EphrinB1 interacts with the transcriptional co-repressor Groucho/xTLE4

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Ephrin signaling is involved in various morphogenetic events, such as axon guidance, hindbrain segmentation, and angiogenesis. We conducted a yeast two-hybrid screen using the intracellular domain (ICD) of EphrinB1 to gain biochemical insight into the function of the EphrinB1 ICD. We identified the transcriptional co-repressor xTLE1/Groucho as an EphrinB1 interacting protein. Whole-mount in situ hybridization of Xenopus embryos confirmed the co-localization of EphrinB1 and a Xenopus counterpart to TLE1, xTLE4, during various stages of development. The EphrinB1/xTLE4 interaction was confirmed by co-immunoprecipitation experiments. Further characterization of the interaction revealed that the carboxy-terminal PDZ binding motif of EphrinB1 and the SP domain of xTLE4 are required for binding. Additionally, phosphorylation of EphrinB1 by a constitutively activated fibroblast growth factor receptor resulted in loss of the interaction, suggesting that the interaction is modulated by tyrosine phosphorylation of the EphrinB1 ICD. [BMB reports 2011; 44(3): 199-204]

INTRODUCTION

The erythropoietin producing hepatoma (Eph) family of receptor tyrosine kinases and their cognate erythropoietin producing hepatoma interactor (Ephrin) ligands are components of a unique bi-directional signaling pathway. This pathway is involved in controlling cell-cell adhesion and repulsion and is also involved in several developmental morphogenetic processes, including neural crest migration, brain segmentation, and vasculogenesis (1). Additionally, aberrant Eph/Ephrin signaling has been associated with tumor progression and angiogenesis (1,2). When an Eph receptor of one cell comes in contact with an Ephrin ligand of an adjacent cell, both a “forward” signal in the Eph expressing cell and a “reverse” signal in the Ephrin expressing cell are induced. Although Eph/Ephrin bidirectional signaling has been an area of intense interest for several years, the focus of many studies has been on Eph receptor signal transduction.

Ephrin molecules are subgrouped into an A-type and B-type, based on sequence similarity and receptor binding specificities. Although both have an extracellular domain capable of binding to Eph receptors, EphrinAs are glycosylphosphatidylinositol (GPI)-anchored to the cell membrane, while EphrinBs are transmembrane proteins with an intracellular domain (ICD). Several conserved tyrosine residues in the ICD of EphrinBs, along with the SH2 domain binding motif YEVK and the carboxy-terminal PDZ binding motif YYKV are important for mediating protein-protein interactions and controlling various morphogenetic processes during development (1-3). Thus, the ICDs of EphrinBs recruit upstream signaling proteins capable of inducing EphrinB phosphorylation as well as downstream EphrinB target proteins often containing SH2 and PDZ domains.

Although the specific mechanism of EphrinB phosphorylation has been elusive, it is generally accepted that Src family kinases are recruited to and phosphorylate Ephrin ligands following binding to Eph receptors (4,5). Ephrin ligands can also be phosphorylated in response to activation of additional tyrosine kinases, including fibroblast growth factor receptor (FGFR) (6) and platelet-derived growth factor receptor (5) or in response to activation of the cell surface molecule claudin (7). EphrinB phosphorylation occurs on conserved tyrosine residues in the carboxy terminus.

Proteins bind to EphrinBs by phosphorylation-dependent and independent mechanisms. Phosphorylation-dependent EphrinB interactors require the phosphorylation of conserved tyrosine residues and include the Src homology 2 adapter protein, growth-factor-receptor-bound protein 4 (Grb4)/Nck adapter protein 2 (Nck2), associated with cell rounding and an increase in focal adhesion kinase catalytic activity (8). Furthermore, the phosphorylation-dependent binding of EphrinB1 to the transcription factor signal transducer and activator of transcription 3 (STAT3) enhances STAT3 activity (9). Phosphorylation-independent interactors bind to EphrinBs in the absence of phosphorytrosine residues, mainly through PDZ domains, and...
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include PDZ-RGS3, a GTP exchange factor involved in cerebellar granule cell migration (10). Syntenin, a PDZ domain-containing scaffold protein that has been implicated in cytoskeletal-membrane organization, cell adhesion, protein trafficking, and the activation of transcription factors (11) also binds in this manner (12). More recently, EphrinB1 was shown to control cell movement and cell-cell adhesions through an interaction with the Dishevelled scaffold protein (13, 14) and the Par polarity complex protein Par-6 (15).

In this study, we performed a yeast two-hybrid screen of a human fetal brain library to identify novel EphrinB interacting proteins and gain insight into the signaling mechanisms of EphrinBs. We identified Groucho, a universal transcriptional corepressor important in regulating Wnt, transforming growth factor-β, and Notch signaling (16). The interaction is characterized biochemically, and putative roles of the interaction are discussed.

RESULTS

EphrinB1 binds hTLE1 in the yeast two-hybrid system

A yeast two-hybrid screen was employed to identify novel EphrinB1 ICD interacting proteins that may provide insight into the signaling mechanisms of EphrinB1. The ICD of murine EphrinB1 (identical to human EphrinB1; Fig. 1A) was used to screen a pretransformed human fetal brain cDNA library under moderate stringency conditions, resulting in the recovery of 2977 putative interacting clones, which were subsequently retested under high stringency conditions, allowing for the detection of moderate and strong levels of reporter activation. A total of 172 putative interacting clones were then verified by retransformation and sequenced. Several candidate interacting molecules were identified along with ten distinct clones of the known EphrinB1 interactor, syntenin (12). Interestingly, the transducin-like enhancer of split 1 (hTLE1), the human ortholog of the Drosophila Groucho transcriptional co-repressor, was recovered (Fig. 1B), corresponding to amino acids 197-770 of the protein (Fig. 1C). hTLE1 exhibited strong reporter activation when co-transformed with the EphrinB1-Gal4 binding domain fusion protein construct but not when co-transformed with the control Gal4 binding domain fusion protein construct, as evidenced by growth on Ade-His-Leu-Trp high stringency selection plates and by a color change with the β-galactosidase assay (Fig. 1B).

EphrinB1 binds hTLE1 in oocytes

To verify the results of the yeast two-hybrid screen, co-immunoprecipitation studies were performed with exogenously expressed proteins in Xenopus oocytes. RNA encoding full-length human TLE1 with an amino-terminal HA epitope tag (hTLE1-HA) was co-injected with RNA encoding Xenopus EphrinB1 containing a FLAG epitope tag in a nonconserved region of the extracellular domain (xB1ncFlag) in Xenopus oocytes. Lysates were prepared and co-immunoprecipitation/Western blot analyses were performed (Fig. 2A). When hTLE1-HA was immunoprecipitated with HA-conjugated agarose beads, xEphrinB1-ncFlag was detected in the immune complexes. This result was validated by reciprocal immunoprecipitations in which xEphrinB1-ncFlag was immunoprecipitated with Flag conjugated beads, and hTLE1-HA was detected in the immune complexes.

EphrinB1 binds Xenopus Groucho ortholog xTLE4

Additional oocyte injections were performed to determine if the three cloned Xenopus Groucho family proteins, xTLE4, xTLE1, and xAES, can also bind xEphrinB1. When xTLE4-HA was immunoprecipitated with HA-conjugated agarose beads, xEphrinB1-ncFlag was detected in the immune complexes, and, similarly, when xEphrinB1-ncFlag was immunoprecipitated with Flag conjugated beads, xTLE4-HA was detected in the im-

![Fig. 1. EphrinB1 Binds hTLE1 in a Yeast Two-Hybrid Screen. (A) The murine C-terminal intracellular domain (ICD) of EphrinB1 (xB1) was used as bait to screen a human fetal brain cDNA library. Amino acid alignment showed 100% identity with the human EphrinB1 ICD (hB1), and 96% identity with Xenopus EphrinB1 ICD (xB1). Schematic diagram of EphrinB1 (bottom panel). (B) Yeast was co-transformed with pGBK7-T7-pCADT7-T (53/T), pGBK7-7-ICD-pCADT7-T (mB1ICD/T), pGBK7-T7-pCADT7-hTLE1 (BK7/hTLE1), or pGBK7-mB1-ICD-pCADT7-hTLE1 (mB1ICD/hTLE1). Retrönformation assays revealed strong reporter activation for both 53/T and mB1ICD/hTLE1, but not mB1ICD/T or BK7/hTLE1, in both the β-galactosidase assay (left) and when plated under high stringency on SD Ade-His-Leu-Trp dropout media plates. (C) DNA sequence analysis of the pCADT7-hTLE1 yeast two-hybrid clone recovered from the screen revealed that the clone contained amino acids 197-770 of hTLE1, which included the CCN, SP, and WD40 domains.

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Fig. 2. hTLE1 and xTLE4 Bind to xEphrinB1 in Xenopus Oocytes. (A) Full-length human TLE1 fused to an amino terminal HA epitope tag (hTLE1-HA) and full-length Xenopus EphrinB1 harboring a FLAG epitope tag in a non-conserved region of the extracellular domain (xEphrinB1-ncFlag) were co-expressed in Xenopus oocytes for co-immunoprecipitation/Western blot analyses. Oocyte lysates were immunoprecipitated and Western blots were performed with the indicated antibodies. (B) Full-length Xenopus TLE orthologs with an amino terminal HA epitope tag (xTLE4-HA, xTLE1-HA, and xAES-HA) were co-expressed with xEphrinB1-ncFlag in Xenopus oocytes for additional co-immunoprecipitation/Western blot analyses. Erk2 expression confirmed equivalent loading of total protein. (C, D) Amino acid homology of human and Xenopus TLE orthologs for each domain and overall protein as compared against hTLE1 (C) and xTLE4 (D).

Fig. 3. The EphrinB1 PDZ Binding Motif is Required For xTLE4 binding
Although EphrinB1 and xTLE4 physically interact, it was important to verify that these two proteins are normally expressed in a manner that provides the opportunity to interact in a developing embryo. Stage 16 and 32 Xenopus embryos were fixed and in situ hybridization was performed (Supplemental Fig. 1A and B). Groucho and EphrinB1 were co-expressed in the neural groove, rhombomeres, forebrain, and branchial arches, consistent with previous studies (17, 18).
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Fig. 4. Tyrosine Phosphorylation of EphrinB1 Inhibits Binding Between EphrinB1 and TLE4. Xenopus oocytes were injected with RNAs as indicated, and lysates were subjected to co-immunoprecipitation/Western blot analyses. Co-expression with constitutively active FGFR1-KE resulted in a loss of binding between xTLE4 and xEphrinB1. However, co-expression with kinase dead FGFR1-KD had no effect on the xTLE4 and xEphrinB1 interaction.

FGFR phosphorylation of xEphrinB1 inhibits xTLE4 binding
Because EphrinB1 can be tyrosine phosphorylated during embryogenesis, we determined whether tyrosine phosphorylation affected the interaction between xEphrinB1 and xTLE4. A constitutively active form of the FGFR1-KE or a kinase dead form (FGFR1-KD) was co-expressed with xEphrinB1-ncFlag and xTLE4-HA in Xenopus oocytes. Co-expression of FGFR1-KE had no effect on the binding between xTLE4-HA and xEphrinB1-ncFlag, as evidenced by reciprocal co-immunoprecipitations, and no detectable tyrosine phosphorylation of EphrinB1 was observed (Fig. 4). Strikingly, co-expression of FGFR1-KE caused a high level of xEphrinB1 phosphorylation and concomitant loss of a co-immunoprecipitation between xEphrinB1-ncFlag and xTLE4-HA (Fig. 4). These data indicate that tyrosine phosphorylation of EphrinB1 inhibits the interaction between EphrinB1 and xTLE4.

DISCUSSION
In this study, we identified the transcriptional co-repressor Groucho as a novel EphrinB1-interacting protein. A common feature of all Groucho proteins is a lack of intrinsic DNA binding activity; thus, they must interact with DNA binding proteins to regulate transcription. Most Grouchos are comprised of five domains (Fig. 1C) important for binding to a variety of proteins: The amino terminal Gln-rich (Q) and carboxy terminal Trp-Asp-repeat (WD) domains are highly conserved, are essential for binding to DNA-binding proteins, and mediate transcriptional repression (19). The internal region is less conserved and contains the Gly-Pro-rich (GP), CcN, and Ser-Pro-rich (SP) domains, which harbor HDAC binding sites, CK2 and cdc2 phosphorylation sites, and a nuclear localization sequence that regulates TLE/Groucho subcellular localization, phosphorylation, and transcription repression activity (20-23). Additional Groucho family members contain only Q and SP domains and act as inhibitors of Groucho-mediated transcriptional suppression (19).

Although binding between xTLE4 and EphrinB1 was robust, binding with the other full-length isoform, xTLE1, was relatively weak (Fig. 2B). Comparing the amino acid sequences between xTLE4 and xTLE1 revealed an overall identity of 68.8%, with the WD domain having 91% identity and the SP domain having only 45.9% identity (Fig. 2D), which correlated with our data that the SP domain of xTLE4 is required exclusively for interaction with EphrinB1. In fact, when comparing the amino acid sequence of xTLE1 and xEphrinB1 with human TLE1 and TLE4, xTLE4 had high homology with hTLE4 (97.4%, Fig. 2D), whereas xTLE1 had a much weaker homology with hTLE1 (69.6%, Fig. 2C). Interestingly, xTLE4 had higher homology to hTLE1 (85.6%) than xTLE1 (Fig. 2C). Thus, although hTLE1 was recovered from the yeast two-hybrid screen, an amino-acid sequence analysis indicated that xTLE4 was more similar to hTLE1 and more likely to bind than xTLE1. It is also very likely that hTLE4 would interact with xEphrinB1 based on...
the high overall homology to xTLE4 (97.4%) and high homology of their SP domains (92.7%, Fig. 2D).

SP domains of the Groucho family of proteins are required for binding to Runt homology transcription factors (Runxs) and downregulating expression of Runt target genes. Runts are required for proper hematopoiesis [Runx1 (24)], osteogenesis [Runx2 (25)], and neurogenesis [Runx3 (26)]. Additionally, transcription factors Pax5 and Hes1 also bind Groucho proteins through the SP domain. Interestingly, binding between EphrinB1 and xTLE4 appears to increase significantly following the loss of the WD40 domain (Supplemental Fig. 2B), suggesting that the WD domain suppresses SP domain binding to EphrinB1. WD domains (aka WD40 domains) are comprised of multiple 40 amino acid repeats thought to form large β-propeller structures (27) and serve as a platform for protein-protein interactions. Thus, the structure of the WD domain itself, or the binding of various molecules at the WD domain may serve to limit the association between the SP domain and EphrinB1.

Notably, although predominantly nuclear, Groucho/TLE has also been found in the cytoplasm (28, 29). Furthermore, TLE1 interacts with the intracellular domain of GP130, a transmembrane receptor (30). It is possible that such interactions lead to nuclear transport and activation of transcription in response to Gp130 stimulation by ligand. Because EphrinB1 co-opts the planar cell polarity pathway through the downstream Wnt mediator Dishevelled (13), it would be interesting to consider whether Groucho’s interaction with EphrinB1 would affect its role in suppressing TCF signaling and/or activating the PCP pathway. Thus, additional studies are needed to determine the true subcellular localization of Groucho/TLEs and what effect EphrinB1 may have on Groucho/TLE activity.

Alternatively, this interaction may be explained by recent evidence suggesting that EphrinBs are processed by a pre-senilin-dependent gamma secretase. Interestingly, in addition to simply being degraded, the resulting EphrinB carboxy-terminal fragment is capable of modulating Src kinase activity by preventing Csk kinase from inhibiting Src autophosphorylation (31). The cleaved fragment may also translocate to the nucleus through a cryptic nuclear localization sequence (32), although the significance of such localization is not yet understood. Thus, a carboxy-terminal fragment of EphrinBs may translocate to the nucleus and bind to specific isoforms of Groucho/TLEs and have important, but, to date, unidentified functions.

MATERIALS AND METHODS

Plasmids and constructs
cDNA image clones encoding full-length hTLE1, xTLE1, xTLE4, and xAES were obtained from the ATCC (Manassas, VA, USA) (Image 3506451, ATCC# MGC-19063; Image 3400863, ATCC# 10646291; Image 7011138, ATCC#10648557; and Image 6863882, ATCC#10530733, respectively). Coding regions were subcloned into pCS107 (a kind gift from Richard Harland) with an amino terminal HA epitope tag by PCR and sequence verified prior to use. Further xTLE4 deletion constructs were generated by PCR. Generation of xEphrinB1-ncFlag constructs and deletion mutants have been described previously (6), as have FGFR1-KD and FGFR1-KD constructs.

Preparation of synthetic RNAs

For all oocyte injections, synthetic capped mRNA was made using the SP6 mMessage mMachine kit (Ambion, Austin, TX, USA), according to the manufacturer’s instructions. Linearized templates were generated as follows: xTLE1 and xTLE4 (full-length and truncation mutants) were linearized with Apgal, hTLE1 and xAES were linearized with Asp718, and pSP64 ATen-containing xEphrinB1-ncFlag constructs (full-length and truncation mutants) were linearized with XbaI. FGFR1-KE in pCS2+ was linearized with NotI, and FGFR1-KD in pSP64T3 was linearized with BamHI.

Xenopus oocyte expression system

Stage 6 Xenopus oocytes were harvested from adult females, manually detached from the ovary, defolliculated with a 2-hour collagenase (1.5 mg/ml) treatment, and incubated in L15 oocyte media at 19°C for at least 12 hours prior to microinjection. Approximately 15 ng of each mRNA was microinjected, as previously described (6). Injection amounts were increased or decreased as necessary after initial expression checks to ensure equivalent expression among all mRNAs. Injected oocytes were incubated in L15 oocyte media at 19°C for 6-18 hours, rinsed in 1× MBS, and harvested in TNSG lysis buffer at 10 μl/oocyte. The lysate was centrifuged, and the clear supernatant was recovered for biochemical analyses.

Immunoprecipitation and Western blot analyses

Oocytes were prepared with ice-cold lysis buffer, as previously described (6). For each immunoprecipitation reaction, 150 μl of lysate (15 oocyte equivalents) was mixed with 650 μl of lysis buffer and 1 μg of antibody (raised against HA [Applied Biological Materials, Vancouver, Canada] or Flag [Applied Biological Materials]) and incubated at 4°C for 1-2 hours, after which 25 μl of protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added to the reaction and rotated in an orbital mixer overnight at 4°C. Washes and immunoblots were performed as previously described (6) using anti-Flag-HRP conjugated (Sigma, St. Louis, MO, USA), anti-FLAG-HRP conjugated (Roche, Indianapolis, IN, USA), or anti-ephrinB1 and anti-Dsh (Santa Cruz Biotechnology) antibodies.

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