Ephrin-B2 is a candidate ligand for the Eph receptor, EphB6

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Abstract No ligand has hitherto been designated for the Eph receptor tyrosine kinase family member, EphB6. Here, expression of an EphB6 ligand in the pro-B leukemic cell line, Reh, is demonstrated by binding of soluble EphB6-Fc fusion protein to the Reh cells. The ligand belongs to the subgroup of membrane spanning ligands, as suggested by the fact that phosphatidylinositol-specific phospholipase C treatment did not abrogate binding of EphB6-Fc. Two transmembrane Eph receptor ligands, ephrin-B1 and ephrin-B2, were identified in Reh cells. Analysis of EphB6-Fc fusion protein binding to ephrin-B1 or ephrin-B2 transfected COS cells revealed a high-affinity saturable binding between EphB6-Fc and ephrin-B2, but not with ephrin-B1. In mice, EphB6 has previously been shown to be expressed in thymus. Here, we show expression of EphB6 in human thymus, as well as the expression of ephrin-B2 in both human and mouse thymus. We conclude that ephrin-B2 may be a physiological ligand for the EphB6 receptor.

Key words: Tyrosine kinase; Ligand; Reh cell line; Scatchard analysis

1. Introduction

Receptor tyrosine kinases and their ligands play a critical role in regulation of cellular survival, proliferation and differentiation [1]. On the basis of predicted structural homologies, sequence conservation and similarity of ligands, receptor tyrosine kinases have been assigned to several subclasses [2]. The largest family of receptor tyrosine kinases, the Eph receptors, consists of at least 14 distinct members highly conserved from *Caenorhabditis elegans* to man (reviewed in [3–6]). Recently, a family of at least eight membrane-bound ligands for Eph receptors, termed ephrins, has been identified (reviewed in [7]). The ephrins share between 23 and 56% identity at the amino acid level and display promiscuous binding to Eph receptors [8]. The ligands can be divided into two classes, those that are attached to the cell membrane by a glycosylphosphatidylinositol (GPI) linkage, ephrin-A1–A5, and those that are membrane spanning molecules, ephrin-B1–B3. The cytoplasmic domains of the transmembrane ligands ephrin-B1 and ephrin-B2 have been shown to be phosphorylated on tyrosine residues after contact with their receptors, suggesting that the transmembrane ligands have receptor-like intrinsic signaling potential [9,10].

The biological function of the interaction of Eph receptors with their corresponding ligands is being unveiled in several different cell systems. Signaling via Eph receptors and ephrins has been implicated in neural and epithelial morphogenesis [11], axon guidance and fasciculation [12–16], control of neural crest cell migration [17–20], formation of rhombomere boundaries [21], inhibition of cell–cell adhesion [22], angiogenesis [23] and morphogenesis of capillary beds [24]. In addition, deregulated expression of the Eph receptors or ligands may play a role in malignant transformation of cells [25–28]. Except for the EphB5 and EphB6 receptors, at least one ligand has been defined for each member of the Eph receptor family [8,29].

In this study we have identified a candidate ligand for the EphB6 receptor.

2. Materials and methods

2.1. Cell lines

All hematopoietic cell lines were grown in RPMI 1640 supplemented with 10% FBS. The monkey cell line COS-1 was grown in DMEM supplemented with 5% FBS. All cell lines were grown at 37°C in a humidified atmosphere with 5% CO_2.

2.2. Construction of vectors

For this study we generated expression vectors for the production of EphB6, EphB1 and CD19 (short), all as fusion proteins with the Fc part of mouse IgG2b. The fusion partner includes, in addition to the CH2 and CH3 domains, also the hinge region of mouse IgG2b (plasmid denoted pcDNA1-Fc). EphB6 ectodomain cDNA was generated by PCR on reverse transcribed first strand cDNA (RT-PCR, HPB-ALL cell line mRNA), using primers that amplified the sequence corresponding to the region encoding amino acids 1–567 in the published EphB6 sequence [30], and further cloned in-frame with the Fc part of mouse IgG2b in pcDNA1-Fc. The recognition sequences of the restriction enzymes *Hin*dIII and *Bgl*II were included in the EphB6 forward and reverse primer respectively, for cloning purposes. EphB1 ectodomain cDNA was generated by RT-PCR (brain mRNA), using primers that amplified the sequence corresponding to the region encoding amino acids 1–538 [31] and further cloned in-frame with the Fc part of mouse IgG2b in pcDNA1-Fc. The recognition sequences of the restriction enzymes *Hin*dIII and *Bam*HI were included in the EphB1 forward and reverse primer respectively, for cloning purposes. The sequence encoding the 51 N-terminal amino acids of the CD19 mRNA was amplified from a CD19 cDNA clone [32] and further cloned in-frame with mouse Fc part of IgG2b in pcDNA1-Fc. For cloning purposes, the recognition sequences of the restriction enzymes *Hin*dIII and *Bam*H1 were included in the CD19 forward and reverse primer respectively.

Ephrin-B1 and ephrin-B2 cDNAs were PCR amplified from Reh first strand cDNA using primers that amplified the open reading frame. Ephrin-B1 was amplified using primers that included amino acids 1–346 [33] and further cloned into the pcDNA1 vector (Invitrogen, CA, USA) using the restriction sites *Hin*dIII and *Xho*I included in the primers. Ephrin-B2 was amplified using primers that included

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amino acids 1–333 [34] and further cloned into the pcDNA1 vector using the restriction sites *Hin*dIII and *Xho*I included in the primers. All constructs were confirmed by sequencing.

2.3. Identification of ephrins by degenerate PCR

Degenerate primers were synthesized based on amino acids that are highly conserved among ephrin-B members. The forward primers (F) were directed against the sequence KFQE (amino acids nos. 113–116 in ephrin-B2) and the reverse primers (R) were directed against the sequence CPHYE (amino acids nos. 301–305 in ephrin-B2). The sequences were: (F) 5′ GGG ATC CAA RTT YCA RGA RT and (R) 5′ GGA ATT CTT YTC RTA RTG NGG RCA (N for A/C/G/T, R for A/G, Y for C/T). First strand cDNA generated from Reh cells was used for the amplification. The PCR conditions were as follows: 42 cycles of 94°C for 30 s, 37°C for 40 s and 65°C for 100 s with the primers F and R. The resulting PCR product was cloned in T-vector (Promega, Madison, WI, USA) and individual clones were sequenced.

2.4. Transfection of COS cells and production of fusion proteins

COS cells were transfected for binding analysis and for fusion protein production. Briefly, COS cells were transfected using a DEAE-dextran protocol [35] with 1 μg/ml plasmid in the transfection medium. For binding analysis, the cells were detached from the plates with 1 mM EDTA in PBS 2 days post-transfection.

Production and secretion of fusion proteins were verified by ^{35}S-methionine labeling of transfected COS cells, and the radiolabeled fusion proteins were subsequently purified from the culture supernatant by protein-G purification and analyzed by SDS–PAGE. For larger scale protein production, transfected COS cells were grown in 225 cm^2 culture flasks (50–100 ml medium) and fusion proteins were affinity purified from the culture supernatants using a protein-G column.

2.5. Cell staining

Reh cells were incubated with the fusion proteins EphB1-Fc, EphB6-Fc and CD19-Fc (2.5 μg/ml) for 1 h at 4°C. The cells were then washed twice and stained with a phycoerytrin-labeled (PE) anti-mouse Ig polyclonal antibody (Ig-RPE, Southern Biotechnology Associates, Birmingham, AL, USA) directed to the Ig tail of the fusion proteins. 10^4 cells were analyzed by flow cytometry.

COS cells transfected with either ephrin-B1 or ephrin-B2 cDNAs were incubated with EphB1-Fc, EphB6-Fc and CD19-Fc fusion proteins. The cells were washed and stained with a PE-labeled anti-mouse Ig polyclonal antibody. 10^4 cells were analyzed by flow cytometry.

2.6. Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment of Reh cells

1×10^6 Reh cells were treated with 1.1 U/ml PI-PLC (Roche Diagnostic, Germany) for 2 h at 37°C in RPMI 1640, followed by washing with PBS. The cells were incubated with either CD19-Fc or EphB6-Fc followed by staining with PE conjugated anti-mouse antibody or anti-CD24-FITC conjugated antibody (Pharmingen, CA, USA). Anti-CD24 serves as a positive control for the PI-PLC treatment. 10^4 cells were analyzed by flow cytometry.

2.7. Scatchard plot analysis

COS cells were transiently transfected with ephrin-B2 expression plasmid, and used in the binding assays 2 days post-transfection. The EphB6-Fc fusion protein was initially bound to the transfectants (1 h, 4°C) followed by binding of a fixed amount of a ^{125}I-labeled anti-mouse antibody (1 h, 4°C). Ephrin-B2 transfectants were analyzed for binding of different concentrations of EphB6-Fc, ranging from 2 μg/ml to 0.0025 μg/ml. To calculate the molar concentration of EphB6-Fc fusion protein, a molecular mass of 190 kDa was assumed. Saturating amounts of ^{125}I-labeled anti-mouse immunoglobulin were used to detect the binding of EphB6-Fc. Cell-bound ^{125}I-labeled antibody was quantified in a Packard autogamma counter. Non-specific binding of ^{125}I-labeled antibody was assayed in the absence of EphB6-Fc, as well as in the presence of a 200-fold molar excess of unlabeled antibody. Both methods gave similar estimates for the non-specific binding, and the figure shows the specific binding, obtained by subtracting non-specific binding from total binding. The specific binding data (total binding minus non-specific binding) were analyzed by non-linear curve fitting using Microsoft Excel 97 with the Solver add-in, and were best fit to the equation $Y = B_{max}*X/(K_d+X)$, indicating the presence of only one type of binding sites. The observed data and the theoretical curve were plotted according to Scatchard [36] using Microsoft Excel 97 (inset).

2.8. Northern blot analysis

Total RNA was extracted from mouse tissues (Balb/c) by standard guanidine thiocyanate methods and mRNA was isolated from tissues using oligo-dT Dynabeads (Dynal, Norway) as previously described [37]. 2 μg mRNA was size-fractionated on a 1% agarose formaldehyde denaturing gel and transferred to nylon membranes. A commercially available multiple tissue Northern blot was purchased from Clontech (Immune blot 1; Clontech, CA, USA). Prehybridization (1 h) and hybridization were performed in hybridization buffer (5×SSPE, 10% dextran sulphate, 0.1% SDS, 50% formamide, 100 μg/ml sheared salmon sperm DNA) at 42°C, and the membranes were hybridized overnight with ^{32}P-dCTP-labeled EphB6 cDNA probe, ephrin-B2 cDNA probe or with a control β-actin probe. After hybridization, the membranes were washed under high stringency in 0.2 SSC/0.1% SDS at 65°C.

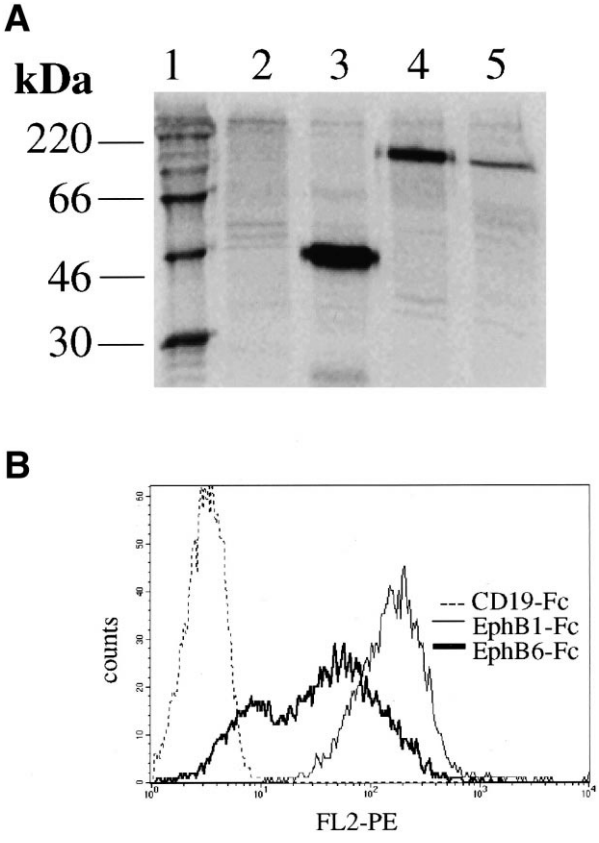

Fig. 1. A: Production and secretion of fusion proteins in transfected COS cells. COS cells were transfected in 6-well plates and the cells were labeled overnight with ^{35}S-methionine in methionine-free medium 2 days post-transfection. Fusion proteins were isolated from the culture medium by incubation with protein-G Sepharose beads and separated by SDS–PAGE under denaturing conditions. Lane 1, radioactive molecular weight standard; lane 2, mock transfected COS cells; lane 3, COS cells transfected with CD19-Fc plasmid; lane 4, COS cells transfected with EphB1-Fc plasmid; lane 5, COS cells transfected with EphB6-Fc plasmid. The protein bands occurring in lanes 3–5 are the CD19-Fc fusion protein, the EphB1-Fc fusion protein and the EphB6-Fc fusion protein, respectively. B: Flow cytometry analysis on fusion protein binding to the Reh cell line. Reh cells were incubated with either CD19-Fc, EphB1-Fc or EphB6-Fc fusion proteins (2.5 μg/ml) followed by staining with a PE conjugated anti-mouse antibody.

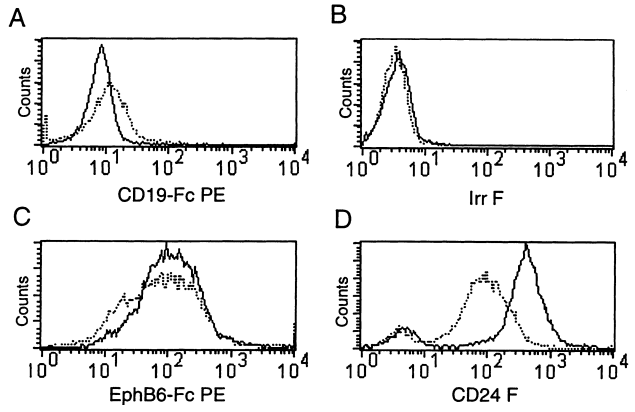

Fig. 2. Flow cytometry analysis on PI-PLC treated Reh cells. The cells were incubated with either CD19-Fc (A) or EphB6-Fc (C) followed by PE conjugated anti-mouse antibody (A and C), irrelevant Ig-FITC (B) or anti-CD24 FITC conjugated (D). Dotted lines represent PI-PLC treated cells, bold line represents untreated cells.

3. Results and discussion

The aim of this study was to identify a candidate ligand for the EphB6 receptor, and to further apply this knowledge in studies of the interaction of the EphB6 receptor with its ligand in hematopoietic tissues. Therefore, a fusion protein of the ectodomain of EphB6 with mouse IgG2b-Fc was generated and applied in binding studies. Corresponding fusion proteins of the ectodomain of EphB1 and of the 51 N-terminal amino acids of the CD19 protein were also produced. Plasmids encoding the fusion proteins were transfected into COS cells, and fusion proteins were isolated from the culture supernatant. Production and secretion of fusion proteins were verified by ^{35}S-methionine-labeling of transfected COS cells followed by SDS–PAGE. The molecular masses of both EphB1-Fc and EphB6-Fc fusion proteins were approximately 100 kDa, and the CD19-Fc fusion protein was approximately 45 kDa (Fig. 1A).

To test the binding characteristics of the fusion proteins, a panel of hematopoietic cell lines were screened for cell surface binding of the EphB6-Fc fusion protein including the B cell lines JKB-2, Tom-1, Reh, Nalm-6, Daudi and Rael, the T cell lines JM, Jurkat and JY, the myeloid cell lines KG1-A and HL60, and the erythroid cell line K562. To this end, only one cell line, the leukemic pro-B cell line Reh, bound the EphB6-Fc fusion protein as shown in Fig. 1B. Therefore, this cell line was used in the following studies to identify a candidate EphB6 ligand. Fig. 1B also shows that the EphB1-Fc fusion protein binds to the Reh cell line. No binding was observed for the CD19-Fc fusion protein which serves as a negative control.

The ligands of the Eph receptors are divided in two classes, ephrin-A1 to ephrin-A5 that are bound to the cell membrane by a GPI linkage, and ephrin-B1 to ephrin-B3 that are membrane spanning molecules [8]. Treatment of cells with phosphatidylinositol-specific phospholipase C (PI-PLC), which releases GPI-linked proteins from their lipid moiety, can be used to test if a given Eph receptor binds to a transmembrane or GPI-linked ligand [38]. As shown in Fig. 2C, the treatment of Reh cells with PI-PLC had minimal effect on the EphB6-Fc fusion protein binding, while a significant reduction in the GPI-linked surface protein CD24 [39] was observed under the same treatment (Fig. 2D) demonstrating that the assay was functional. We conclude from this observation that the EphB6 receptor most likely binds to a member of the transmembrane class of Eph ligands (ephrin-B). This verifies the assumption made by the Eph Nomenclature Committee who has placed the EphB6 receptor in the subclass of receptors (EphB1–B6) that preferentially bind transmembrane ligands based on the relatedness of their extracellular domains [40]. The other Eph receptor subgroup (EphA1–A8) preferentially binds GPI-linked ephrin ligands.

Based on the results with PI-PLC treatment, we established a PCR-based screen to identify the transmembrane ligand(s) that are expressed in the Reh cell line. The intracellular domain of the three transmembrane ligands, ephrin-B1–B3, are highly conserved, in particular at the 33 most C-terminal amino acids [41]. In addition, there are conserved amino acid stretches in the extracellular domain. Degenerated oligonucleotides were generated based on a conserved sequence in the extracellular domain, amino acids KFQE and a conserved region in the intracellular domain, CPHYE. The primers were employed in RT-PCR using Reh mRNA. The resulting product was cloned and subsequently sequenced. Initially, DNA from five separate clones was sequenced. Four of the sequences were identical to ephrin-B1 and one sequence was identical to ephrin-B2. No ephrin-B3 sequence was identified. Northern blot analysis confirmed the expression of both ephrin-B1 and ephrin-B2 in the Reh cells (data not shown).

RT-PCR analysis of Reh cell mRNA with ephrin-B3-specific primers revealed no expression of ephrin-B3 in these cells (data not shown). Based on this information, cDNAs, including the complete open reading frames of both ephrin-B1 [33] and ephrin-B2 mRNA [34], were amplified by RT-PCR from Reh cell mRNA and further cloned into the expression vector pcDNA1 for transfection and subsequent binding analysis.

Ephrin-B1 and ephrin-B2 cDNAs in pcDNA1 were transfected into COS cells and EphB6-Fc binding was evaluated by flow cytometry. As shown in Fig. 3A, binding of EphB6-Fc to the ephrin-B2 transfectants was demonstrated in the concentration range of 2.5 μg/ml to 0.05 μg/ml of EphB6-Fc fusion protein. In contrast, binding to the ephrin-B1 transfectants was much weaker and only observed at the highest concentration of EphB6-Fc (2.5 μg/ml, Fig. 3B) as revealed by both fewer positive cells and less fluorescence intensity compared with ephrin-B2. No binding was observed to the ephrin-B1 transfectants at lower EphB6-Fc concentrations (0.5–0.05 μg/ml), in contrast to the binding observed to the ephrin-B2 transfectants (Fig. 3). Both the ephrin-B1 and the ephrin-B2 ligands have previously been shown to bind to the EphB1 receptor with approximately the same binding affinity [8]. We also found comparable binding of EphB1-Fc fusion protein to both the ephrin-B1 and ephrin-B2 transfectants (Fig. 3B), in the range of 1.25 to 0.02 μg/ml. To confirm that the ephrin-B2 ligand bound to the EphB6 receptor, we demonstrate that ephrin-B2-Fc fusion protein bound to COS cells transfected with full length EphB6 cDNA (data not shown).

Previously, the ephrin-B2 ligand has been shown to bind the receptors EphB1, EphB2, EphB3 and EphB4 and subsequently induce auto-phosphorylation of these receptors [8,42–44]. The ephrin-B1 ligand also binds and induces auto-phosphorylation of the EphB1, EphB2, EphB3 receptors [11], but it binds with very weak affinity to EphB4 [44]. In vitro clus-

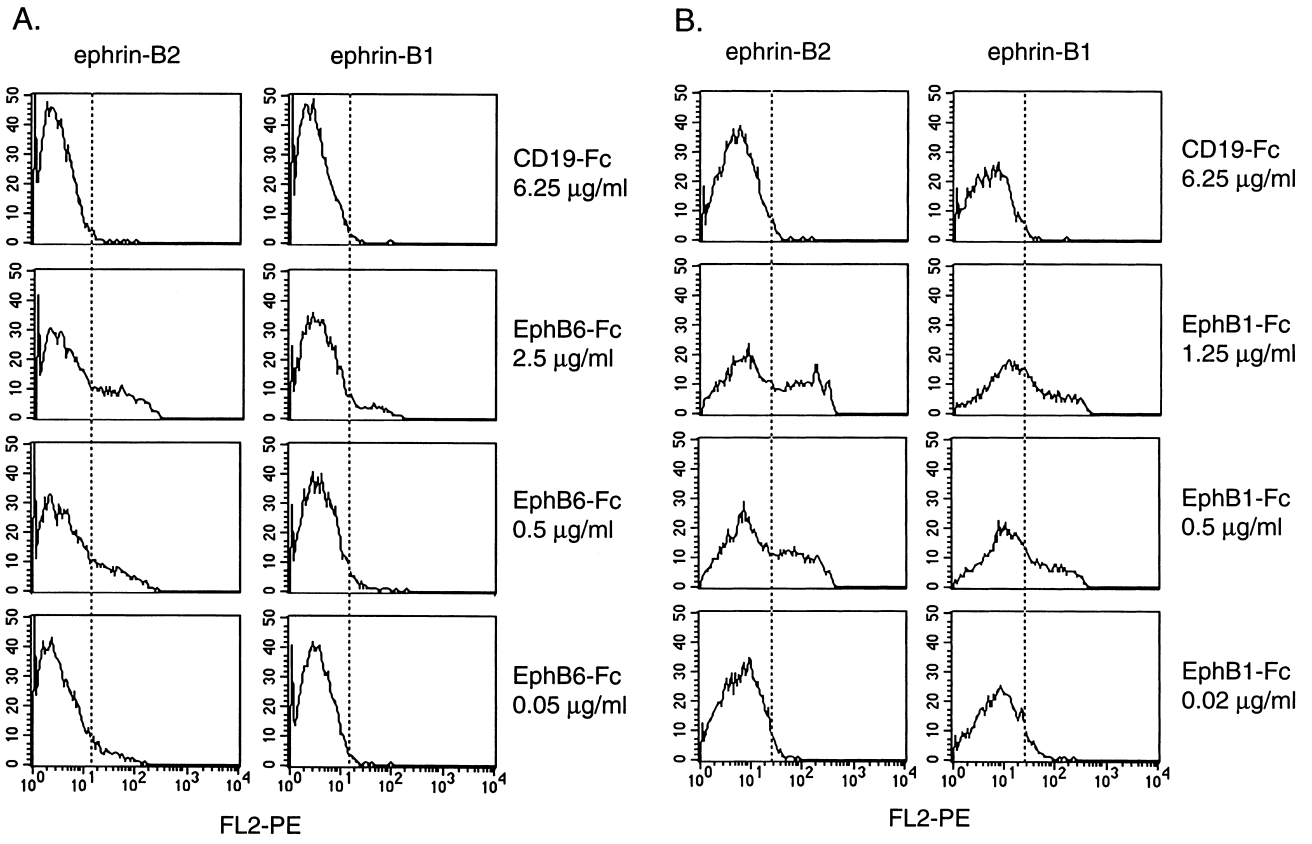

Fig. 3. Flow cytometry analysis on binding of EphB6-Fc or EphB1-Fc fusion proteins to ephrin-B1 and ephrin-B2 transfected COS cells. The cells were incubated with either EphB6-Fc or EphB1-Fc followed by staining with PE conjugated anti-mouse antibody. Dead cells were excluded by propidium iodide staining. A: Flow cytometry analysis on EphB6-Fc binding to ephrin-B2 (left panel) and ephrin-B1 (right panel) transfected COS cells, using decreasing concentrations of EphB6-Fc fusion protein. The upper panel shows binding of the negative control protein (CD19-Fc) to the transfected cells. B: Flow cytometry analysis of EphB1-Fc binding to ephrin-B2 (left panel) and ephrin-B1 (right panel) transfected COS cells, using decreasing concentrations of EphB1-Fc fusion protein. The upper panel shows binding of the negative control protein (CD19-Fc) to the transfected cells. The *Y* axis shows the cell counts.

tering of ephrin-B1 ligand does not induce auto-phosphorylation of EphB4 like it does for EphB1–B3, but intriguingly, coculturing of EphB4 and ephrin-B1 transfectants leads to auto-phosphorylation of EphB4, although to a lower extent than coculturing with the ephrin-B2 ligand [44]. It remains unclear if ephrin-B1 is a physiological ligand for the EphB4 receptor. Here we show that EphB6, like EphB4, preferentially binds to ephrin-B2 in comparison to ephrin-B1.

In addition, we have performed binding studies of EphB6-Fc to COS cells transfected with two members of the ephrin-A class of ligands (ephrin-A2 and ephrin-A4) showing no binding to these ligands (data not shown).

Further, to evaluate the affinity of the interaction between EphB6 and ephrin-B2, we performed quantitative analysis of the binding between EphB6-Fc fusion protein and COS cells expressing ephrin-B2, using a two-step screening protocol [45]. COS cells were transfected with ephrin-B2 expression plasmid, and used in the binding assays 2 days post-transfection. The EphB6-Fc fusion protein was initially bound to the transfectants followed by binding of a fixed amount of a ^{125}I-labeled anti-mouse antibody. Our calculations are based on the assumption that one molecule of EphB6-Fc was bound per ligand molecule at saturation, and that one molecule of ^{125}I-labeled antibody was bound per EphB6-Fc molecule. Ephrin-B2 transfectants were analyzed for binding of different concentrations of EphB6-Fc. Saturating amounts of ^{125}I-labeled

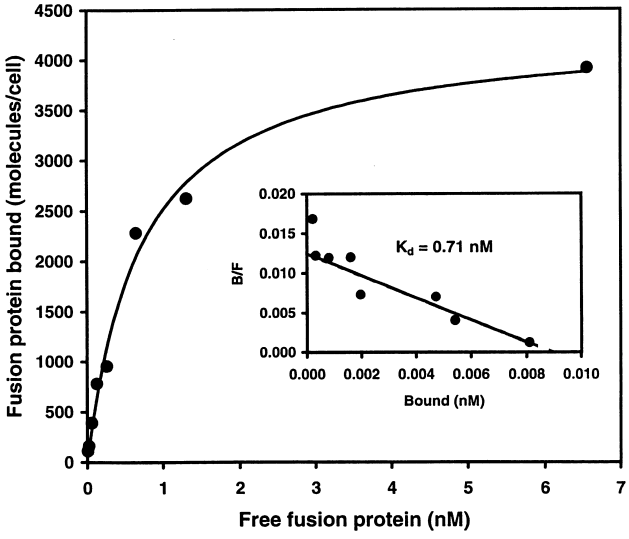

Fig. 4. Quantitative analysis of the binding between EphB6-Fc fusion protein and COS cells expressing ephrin-B2. COS cells were transiently transfected with ephrin-B2 expression plasmid, and used in the binding assays 2 days post-transfection. Binding of the EphB6-Fc fusion protein was assayed as described in Section 2. Insets show Scatchard representations of specific binding. The figure shows one representative experiment of three, yielding similar results.

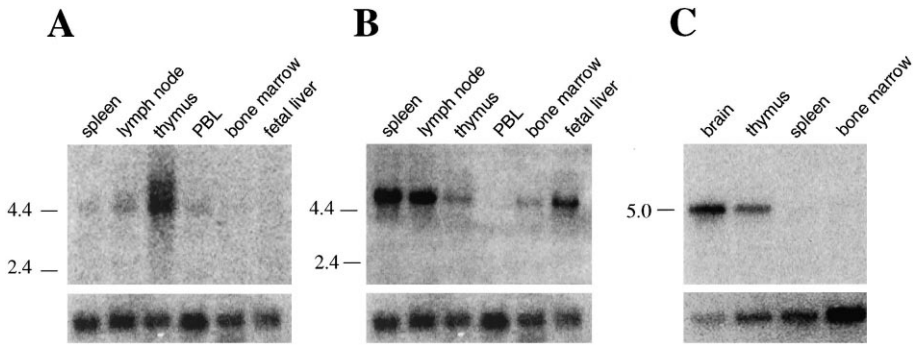

Fig. 5. mRNA expression of EphB6 and ephrin-B2 in different human and mouse tissues. A: Expression of EphB6 (upper panel) in different human hematopoietic tissues. B: Expression of ephrin-B2 (upper panel) in different human hematopoietic tissues. C: Expression of ephrin-B2 (upper panel) in different mouse hematopoietic tissues and brain. Lower panels in A, B and C represent hybridization with a β-actin probe. Molecular weight in kb indicated to the left.

anti-mouse immunoglobulin were used to detect the binding of EphB6-Fc to COS cells. The specific binding data were analyzed by non-linear curve fitting, and were best fit to the equation $Y = B_{max} * X/(K_d + X)$, indicating the presence of only one type of binding sites. Fig. 4 shows the specific binding data as well as the Scatchard [36] transformation of the observed data and the theoretical curve, for illustration (Fig. 4, inset) of one of three experiments yielding similar results. The results indicate a dissociation constant (K_d) of 0.5–1 nM, which is comparable to data presented for binding of other ligands to the Eph receptors [8,46,47].

A true receptor-ligand interaction would imply binding of the ligand followed by auto-phosphorylation of the receptor leading to intracellular signaling. In both mouse and man the intracellular tyrosine kinase domain of EphB6 has been shown to be defective [48,30] and therefore such a ligand interaction would not induce auto-phosphorylation by the receptor. Previously, it has been shown that also the transmembrane ephrin ligands can be phosphorylated on tyrosine residues after receptor interaction [9,10]. Thus, it is proposed that transmembrane ligands of Eph receptors act not only as conventional RTK ligands, but also as receptor-like signaling molecules, suggesting that transmembrane ligands and their receptors mediate bidirectional signaling. In the case of the EphB6 receptor one can speculate that, due to the defective kinase domain, intracellular signaling after receptor-ligand interaction is not bidirectional, but unidirectional and occurs only in the cells carrying the membrane spanning ligand. In our hands, we have neither succeeded in detecting phosphorylation of the ephrin-B2 ligand after interaction with EphB6-Fc, nor of ephrin-B1 after EphB1-Fc interaction as previously shown by others [9,10].

Lastly, we performed EphB6 and ephrin-B2 mRNA expression analysis in hematopoietic tissues. Human EphB6 expression in such tissues has previously not been demonstrated. We show here that, as in mice [30], this receptor is strongly expressed in human thymus and barely detectable in other hematopoietic tissues (Fig. 5A). Furthermore, ephrin-B2 mRNA was detected in human spleen and lymph node, and with weaker expression in thymus, bone marrow and fetal liver (Fig. 5B). A different expression pattern was observed in mice, with high ephrin-B2 mRNA level in thymus (comparable to brain) and absence of ephrin-B2 mRNA in spleen and bone marrow (Fig. 5C). Thus, in both man and mouse, the EphB6 receptor and its candidate ligand, ephrin-B2, can both be found in thymus, although the ephrin-B2 expression in mouse thymus is more pronounced than in humans. We therefore speculate that this receptor-ligand couple serves a physiological role in thymus.

In conclusion, we report here that ephrin-B2 is a candidate ligand for the EphB6 receptor. The interaction of EphB6 and ephrin-B2 was shown to be of high affinity by Scatchard analysis. EphB6 has a limited expression in both human and mouse tissues, and in hematopoietic tissues expression is mainly in thymus. Ephrin-B2 expression is also observed in thymus, although weak in humans. The next challenge in the study of the biological function of this receptor-ligand interaction will be to define the cell populations in the micro-environment, where these genes are expressed, for further functional analysis.

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