Tyr-298 in ephrinB1 is critical for an interaction with the Grb4 adaptor protein

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The Eph family of receptor tyrosine kinases and their membrane-bound ligands, the ephrins, are thought to play a role in the regulation of cell adhesion and migration during development by mediating cell-to-cell signalling events. The transmembrane ephrinB protein is a bidirectional signalling molecule that sends a forward signal through the activation of its cognate receptor tyrosine kinase residing on another cell. The reverse signal is transduced into the ephrinB-expressing cell via tyrosine phosphorylation of its conserved C-terminal cytoplasmic domain. Previous work from our laboratory has implicated the activated FGFR1 (fibroblast growth factor receptor 1) as a regulator of a de-adhesion signal that results from overexpression of ephrinB1. In the present study, we report the isolation of *Xenopus* Grb4 (growth-factor-receptor-bound protein 4), an ephrinB1-interacting protein, and we show that when expressed in *Xenopus* oocytes, ephrinB1 interacts with Grb4 in the presence of an acti-

vated FGFR1. Amino acid substitutions were generated in Grb4, and the resulting mutants were expressed along with ephrinB1 and an activated FGFR in *Xenopus* oocytes. Co-immunoprecipitation analysis shows that the FLVR motif within the Src homology 2 domain of *Xenopus* Grb4 is vital for this phosphorylation-dependent interaction with ephrinB1. More importantly, using deletion and substitution analysis we identify the tyrosine residue at position 298 of ephrinB1 as being required for the physical interaction with Grb4, whereas Tyr-305 and Tyr-310 are dispensable. Moreover, we show that the region between amino acids 301 and 304 of ephrinB1 is also required for this critical tyrosine-phosphorylation-dependent event.

Key words: ephrinB1, Grb4 (growth-factor-receptor-bound protein 4), tyrosine phosphorylation, *Xenopus*.

INTRODUCTION

EphrinB molecules are membrane-anchored proteins that are known to be bidirectional signalling molecules regulating many critical cellular events during development [1–3]. After cell–cell contact, the transmembrane ephrinB proteins not only send a forward signal in cells expressing the EphB family receptor, but also transmit a reverse signal into ephrinB-ligand-expressing cells. Recent studies have suggested that the ephrinB reverse signalling is important for transducing signals during development. At the segmental boundaries within the vertebrate hindbrain, the cytoplasmic domain of ephrinB1 is required for restricting the intermingling of adjacent cell populations that express either ephrinB1 or the cognate Eph receptor [4,5]. Similarly, ephrinB3 acts as a midline barrier to prevent axonal crossing during development of the vertebrate nervous system [6]. In addition, reverse signalling by ephrinB2 is required for vasculogenesis, angiogenesis and vessel maturation [7–9].

To date, 15 members of the Eph family of receptor tyrosine kinases have been identified in vertebrates. These proteins can be divided into two subsets, EphA and EphB, on the basis of sequence similarity and their preference for a subset of ligands with which they bind. EphA receptors (EphA1–A9) bind glycosylphosphatidyl-anchored ephrinA ligands (ephrinA1–A5), whereas EphB receptors (EphB1–B6) bind transmembrane ephrinB ligands (ephrinB1–B3) [10–12]. EphrinB proteins (B1–B3) have well-conserved cytoplasmic domains that include 33 amino acids

at the C-terminus showing nearly 100 % identity among the ephrinB proteins [13–18]. Five conserved tyrosine residues reside within the C-terminal 33 amino acids that may mediate or modulate reverse signalling events. On stimulation by a clustered soluble form of the Eph receptor or on contact of EphB-receptor-expressing cells with ligand-expressing cells, the cytoplasmic domains of ephrinB1 proteins undergo rapid phosphorylation on well-conserved tyrosine residues [19,20]. In ephrinB1, tyrosine residues at positions 312, 317 and 331 are phosphorylated after engagement with the Eph receptor ectodomain *in vivo*. Interestingly, Tyr-331 in the PDZ (PSD-95, Dlg and ZO-1)-binding motif has been determined to be the major *in vivo* phosphorylation site of ephrinB1 bound to EphB2, and is proposed to be involved in multimerization of the ephrinB molecule [21].

The signalling pathway mediated by ephrinB activation can be classified into two pathways, phosphotyrosine-dependent and -independent pathways. The C-terminal PDZ-binding motif of ephrinB1 molecules can mediate a tyrosine-phosphorylation-independent signalling event. An interaction between ephrinB1 and PDZ domains has been demonstrated using the ephrinB1 cytoplasmic domain in the yeast two-hybrid system [22–24]. Most of the binding proteins identified to date contain PDZ domains that serve as adaptors or scaffolds for multiple signalling complexes [25–27]. PDZ-RGS3 (where RGS stands for regulator of G-protein signalling), a cytoplasmic protein that contains a regulatory domain for heterotrimeric G-protein signalling, also binds ephrinB1 through its PDZ domain and regulates the migration

Abbreviations used: FGF, fibroblast growth factor; FGFR, FGF receptor; Grb4, growth-factor-receptor-bound protein 4; HA, haemagglutinin; HRP, horseradish peroxidase; JNK, c-Jun N-terminal kinase; RGS, regulators of G-protein signalling; SFK, Src family kinase; SH2 domain, Src homology 2 domain.

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The sequence representing the predicted *Xenopus* homologue of the Grb4 protein reported in the present study has been deposited in the GenBank® under the accession number AY354455.

of cerebellar granule cells [28]. Furthermore, the ectopic overexpression of ephrinB1 in HEK-293 cells results in the activation of JNK (c-Jun N-terminal kinase) activity and induces morphological changes through the novel ephrinB1 reverse signalling pathway [29]. Interestingly, the activation of JNK by ephrinB1 reverse signalling is not dependent on its PDZ-binding motif, but requires the ephrinB1 cytoplasmic region between amino acids 264 and 312 (ephrinB1 Δ C82 to ephrinB1 Δ C33). Moreover, overexpression of ephrinB1 during early *Xenopus* embryogenesis affects cell adhesion, resulting in cell dissociation in a phosphorylation-independent manner [30].

In addition to the PDZ proteins, another important regulator of cell adhesion and movement has been discovered to interact with ephrinB1 in a phosphorylation-independent manner. The *Xenopus* dishevelled protein has been recently shown to form a complex with the ephrinB ligand and to mediate cell repulsion via modification of RhoA activity [31].

Recent evidence suggests that ephrinB1 is phosphorylated by SFKs (Src family kinases) after interaction with EphB receptors. The formation of a complex between ephrinB1 and SFKs activates SFK activity, which in turn phosphorylates tyrosine residues of ephrinB1 that are required for the angiogenic sprouting of primary endothelial cells [32]. An unusual characteristic of the tyrosine-phosphorylation-dependent pathway was noted when it was revealed that 'cross-talk' can exist between the ephrinB cytoplasmic domain and other receptor tyrosine kinase pathways. Serum- or platelet-derived growth factor stimulation of ephrinB ligand-expressing cells results in the phosphorylation of ephrinB1. Tyrosine phosphorylation induced by serum treatment is unaffected by inhibition of SFKs, indicating that kinases other than SFKs can mediate the serum induction of ephrinB1 tyrosine phosphorylation. The tie-2 receptor tyrosine kinase has been shown to phosphorylate ephrinB2 *in vitro* [33]. *In vivo*, ligand stimulation of the FGFR (fibroblast growth factor receptor) induces tyrosine phosphorylation of ephrinB molecules in chick primary retina culture, indicating that the FGFR may 'cross-talk' with ephrinB1 [34]. Moreover, the phosphorylation-independent induction of cell dissociation by ephrinB1 in *Xenopus* embryos was rescued by an activated FGFR. This rescue required the tyrosine residues at positions 305 and 310 within the conserved C-terminus of *Xenopus* ephrinB1. These tyrosine residues are equivalent to the phosphorylation sites Tyr-312 and Tyr-317 discovered *in vivo* [21]. Whereas the activated FGFR was capable of interacting with and inducing the tyrosine phosphorylation of ephrinB1, it may not directly phosphorylate the transmembrane protein, since an ephrinB1 mutant lacking Tyr-305 and Tyr-310, critical for association with FGFR, is still phosphorylated in the presence of an activated FGFR [34].

Recently, an SH2 (Src homology 2)-domain-containing signalling molecule that binds to the intracellular domain of ephrinB1 in a phosphorylation-dependent manner was identified. Tyrosine-phosphorylated ephrinB1 molecules recruit the adaptor protein Grb4 (growth-factor-receptor-bound protein 4), which consists of one SH2 and three SH3 domains and can increase FAK (focal adhesion kinase) catalytic activity as well as cell rounding [35]. Binding of Grb4 to ephrinB1 requires 22 amino acids of the cytoplasmic region, which is identical between ephrinB1 and ephrinB2 and contains three of the five conserved tyrosine residues. An NMR binding study identified two tyrosine residues, Tyr-298 and Tyr-310, within the 22-residue hairpin of ephrinB2 as phosphorylation sites that may be relevant for high-affinity binding to the Grb4 SH2 domain [36]. However, the critical tyrosine residue involved in the binding of Grb4 SH2 domain is not yet clear.

In the present study, we show that an FGFR can induce the phosphorylation of ephrinB1 and promote the formation of a com-

plex between ephrinB1 and Grb4. In addition, we define the critical region within ephrinB1 required for binding Grb4 and identify Tyr-298 as vital for binding the Grb4 SH2 domain *in vivo*. Taken together, our results suggest that ephrinB1 reverse signalling may be modulated by FGF (fibroblast growth factor) signalling and that the Grb4 adaptor protein may participate in this signalling as a result of being recruited to the phosphorylated Tyr-298 site.

EXPERIMENTAL

Isolation of *Xenopus* Grb4

Primers representing the C-terminal sequence of *Xenopus* Grb4 were created based on the EST (expressed sequence tag) sequence (*Xenopus* EST project at Washington University, St. Louis, MO, U.S.A.) and a 360 bp fragment was generated from a stage 30 *Xenopus* head library. This fragment was used as a probe to screen 500 000 recombinant phage for full-length *Xenopus* isolates. Two independent isolates representing the predicted *Xenopus* homologue of the Grb4 protein were isolated and sequenced (GenBank® accession number AY354455).

Plasmids and mutagenesis of cDNA

cDNA encoding wild-type x-ephrinB1 (*Xenopus* ephrinB1) was subcloned into pSP64A as described previously [34]. C-terminal truncation mutants of wild-type x-ephrinB1 were generated by PCR using the plasmid pSP64A x-ephrinB1 as the template and the sense primer 5'-GGACGGCTTCTTCAACTCC-3', in conjunction with antisense primers that generated a stop codon at the appropriate positions. PCR products were purified with the agarose-gel extraction kit according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN, U.S.A.), digested with *PacI* and *EcoRV*, re-purified and ligated into the *PacI* and *EcoRV* sites of pSP64Aten (-*HindIII*) x-ephrinB1. Single-point mutants were also generated by the PCR method. Mutants Y298F (Tyr-298 $\rightarrow$ Phe), S302A, Y305F and Y310F were prepared using pSP64Aten-x-ephrinB1 as a template. Both the double mutants Y305F/Y310F and Y298F/Y305F were prepared using Y305F as a template to generate a second mutation at positions 298 and 310 respectively. The double mutant S302A/Y310F was prepared using Y310F as a template to generate a second mutation at position 302. The triple mutants S302A/Y305F/Y310F and Y298F/Y305F/Y310F were prepared using Y305F/Y310F as a template to generate a second mutation at positions 298 and 302 respectively. All ephrinB1 mutants were tagged with FLAG epitope (DYKDDDDK) at the C-terminus of x-ephrinB1. PCR products were purified, digested with *BamHI* and *EcoRI*, re-purified and subcloned into the *BamHI* and *EcoRI* sites of pCS2+. DNA sequencing was performed to confirm the identity of each mutant.

cDNA encoding x-Grb4 (*Xenopus* Grb4) was subcloned into plasmid pCS2+, and HA (haemagglutinin) epitope was introduced to the C-terminus of x-Grb4. Single-point mutants of x-Grb4 were generated by the PCR method. To produce various x-Grb4 mutants, either the conserved arginine residue within the 'FLVRES' region of the SH2 domain was substituted with a leucine residue or the first (absolutely conserved) tryptophan residue of the characteristic tryptophan doublet (WW doublet) of the SH3 domains was substituted with a lysine residue, as described in [37,38]. PCR products were purified, digested with *BamHI* and *EcoRI*, re-purified and subcloned into the *BamHI* and *EcoRI* sites of pCS2+ plasmid vector. cDNA encoding mutants were verified by DNA sequencing. cDNA encoding *Xenopus* Nck1 [39] was tagged with HA epitope at the C-terminus. cDNAs

encoding the wild-type mutant forms of *Xenopus* FGFR1 were subcloned into plasmid pCS2+.

Preparation of synthetic RNA

For all oocyte injections, a synthetic capped mRNA was made by using the SP6 mMessage mMachine kit according to the manufacturer's instructions (Ambion, Austin, TX, U.S.A.). pSP64A plasmid-containing x-ephrinB1 constructs were linearized with *Eco*RI, and pSP64Aten (-*Hind*III) plasmids were linearized with *Xba*I. pCS2+ plasmids containing the x-ephrinB1 constructs were linearized with Asp-718. Both x-Grb4 wild-type and mutant constructs in pCS2+ were linearized with *Not*I. FGFR constructs in pCS2+ [40] were also linearized with *Not*I, and pSP64T3 containing FGFR1C289R/K420A was linearized with *Bam*HI.

Preparation of *Xenopus* oocytes and microinjection

Female *Xenopus laevis* oocytes were purchased from Nasco (Fort Atkinson, WI, U.S.A.). *X. laevis* oocytes were obtained by incubating fragments of ovary in MBS (modified Barth's solution; 88 mM NaCl/1 mM KCl/2.5 mM NaHCO₃/0.7 mM CaCl₂/1 mM MgSO₄/5 mM Hepes, pH 7.8) to which was added 1.5 mg/ml collagenase (Roche, Indianapolis, IN, U.S.A.). After gentle shaking for 1.5 h at 20 °C, the oocytes were rinsed 3–5 times in MBS and incubated in L-15 oocyte medium containing 1 % (w/v) BSA (Type V; Sigma). After maintaining for 20–30 min at 19 °C, the oocytes were rinsed at least three times in MBS, and stage V–VI oocytes [41] that were completely devoid of follicle cell investments were collected and maintained at 19 °C in L-15 oocyte medium. Microinjections were performed, 18 h after oocyte preparation, using the Drummond Nanoject with an injection volume of 18.4 nl.

Specific amounts of various RNAs were injected into each oocyte: x-FGFR1K562E and x-FGFR1C289R/K420A at 10 ng, x-ephrinB1 wild-type and all mutants at 15 ng and x-Grb4 wild-type, x-Grb4 mutants and Nck1 at 7.5 ng. Oocytes were cultured for 16–18 h at 19 °C.

Immunoprecipitation and Western-blot analysis

Oocytes were homogenized with 10 µl/oocyte of cold lysis buffer [20 mM Tris/HCl (pH 8.0), 137 mM NaCl, 1 % Nonidet P40, 10 mM PMSF, 40 mM Na₃VO₄ and 15 mM β-glycerophosphate containing 1:50 diluted Calbiochem Protease Inhibitor Cocktail Set III], as described previously [34]. Insoluble material was removed by centrifugation at 14000 *g* for 10 min, followed by extraction with Freon (Sigma) at a 1:1 (v/v) ratio. Immunoprecipitation was conducted on 15 oocyte equivalents with the anti-FLAG antibody (Sigma) for 1 h, then Protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 4–12 h at 4 °C. All immunoprecipitates were washed with lysis buffer. Two oocyte equivalents were examined for direct lysate analysis. Immunocomplexes and lysates were resolved by SDS/PAGE and electrophoretically transferred on to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.). After the proteins were transferred on to a membrane, the membrane was probed in TBST buffer (10 mM Tris/HCl, pH 8/150 mM NaCl/0.05 % Tween-20) containing 5 % (w/v) milk. Primary antibodies were used at the following dilutions: anti-FLAG at 1:1000, anti-HA at 1:1000, anti-FGFR at 1:1000 and anti-phosphotyrosine at 1:1000. For anti-phosphotyrosine blotting, 5 % (w/v) BSA in TBST was used. The membrane was washed three times with TBST, incubated for 1 h with an appropriate secondary antibody coupled with HRP (horseradish peroxidase), washed

three times with TBST, followed by application of ECL[®] reagents according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ, U.S.A.).

For detection of immunocomplexes, the HRP-conjugated anti-HA antibody (Roche) was directly used at a dilution of 1:1000.

RESULTS

EphrinB1 physically interacts with Grb4 in a phosphorylation-dependent manner

We have found previously that an activated FGFR associated with and induced the phosphorylation of ephrinB1 on tyrosine residues [34]. Moreover, this phosphorylation decreased the ability of overexpressed ephrinB1 to cause cell dissociation. Our work suggested that cross-talk between the FGFR and ephrinB1 can exist in both neuronal and non-neuronal tissues. On the basis of this result, we hypothesized that an activated FGFR may induce the phosphorylation of conserved tyrosine residues, thus recruiting signalling molecules containing the SH2 domain to ephrinB1 and mediating or modulating reverse signalling. Since the Grb4 adaptor protein has been discovered to interact with ephrinB1 [35], we isolated the full-length *Xenopus* Grb4 cDNA. This cDNA encodes 381 deduced amino acids displaying 82 % identity with the human Nck2 and only 65 % identity with the *Xenopus* Nck1 (Figure 1; see the Experimental section). A synthetic RNA encoding C-terminally FLAG-tagged *Xenopus* ephrinB1 was co-expressed with HA-tagged Grb4 (Grb4-HA) in *Xenopus* oocytes in the presence of constitutively active FGFR.

Oocyte lysates were immunoprecipitated with anti-FLAG antibody and Western-blot analysis was performed with HRP-conjugated anti-HA antibody. The tyrosine phosphorylation state of *Xenopus* ephrinB1 was confirmed with HRP-conjugated anti-phosphotyrosine antibody. Oocyte lysates were also subjected to Western-blot analysis with anti-HA, anti-FLAG and anti-FGFR antibodies to determine the levels of protein expression. Similar protein expression levels were observed for ephrinB1, Grb4 and FGFR1 (Figure 2). As shown in Figure 2, ephrinB1 does not bind Grb4 in the presence of kinase-inactive FGFR. However, ephrinB1 is capable of binding Grb4 in the presence of constitutively active FGFR, which effectively phosphorylates ephrinB1, as revealed by Western-blot analysis with an anti-phosphotyrosine antibody. Phosphatase treatment of immunoprecipitates completely diminishes the association between ephrinB1 and Grb4, indicating that this interaction is dependent on phosphorylation. Although the SH2 domain of *Xenopus* Nck1 shares 65 % identity with that of Grb4, the full-length Nck1 shows little or no binding with tyrosine-phosphorylated ephrinB1, suggesting that Grb4 and Nck1 are not functionally interchangeable. Taken together, these results indicate that *Xenopus* Grb4 physically interacts with ephrinB1 in a phosphorylation-dependent manner. Moreover, a constitutively active FGFR induces the Grb4 and ephrinB1 interaction, similar to an interaction that is also initiated as a result of ephrinB binding to the ectodomain of the EphB receptor. FGFR and ephrinB1 have overlapping expression patterns during development and, therefore, have the opportunity to interact, raising the possibility that FGFR signalling may be an alternative pathway affecting ephrinB1-mediated reverse signalling *in vivo*.

Formation of a complex between ephrinB1 and Grb4 depends on the SH2 domain of Grb4

To verify that the *Xenopus* Grb4 and ephrinB1 proteins display interaction requirements similar to those reported for the human and mouse proteins in the yeast two-hybrid system, we examined

Figure 1 Comparison of the amino acid sequences of human Grb4, mouse Nck2, *Xenopus* Grb4 and *Xenopus* Nck1

The indicated amino acid sequences are aligned. Identical amino acids and residues are highlighted in grey boxes and individual SH2 and SH3 domains are underlined in black. The arrowheads indicate the first tryptophan residue of a conserved tryptophan doublet within the SH3 domains and the arginine residue of the FLVR motif within the SH2 domain. h, human; m, mouse; x, *Xenopus*. Sequences were aligned using ClustalW (<http://cbcsrv.watson.ibm.com/Tmsa.html>).

Figure 2 Phosphorylation-dependent x-ephrinB1/x-Grb4 binding

Oocytes were left uninjected (–) or injected (+) with the indicated RNA(s). Oocyte lysates were immunoprecipitated (IP) with anti-FLAG antibody. For treatment with calf intestinal phosphatase (CIP) (New England Biolabs, Beverly, MA, U.S.A.), immunoprecipitates were washed three times with a buffer (100 mM NaCl/50 mM Tris/HCl, pH 7.9/10 mM MgCl₂), and then incubated with 1 unit of CIP. SDS/PAGE and Western-blot analysis of immunocomplexes were conducted with an anti-HA antibody (top panel). The phosphorylation state was revealed by an anti-phosphotyrosine antibody (second panel). This blot was stripped for reprobing with anti-FLAG antibody (third panel). Oocyte lysates were analysed directly by SDS/PAGE and Western-blot analysis with anti-FLAG (fourth panel), anti-HA (fifth panel) and anti-FGFR1 (sixth panel) antibodies. FGFR1 KE, constitutively active *Xenopus* FGFR1 (FGFR1K562E); KD, kinase-dead *Xenopus* FGFR1 (FGFR1C289R/K420A); ephrinB1-FLAG, FLAG-tagged *Xenopus* ephrinB1; Grb4-HA, HA-tagged *Xenopus* Grb4; Nck1, HA-tagged *Xenopus* Nck1.

whether the SH2 or SH3 domains of *Xenopus* Grb4 are sufficient for phosphorylation-dependent binding to ephrinB1 in the *Xenopus* oocyte system. The Grb4 protein consists of three SH3 domains and one SH2 domain that are critical for mediating protein–protein interactions. SH3 domains have a characteristic

conserved tryptophan doublet, and SH2 domains have a conserved FLVR motif. Mutation of the first tryptophan residue in the WW doublet within an SH3 domain is known to affect binding with a target protein in a phosphotyrosine-dependent manner [37]. In addition, mutation of the arginine residue in the FLVR motif of SH2 domains precludes binding to the target protein in a phosphotyrosine-dependent manner [38].

We generated four point mutations (W38K, W149K, W235K and W38K/W149K/W235K) in Grb4 where each of the individual tryptophan residues is replaced by a lysine residue or all three tryptophan residues are replaced by lysine residues. We also created one mutation (R312L) in Grb4 where an arginine residue in the FLVR motif is replaced by a leucine residue. To examine the interaction of these Grb4 mutants with ephrinB1, synthetic RNAs encoding the above mutants were co-expressed with ephrinB1 in *Xenopus* oocytes along with a constitutively active *Xenopus* FGFR. As shown in Figure 3, mutations in either one or all three SH3 domains have no effect on the ability to bind ephrinB1. However, a mutation within the SH2 domain of Grb4 completely inhibits the interaction with ephrinB1, indicating that the SH2 domain of Grb4 is critical for binding tyrosine-phosphorylated ephrinB1. Taken together, these results support the concept that the formation of a complex between ephrinB1 and Grb4 depends specifically on the SH2 domain of Grb4.

The region between amino acids 301–308 in ephrinB1 is required for binding Grb4

EphrinB proteins consist of several functional domains, including an extracellular receptor-binding domain, a transmembrane domain and an intracellular cytoplasmic domain containing a PDZ-binding motif at the C-terminus. Within their intracellular domain, ephrinB proteins have five conserved tyrosine residues at positions 298, 305, 310, 324 and 325. In addition, ephrinB1 possesses an additional tyrosine residue at position 294 that is not conserved among other ephrinB proteins (B2 and B3). To determine the exact region of ephrinB1 that is required for binding Grb4, we generated a series of FLAG-tagged mutants that sequentially delete the conserved tyrosine residues from the C-terminus of ephrinB1.

Figure 3 Formation of a complex between x-ephrinB1 and x-Grb4 requires the SH2 domain

(A) Schematic representation of wild-type (WT) and mutant *Xenopus* Grb4. The SH2 and SH3 domains are represented by boxes; mutant domains in which ligand binding activity was ablated are indicated by a large 'X'. (B) Mapping of the x-ephrinB1-binding site on x-Grb4. A synthetic RNA encoding x-ephrinB1 was co-injected into *Xenopus* oocytes with each Grb4 mutant in the presence of constitutively active x-FGFR1 or a kinase-dead x-FGFR1. Lysates were immunoprecipitated with an anti-FLAG antibody, transferred and immunoblotted with an anti-HA antibody (uppermost panel). Lysates were analysed directly by SDS/PAGE followed by Western-blot analysis with anti-FLAG (second panel), anti-HA (third panel) and anti-FGFR1 (bottom panel) antibodies. Grb4 W38K, W149K, W235K and W38/149/235K, HA-tagged *Xenopus* Grb4 mutants having Trp residues replaced by Lys. Grb4 R312L has a substitution of Lys in place of Arg. Other abbreviations are the same as in Figure 2.

EphrinB1 Δ 60 is a deletion mutant lacking the entire cytoplasmic domain. The ephrinB1 Δ 40 deletion mutant lacks the coding sequence that includes all six tyrosine residues. EphrinB1 Δ 33 deletion mutant only retains the tyrosine residue at amino acid position 294. EphrinB1 Δ 26 is a truncation mutant containing tyrosine residues at positions 294 and 298. EphrinB1 Δ 19 retains tyrosine residues at positions 294, 298 and 305. EphrinB1 Δ 15 retains tyrosine residues at positions 294, 298, 305 and 310, but lacks the possible binding site (PXXP) for SH3 domain proteins and the PDZ-binding motif (YYKV). EphrinB1 Δ 6 is a PDZ-binding-motif deletion mutant.

To examine the interaction of these mutants with Grb4, synthetic RNAs encoding the above mutants were co-expressed with

Figure 4 Requirement of the region between amino acids 301 and 308 of x-ephrinB1 for binding to x-Grb4

(A) Schematic representation of wild-type and mutant *Xenopus* ephrinB1. The solid line indicates the extracellular receptor-binding domain. The hatched box and dotted box represent the transmembrane domain and cytoplasmic region respectively. x-EphrinB1 deletion mutants are constructed by the serial deletion of the C-terminal region. The C-terminal 40 amino acids are represented. (B) Synthetic RNAs encoding wild-type and C-terminally truncated x-ephrinB1 proteins were injected into oocytes together with x-Grb4 and an activated x-FGFR1 or a kinase-dead x-FGFR1. Equal amounts of lysates were subjected to immunoprecipitation with anti-FLAG antibody, then transferred and blotted with anti-HA antibody (upper panel). Oocyte lysates were probed by immunoblotting as indicated. ephrinB1 FLAG Δ 6– Δ 60, FLAG-tagged *Xenopus* ephrinB1 mutants lacking the indicated number of C-terminal amino acids. Other abbreviations are the same as in Figure 2.

Grb4 and a constitutively active FGFR in *Xenopus* oocytes. Co-immunoprecipitations were performed on oocyte lysates using an anti-FLAG antibody. Bound Grb4 protein was detected with the HRP-conjugated anti-HA antibody. As shown in Figure 4, three deletion mutants (ephrinB1 Δ 6, ephrinB1 Δ 15 and ephrinB1 Δ 19) are capable of interacting with Grb4, indicating that the tyrosine residues within the PDZ-binding motif and the tyrosine residue at position 310 are not required for binding to Grb4. Furthermore, the ephrinB1 Δ 15 mutant provides evidence that the possible SH3-binding motif within the C-terminus is not necessary for an interaction with Grb4. In contrast, the ephrinB1 Δ 60, ephrinB1 Δ 40, ephrinB1 Δ 33 and ephrinB1 Δ 26 deletion mutants all fail to interact with Grb4. These results demonstrate that the region between amino acids 301 and 308 in ephrinB1 is critical for the interaction with Grb4.

The tyrosine residue at position 305 is not involved in the interaction with Grb4

The observation that the ephrinB1 Δ 19 mutant retains the ability to bind Grb4 raises the possibility that the tyrosine residue

Figure 5 Tyr-305, Tyr-310 and Ser-302 of x-ephrinB1 are dispensable for x-Grb4 interaction

(A) Schematic representation of x-ephrinB1 cDNA harbouring mutations at Tyr-298, Ser-302, Tyr-305 and Tyr-310 in the conserved cytoplasmic domain. The C-terminal 33 amino acids of x-ephrinB1 are shown. Numbers indicate positions of the five conserved tyrosine residues and the corresponding amino acids in mouse ephrinB1 are shown in parentheses. (B) Tyr-305 and Tyr-310 are not required for the binding of Grb4. *Xenopus* oocytes were injected with the indicated combinations of x-ephrinB1 mutants, Grb4 and an activated or kinase-dead FGFR1. Lysates were immunoprecipitated using an anti-FLAG antibody. Co-immunoprecipitation of HA-tagged x-Grb4 was detected by Western-blot analysis with an anti-HA antibody (uppermost panel). Expressions of x-ephrinB1, x-Grb4 and x-FGFR1 proteins in lysates was determined by Western-blot analysis with an indicated antibody (second, third and bottom panels). FLAG-tagged *Xenopus* ephrinB1 mutants have single, double or triple mutations as indicated. Abbreviations are the same as in Figures 2 and 4.

at position 305 in ephrinB1 may participate in Grb4 binding. Moreover, the tyrosine phosphorylation of ephrinB1 in response to stimulation by a soluble form of the EphB2 ectodomain is known to occur on Tyr-305, Tyr-310 and Tyr-324 within its cytoplasmic domain [21]. In the following experiment, three point mutations (Y305F, Y310F and Y305F/Y310F) in ephrinB1 were generated where each of the tyrosine residues is replaced by a phenylalanine residue. The ability of these mutants to interact with Grb4 was tested by co-immunoprecipitation assays. The results show a robust interaction between Grb4 and either the wild-type or mutant ephrin proteins, indicating that the mutation at Tyr-305 does not affect Grb4 binding (Figure 5). Expression levels appear to be equivalent among the different proteins. These results suggest that the interaction between Grb4 and ephrinB1 may not involve the tyrosine residues at positions 305 and 310

in ephrinB1. Alternatively, the binding may not occur via a classical SH2-phosphotyrosine interaction. Recent studies reveal that several SH2-domain-containing signalling proteins interact with their binding partners in a phosphotyrosine-independent fashion [42,43].

After stimulation by the EphB receptor, the cytoplasmic domain of ephrinB1 is predominantly phosphorylated at serine and tyrosine residues *in vivo* [21,32]. Since the ephrinB1Δ26 mutant has a deletion at Ser-302 and loses its ability to bind Grb4, we examined whether Ser-302 of ephrinB1 is involved in binding Grb4. Several mutants were constructed that replace Ser-302 with an alanine residue either singly (S302A) or in combination with tyrosine-to-phenylalanine substitutions at positions 310 (S302A/Y310F) and 305 (S302A/Y305F/Y310F). These mutants were co-injected with Grb4-HA and the interaction was assayed by co-immunoprecipitation analysis. As shown in Figure 5, the serine-to-alanine substitution does not affect Grb4 binding regardless of whether the tyrosine residues at 305 and 310 are also mutated, indicating that the serine residue is not involved in the formation of a complex between ephrinB1 and Grb4. These results suggest that upstream tyrosine residues (Tyr-294 and Tyr-298) may be critical to the phosphorylation-dependent binding of ephrinB1 with Grb4. This possibility is strongly supported by an experiment involving the ephrinB1Δ24 mutant. Although ephrinB1Δ24 loses four conserved tyrosine residues at positions 305, 310, 324 and 325, the interaction between ephrinB1 and Grb4 is maintained. Collectively, these results demonstrate that the tyrosine residues at positions 305 and 310 in ephrinB1 are not necessary for binding Grb4 and that Ser-302 is also not necessary for this interaction.

Tyr-298 of ephrinB1 is critical for binding Grb4

To define further the residues necessary for the interaction with Grb4, we tested whether the tyrosine residue at position 298 within ephrinB1 is important for binding Grb4. Three ephrinB1 point mutants were created (Y298F, Y298F/Y305F and Y298F/Y305F/Y310F), where each tyrosine residue was replaced by a phenylalanine residue. Synthetic RNAs encoding these mutants were co-expressed with Grb4-HA, and examined for the ability to interact with Grb4 by co-immunoprecipitation and Western-blot analysis. Interestingly, the Tyr-298 mutant (Y298F) is incapable of binding Grb4, indicating that Tyr-298 is a critical residue for ephrinB1/Grb4 binding (Figure 6). It is worth noting that substitution of negatively charged amino acids (glutamic or aspartic acid residue) for Tyr-298 does not rescue Grb4 binding, supporting the concept that this interaction requires a phosphorylated tyrosine residue at position 298 rather than a mere structural alteration caused by a negative charge (Figure 6). Recent phosphopeptide mapping experiments suggested that Tyr-310 of ephrinB1 might be involved in binding Grb4 [36]. However, our *in vivo* binding experiment shows that although the ephrinB1 double mutant (Y298F/Y305F) has a tyrosine residue at 310, it will not associate with Grb4. This result, in concert with our co-immunoprecipitation experiments using the ephrinB1 Y310F mutant (Figure 6), implies that Tyr-310 of ephrinB1 is not required for binding Grb4 *in vivo*. The observation that the Y298F mutant does not associate with Grb4 raises the formal possibility that ephrinB1 may not be effectively phosphorylated by an activated FGFR. To examine this possibility, the phosphorylation state of each mutant was verified by probing the blot with an HRP-conjugated anti-phosphotyrosine antibody. All mutants show equivalent levels of phosphorylation, suggesting that the ephrinB1 protein is indeed effectively phosphorylated by the FGFR.

Figure 6 Tyr-298 of x-ephrinB1 is critical for x-Grb4 interaction

Synthetic RNAs encoding x-ephrinB1 or x-ephrinB1 mutants were co-injected with x-Grb4 and an activated or kinase-dead x-FGFR1 as indicated. Immunoprecipitation was conducted with an anti-FLAG antibody. Associated proteins were separated by SDS/PAGE and analysed by immunoblotting with anti-HA antibody (top panel). Anti-phosphotyrosine antibody was used to visualize the phosphorylation state of ephrinB1 and each x-ephrinB1 mutant (second panel). Oocyte lysates were analysed by SDS/PAGE and Western-blot analysis with anti-FLAG (third panel), anti-HA (fourth panel) and anti-FGFR1 (bottom panel) antibodies respectively. EphrinB1-FLAG Y298F, Y298D and Y298E represent FLAG-tagged *Xenopus* ephrinB1 mutants having Tyr-298 replaced by Phe, Asp and Glu as indicated. All other mutants have single or combination mutations as indicated. Other abbreviations are the same as in Figure 2.

Amino acids 301–304 of ephrinB1 are essential for an interaction with Grb4

The fact that ephrinB1 Δ 26 is not capable of binding Grb4 appeared to be in contrast with our results obtained with the Y298F mutant, because ephrinB1 Δ 26 still possesses two possible phosphorylation sites at 294 and 298. The inability of ephrinB1 Δ 26 to interact with Grb4 suggested two likelihoods: (1) the additional residues C-terminal to Tyr-298 are critical components of the Grb4-binding site, and (2) Tyr-298 may remain unphosphorylated in this mutant, perhaps owing to loss of either the bound kinase or the kinase recognition site. To assess these probabilities, three deletion mutants (ephrinB1 Δ 24, ephrinB1 Δ 22 and ephrinB1 Δ 20) that truncate this region were constructed. Synthetic RNAs encoding these mutants were co-expressed with Grb4-HA, and examined for the ability to interact with Grb4 using immunoprecipitation and Western-blot analysis. The tyrosine phosphorylation state was assessed using an HRP-conjugated anti-phosphotyrosine antibody. As shown in Figure 7, although ephrinB1 Δ 26 displays a basal level of tyrosine phosphorylation, it does not bind Grb4. Furthermore, the phosphorylation state markedly increases and the gel mobility correspondingly shifts with the addition of C-terminal residues, as demonstrated by ephrinB1 Δ 24, ephrinB1 Δ 22 and ephrinB1 Δ 20. Interestingly, the ephrinB1 Δ 24 mutant associates with Grb4, implying that the region between amino acids 301 and 304 may be required for FGFR-induced phosphorylation of ephrinB1. Taken together, these results suggest that the tyrosine residue at position

Figure 7 Tyr-298 phosphorylation is dependent on amino acids 301–304

Synthetic RNAs encoding wild-type ephrinB1 or a series of ephrinB1 deletion mutants were injected into oocytes with x-Grb4 and an activated or kinase-dead FGFR1. Lysates were immunoprecipitated using an anti-FLAG antibody. Co-immunoprecipitation of HA-tagged x-Grb4 was detected by Western-blot analysis using an anti-HA antibody (uppermost panel) and the tyrosine phosphorylation level was detected using an anti-phosphotyrosine antibody (second panel). Expressions of x-ephrinB1, x-Grb4 and x-FGFR1 proteins were determined by Western-blot analysis with an indicated antibody (second, third and bottom panels respectively). Other abbreviations are the same as in Figures 2 and 4.

298, and the region that includes amino acids 301–304 within ephrinB1 are absolutely necessary for binding Grb4.

DISCUSSION

As bidirectional signalling molecules, ephrinB proteins play an important role in regulating numerous cellular events during development. Particularly, ephrinB reverse signalling has been implicated in cell migration, segmentation and vasculogenesis [1–3].

On the basis of recent studies, reverse signalling may be classified into four distinct pathways that specifically require the intracellular domain of ephrinB: (1) JNK activation that correspondingly causes cell rounding, (2) inhibition of heterotrimeric G-proteins via the binding of PDZ-RGS3 to the PDZ-binding motif (YYKV) in the C-terminus of ephrinB molecules, thus affecting cerebellar granule cell migration, (3) activation of RhoA via binding of Dishevelled and (4) modulation of cytoskeletal dynamics via phosphotyrosine-dependent binding of Grb4 that requires conserved tyrosine residues within 33 amino acids of the C-terminus [28,29,31,35].

We isolated the *Xenopus* Grb4 protein that displays 82% identity with the human Nck2 and only 65% identity with the *Xenopus* Nck1 (Figure 1). Grb4 and Nck1 are structurally related and share several common protein targets. Recent evidence indicates that mouse Grb4 and Nck1 have overlapping and mutually compensatory functions regarding embryonic survival [44]. Although these two proteins appear to have many overlapping functions, there is also evidence to suggest that they may have divergent functions. A previous study has shown that Nck2, but not Nck1, interacts with the LIM (Lin-11, Isl-1, Mec-3)-only adaptor protein PINCH (particularly interesting new Cys-His protein) through its SH2 domain [45]. Furthermore, our observation that *Xenopus* Nck1 showed little or no binding with tyrosine-phosphorylated ephrinB1 suggests that *Xenopus* Grb4 and Nck1 are not functionally interchangeable (Figure 2). These results support similar findings indicating that the human Nck1 SH2 domain does not bind mouse ephrinB1 [35]. It is

also worth noting that tyrosine-phosphorylated ephrinB1 is specifically recognized by the SH2 domain of Grb4. Although ephrinB1 has a characteristic PXXP motif for binding SH3 domain proteins, mutation of all three SH3 domains has no effect on the formation of a complex between ephrinB1 and Grb4, indicating that Grb4 binding absolutely depends on its SH2 domain (Figure 3).

Although it has been shown that contact with either clustered EphB receptors or EphB-receptor-expressing cells induces the phosphorylation of ephrinB1 and the recruitment of the Grb4 adaptor protein, the tyrosine kinase that phosphorylates ephrinB molecules is not yet definitive. A recent study demonstrated that SFKs are positive regulators of ephrinB1 phosphorylation and phosphotyrosine-mediated reverse signalling [32]. However, it is not clear whether the tyrosine phosphorylation of ephrinB1 by SFKs can recruit adaptor proteins having SH2 domains such as Grb4. It is noteworthy that ephrinB1 phosphorylation induced by calf serum was not affected by a specific SFK inhibitor, indicating that kinases other than SFKs can mediate the serum response that results in phosphorylation of ephrinB1 and can trigger or modulate its reverse signal.

Previously, we showed that an activated FGFR tyrosine kinase can induce the phosphorylation of ephrinB1 within its conserved 33-amino-acid C-terminus [34]. In the present study, we show that tyrosine phosphorylation induced by the FGFR affects the ability of ephrinB1 to complex with Grb4. We initially postulated that the interaction between ephrinB1 and Grb4 might depend on Tyr-305 and Tyr-310 within ephrinB1, because these two tyrosine residues were major phosphorylation sites *in vivo*. Moreover, these two tyrosine residues reside within the 22-residue cytoplasmic region of ephrinB1 that is required for Grb4 binding [35]. Strikingly, mutation and co-immunoprecipitation analysis indicates that both Tyr-305 and Tyr-310 have little effect on Grb4 binding, suggesting a different role for these two tyrosine residues in reverse signalling (Figure 5). In addition, although both Tyr-305 and Tyr-310 may be important for the association of ephrinB1 with the FGFR [34], either single (ephrinB1Y305F and ephrinB1Y310F) or double substitutions of both tyrosine residues (ephrinB1Y305F/Y310F) does not affect the ability of ephrinB1 to bind Grb4. This suggests that ephrinB1 may not be directly phosphorylated by FGFR, since ephrinB1 is still phosphorylated in the absence of these two tyrosine residues (Figure 7; [34]).

Earlier work has demonstrated that PMA, a general activator of protein kinase C, promotes tyrosine phosphorylation and oligomerization of endogenous ephrinB1 [46]. This finding raises the possibility that both Tyr-305 and Tyr-310 might be required for the oligomerization of ephrinB1 proteins [46]. Alternatively, tyrosine phosphorylation may result in the recruitment of a set of SH2-domain-containing proteins that are yet to be identified.

We further demonstrated that the cytoplasmic region between amino acids 301 and 304 is required for an interaction between ephrinB1 and Grb4. Although we are unable to preclude the possibility that this region is a component of the Grb4-binding site, our results provide evidence that these residues may be important for the interaction with, or recognition by, a tyrosine kinase that phosphorylates ephrinB1. Specifically, ephrinB1 Δ 26 retains only one of the five conserved tyrosine residues and fails to bind Grb4. In contrast, ephrinB1 Δ 24 maintains the same tyrosine residue, but associates with Grb4. Moreover, although a basal level of tyrosine phosphorylation is observed in ephrinB1 Δ 26, a mobility shift that accompanies a markedly higher phosphorylation state is achieved when more C-terminal residues are present (ephrinB1 Δ 24, ephrinB1 Δ 22 and ephrinB1 Δ 20). Collectively, these results indicate that amino acid 304 represents the most C-terminal residue necessary for tyrosine phosphorylation of Tyr-298 and the requisite binding of Grb4.

We have also identified Tyr-298 of ephrinB1 as a critical residue for an interaction with Grb4. Indeed, mutation of Tyr-298 abrogates the formation of a complex between ephrinB1 and Grb4, suggesting that Tyr-298 may be phosphorylated *in vivo* and recruits SH2 domain proteins such as Grb4. Whereas a previous *in vivo* mapping study did not identify Tyr-298 as a major phosphorylation site [21], another recent study using NMR and phosphorylated peptides supports the concept that Tyr-298 can bind the Grb4 SH2 domain [36]. However, in contrast with the previous report, it is clear that in the *Xenopus* system *in vivo*, Grb4 requires the phosphorylation of Tyr-298, but not the tyrosine residue at position 305 or 310. Thus, it is possible that this phosphorylation event is context-dependent and both spatially and temporally restricted.

In summary, our results indicate that the FGFR tyrosine kinase can induce the phosphorylation of ephrinB1 on Tyr-298. This specific event allows ephrinB1 to associate with the downstream signalling molecule Grb4 that can mediate or modulate ephrinB reverse signalling.

We thank Bruce Mayer (Department of Genetics and Developmental Biology, University of Connecticut Health Center, Farmington, CT, U.S.A.) for providing x-Nck1 cDNA and Robert Friesel (Center for Molecular Medicine, Maine Medical Center Research Institute, South Portland, ME, U.S.A.) for x-FGFR antibodies. We also thank Peter Johnson from our laboratory for a critical reading of the manuscript.

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Received 22 September 2003; accepted 10 October 2003

Published as BJ Immediate Publication 10 October 2003, DOI 10.1042/BJ20031449