), for lymphocyte development and activation (38, 39), and for integrin signaling in hematopoietic cells

![Fig. 4. The SH2 domain of SHEP1 mediates interactions with Eph receptors](image)

![Fig. 5. SHEP1 co-precipitates with EphB2 and is tyrosine-phosphorylated in cells expressing activated EphB2.](image)
SHEP1 Couples Eph Receptors to Ras Proteins

The GEF domain of SHEP1 exhibits specificity for R-Ras and Rap1A. A, a GST-SHEP1 fusion protein containing the GEF domain binds preferentially to the FLAG-tagged GTPases R-Ras and Rap1A. Extracts of 293T cells transfected with FLAG-tagged GTPase constructs or untransfected 293T cells as a control were incubated in radioimmune precipitation buffer with GST-SHEP1 (aa 474–854) bound to glutathione-agarose beads. Bound GTPase proteins were detected by immunoblotting with a polyclonal anti-Flag antibody. Equal amounts of lysates from the transfected cells were also probed with anti-Flag antibodies to show the expression levels of the different GTPases. B, a GST-SHEP1 protein containing the GEF domain binds wild type R-Ras from transfected 293T cells and, to a much lesser extent, constitutively active, GTP-bound, R-Ras38V. Extracts of 293T cells transfected with Myc-tagged wild type R-Ras or R-Ras38V were incubated in 1% Triton X-100 buffer with GST-SHEP1 (aa 474–854) or GST bound to glutathione beads. The samples were washed, and the material remaining associated with the beads and the extracts of the transfected cells were separated by SDS-PAGE and probed with anti-Myc antibodies. C, a GST-SHEP1 protein containing the GEF domain (aa 474–854) does not promote in vitro GDP/GTP exchange. The results are expressed as counts of 3H-labeled GDP remaining bound to GST-R-Ras and GST-Ha-Ras divided by the average counts remaining bound when GST was added instead of an exchange factor. For the 2-h time point, to reveal the weak activation of R-Ras exchange activity induced by the CDC25 GEF domain, the ratios of GST-CDC25 and GST-SHEP1 to GST-R-Ras were 2-fold higher than used for the 1-h time point. Each value is the average of three independent measurements (except for the 2-h time point with GST-CDC25, in which two independent measurements were made). Experimental points are indicated by filled squares.

This is further supported by the recent work of van Agthoven et al. (28), who demonstrate that aberrant expression of the BCAR3/SHEP2 protein in breast cancer cells results in a bypass of estrogen dependence for proliferation, thereby inducing resistance to the anti-estrogen drug tamoxifen. BCAR3/SHEP2 may thus stimulate an alternate growth pathway and/or promote cell survival. The high degree of homology (46% amino acid identity) of BCAR3/SHEP2 with SHEP1 suggests that these two proteins may function in similar pathways involving small Ras GTPases.

Although our assays have not shown a GTP exchange activity for the GEF domain of SHEP1, this may be due to a shortcoming in our understanding of the regulation of SHEP1 catalytic activity. It has been demonstrated that many GEF proteins have negatively regulating regions outside the catalytic domain (41–43). In particular, both Vav and the Rap1A exchange factor, C3G, are activated by phosphorylation of specific tyrosine residues outside the catalytic region (44, 45). Since SHEP1 contains many potential target tyrosines and is heavily phosphorylated when co-expressed with activated EphB2, such a regulatory mechanism may control the GTP exchange activity of SHEP1 in vivo.

It is also possible that R-Ras and Rap1A, although readily bound by SHEP1, are not its physiological targets. Recent studies of GTPase-GEF complexes have highlighted the structural motifs essential for catalytic activity (46–48). Most GEF proteins contain a stabilizing SCR/Ras effector motif region that seems to be absent in SHEP1. The co-crystal of SOS/Ras has shown that a protruding helical hairpin formed in part by the SCR3 region (see Fig. 1A) plays an important role in the nucleotide exchange mechanism (46). The SHEP1 GEF domain is divergent from other GEF proteins in this region, perhaps indicating that either the mechanism of action of SHEP1 is different or that the target may not be a “classical” Ras protein.

Alternatively, the ability of SHEP1 to bind R-Ras and Rap1A without activating them suggests the intriguing possibility that SHEP1 is not a functional GEF but rather a competitive inhibitor of nucleotide exchange. Finally, SHEP1 may serve as an adaptor, controlling the subcellular localization of certain Ras family GTPases. In the absence of an activated Eph receptor, the PXPF motifs of SHEP1 may interact with SH3 domains in other proteins. For example, SHEP1 may localize R-Ras to focal contacts, where this small GTPase may maintain integrin activation (31), and at other intracellular locations where R-Ras regulates apoptotic signaling pathways (49). Following Eph receptor activation, SHEP1 would become associated with Eph receptors through its SH2 domain. Once associated with Eph receptors, SHEP1 and R-Ras would become tyrosine-phosphorylated. Recent work by Zou et al. (40) has confirmed the expectation that proteins containing both SH2 and GEF domains should have a crucial importance in development and cell transformation.

During the preparation of this manuscript, the partial sequence of human SHEP1 was published as NSP3 (GenBank™ accession number AF124251). Although no functional data were presented for this protein, a new relative, termed NSP1 (GenBank™ accession number AF124249) (36% amino acid identity with SHEP1), was found to be associated with tyrosine kinase receptors of the epidermal growth factor and insulin receptor families (50). A connection with integrin-mediated adhesion has also been proposed for NSP1, because of its reg-
ulated association with p130

Ras, GST-CDC25, and FLAG-tagged GTPase plasmids and for helpful
downstream of Eph receptors and other receptor tyrosine ki-

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