A new ephrin-A1 isoform (ephrin-A1b) with altered receptor binding properties abrogates the cleavage of ephrin-A1a

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Ephrins are ligands for the Eph receptor tyrosine kinases, which play important roles in patterning nervous and vascular systems. Ephrin-A1 is a glycosylphosphatidylinositol-anchored ligand that binds to the EphA receptor tyrosine kinases. In the present study, we have identified a new ephrin-A1 isoform, denoted ephrin-A1b (ephrin-A1 isoform b). Compared with the originally described ephrin-A1 sequence, ephrin-A1a [Holzman, Marks and Dixit (1990) *Mol. Cell. Biol.* **10**, 5830–5838], ephrin-A1b lacks a segment of 22 amino acids (residues 131–152). At the transcript level, exon 3 is spliced out in the transcript encoding ephrin-A1b. Transfection of HEK-293T cells (human embryonic kidney 293 cells) with an ephrin-A1b-expressing plasmid resulted in a signi-

ficant expression of the protein on the cell surface. However, soluble EphA2 receptor (EphA2-Fc) bound weakly to ephrin-A1b-expressing transfectants, but bound strongly to ephrin-A1a-expressing transfectants. Ephrins have been shown to undergo regulated cleavage after interaction with their receptors. This process is inhibited by co-expression of ephrin-A1a and ephrin-A1b, indicating that ephrin-A1b influences the cleavage process. Taken together, these findings indicate that this newly described isoform may regulate the function of its ephrin-A1a counterpart.

Key words: cleavage, ephrin, isoform, phosphorylation, receptor, transfectant.

INTRODUCTION

Eph receptor tyrosine kinases and their ligands, known as ephrins, can orchestrate cell movements by transducing bidirectional tyrosine kinase-mediated signalling both into cells expressing the receptors and into cells expressing the ligands [1,2]. Therefore Eph receptors and ephrins can act both as receptors and ligands. Bidirectional signalling mediated by Eph receptors and ephrins is supposed to transduce signals that regulate the cytoskeleton during axon path finding, cell migration, cell adhesion and vascular remodelling [3–6]. One prominent function for Eph receptors is to establish cell positioning and maintain cellular organization [7].

Ephrins can be divided into two subgroups. The ephrin-A class consists of six members that are linked to the plasma membrane through a GPI (glycosylphosphatidylinositol) anchor, whereas the ephrin-B class consists of three members and they are transmembrane proteins. In general, an ephrin-A binds promiscuously to EphA receptors and an ephrin-B binds promiscuously to EphB receptors, with the exception of some ephrin-B ligands binding EphA4 [8] and EphB4 that binds preferentially the ephrin-B2 ligand [9,10]. Reverse signalling through ephrins has been observed for both subgroups. The signalling cascade through ephrins has been shown to affect adhesion, migration, boundary formation, chemotaxis and vascularization [7]. Members of the ephrin-B subgroup become tyrosine-phosphorylated after receptor interaction and can serve either as a scaffold for adapter proteins, such as Grb4 [11] or as a substrate for a phosphatase PTB-B1 (protein tyrosine phosphatase BAS-like) [12]. Ephrin-A has been shown to recruit the Src family kinase Fyn into lipid rafts, accompanied by redistribution of vinculin, activation of the mitogen-activated protein kinase pathway, tyrosine phosphorylation of a 120 kDa protein and increased cell-substrate adhesion [13,14]. Interestingly, ephrin-A reverse signalling may be modulated by cell-surface cleavage of the ligand through association with the metalloproteinase ADAM10/Kuzbanian [15].

This could serve a dual function, namely termination of signal and repulsion instead of adhesion.

We were interested in examining the functions of ephrins and Eph receptors, particularly in relation to the immune system [16–18]. During our studies, we discovered a new ephrin-A1 isoform, denoted ephrin-A1b (ephrin-A1 isoform b) with altered receptor binding properties. When co-expressed with ephrin-A1a, it inhibited cleavage of the ephrin-A1a isoform mediated through receptor cross-linking.

EXPERIMENTAL

Cells and antibodies

HEK-293T cells (human embryonic kidney 293 cells; A. T. C. C. CRL 1573) were grown in RPMI 1640 (Gibco BRL, Paisley, Scotland, U.K.), supplemented with 10 % (v/v) foetal calf serum (Gibco BRL), at 37 °C in a humidified atmosphere containing 5 % CO₂. The antibodies used were anti-ephrin-A1 (catalogue no. Sc-911; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-ephrin-A4-biotin (catalogue no. BAF369; R&D Systems, Minneapolis, MN, U.S.A.), anti-phosphotyrosine PY99 (catalogue no. Sc-7020; Santa Cruz Biotechnology), anti-phosphotyrosine 4g10 biotin (catalogue no. 16-103; Upstate Biotechnology, Lake Placid, NY, U.S.A.), anti-EphA2 (catalogue no. Sc-924; Santa Cruz Biotechnology) and anti-actin (catalogue no. Sc-1616; Santa Cruz Biotechnology).

Identification of an ephrin-A1 spliced variant

PCR was performed on tonsil cells with specific ephrin-A1 primers. The resulting PCR product was separated on a 1 % (w/v) agarose gel. Two bands were observed, one with the expected size and the other lower, indicating the presence of a spliced variant.

Abbreviations used: EST, expressed sequence tag; GPI, glycosylphosphatidylinositol; DTT, dithiothreitol.

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The lower band was excised, cloned into pGEMT Easy vector (Promega, Madison, WI, U.S.A.) and sequenced. The sequence revealed a deletion in the nucleotide sequence of ephrin-A1 corresponding to the amino acids 131–152. A BLAST search was performed and the corresponding EST (expressed sequence tag) sequences were identified. One of these ESTs (GenBank® accession no. BI754211) was obtained from HGMP (Human Genome Mapping Project) Resource Centre UK (Hinxton Hall, Cambridge, U.K.) and fully sequenced.

PCR was performed on cDNA generated from mRNA from different tissues (Clontech, Palo Alto, CA, U.S.A.) to evaluate the relative expression levels of the two ephrin-A1 transcripts.

Generation of ephrin-A1 cDNA constructs

For transfection purposes, full-length cDNAs corresponding to ephrin-A1a (GenBank® accession no. NM_004428) and ephrin-A1b (NM_182685) were amplified by PCR using specific primers. Tonsil RNA was used as a template for ephrin-A1a, whereas an EST cDNA clone (GenBank® accession no. BI754211) was used as a template for generating ephrin-A1b constructs. Full-length cDNAs, including the complete open reading frame of the two ephrin-A1 variants, was cloned into the expression vector pCDNA1 (Invitrogen, Carlsbad, CA, U.S.A.).

Generation of an ephrin-A4 deletion mutant

An ephrin-A4 deletion mutant, lacking the amino acids corresponding to exon 3 in ephrin-A1b, was generated by a two-step PCR approach. In the first PCR, a reverse primer was used that included the nucleotide sequences 407–429 and 495–515 of ephrin-A4 (accession no. NM_005227), thus excluding the sequence corresponding to amino acid residues 135–156 (nucleotide sequence 430–494). This was combined with a forward vector-specific primer. The template for the PCR was an ephrin-A4 cDNA clone described previously (accession no. NM_005227). A second PCR was performed that amplified the ephrin-A4 nucleotide sequence 496–633, including the stop codon and a restriction site (*Xho*I). Products from both these reactions were pooled and used as the template in a final PCR using the same forward vector-specific primer in combination with the reverse primer that included the stop codon. The resulting product, where the nucleotide sequence from amino acids 430–494 was deleted, was gel-isolated and cloned into pCDNA1 expression vector using the restriction sites *Hind*III and *Xho*I. Sequence analysis confirmed that the sequence was correct and lacked the nucleotide sequence 430–494. This cDNA clone was termed ephrin-A4del (ephrin-A4 deletion mutant).

Generation of fusion proteins, transfection of cells and flow cytometry

The control fusion protein CD19-Fc was generated as described previously [17]. Construction of EphA2-Fc was performed, in principle, as described previously for EphB6-Fc and EphB1-Fc [18]. EphA2 ectodomain was amplified by PCR using specific primers and the resulting product was cloned in front of the Fc part of mouse IgG2b, thereby generating EphA2-Fc. HEK-293T cells were transfected as described previously [19] using LIPOFECTAMINE™ (Invitrogen). Soluble EphA2-Fc and CD19-Fc were purified from the culture supernatant by affinity chromatography on a Protein G column (Amersham Biosciences, Uppsala, Sweden) as described previously [17]. For detection of surface-bound ephrin molecules, transfected HEK-293T cells were incubated with anti-ephrin-A1 or anti-ephrin-A4 antibody or EphA2-Fc and then stained with a PE (phycoerythrin)-labelled

anti-mouse Ig polyclonal antibody or PE-labelled anti-rabbit antibody (Southern Biotechnology Associates, Birmingham, AL, U.S.A.) or PE-labelled streptavidin (BD Transduction Laboratories, Lexington, KY, U.S.A.). Surface expression was analysed using a FACSCalibur flow cytometer (Becton Dickinson).

Subcellular fractionation

Transfected cells were washed with ice-cold PBS, resuspended in buffer A [5 mM Tris/HCl (pH 8)/5 mM EDTA/75 mM sucrose, proteinase and phosphatase inhibitors] and then sonicated four times for 15 s. Nuclei were pelleted by centrifugation at 400 g for 5 min at 4 °C using a microcentrifuge. The supernatants were centrifuged at 32 000 g for 30 min at 4 °C using a Beckman centrifuge, collected and used as the cytosolic fraction. The membrane pellets were washed three times with PBS, solubilized in buffer A containing 1 % Triton X-100 for 15 min at 4 °C and then centrifuged at 10 000 g for 10 min at 4 °C using a microcentrifuge. The supernatants were used as the membrane fraction. Alternatively, to detect phosphorylated proteins, the pellets were dissolved in buffer A containing 1 % Triton X-100 and sample buffer (3 × SDS/0.1 M DTT) was added; the samples were then boiled before separation by SDS/PAGE.

Biotinylation and isolation of cell-surface proteins

The transfected cells were washed twice with ice-cold PBS and resuspended in bicarbonate buffer (10 mM NaHCO₃/150 mM NaCl, pH 8.5). We added 50 µg/ml biotin-X-NHS (where NHS stands for *N*-hydroxysuccinimido; Calbiochem, Darmstadt, Germany), and incubated the cells on ice for 30 min. Cells were then washed once with bicarbonate buffer and twice with ice-cold PBS before lysis of the cells in PBS/1 % Nonidet P40. For the isolation of biotinylated proteins, cell lysates were incubated with magnetic streptavidin beads (Dynal, Oslo, Norway) for 30 min at 4 °C, followed by magnetic separation. The proteins bound to streptavidin beads were dissolved in sample buffer, boiled and separated by SDS/PAGE.

EphA2-Fc stimulation

HEK-293T cells were transfected with either ephrin-A1a- or ephrin-A1b-expressing plasmids alone or co-transfected with ephrin-A1a + ephrin-A1b (1:1 or 5:1). The cells were serum-starved overnight in RPMI 1640 24 h after transfection. For the phosphorylation experiments, EphA2-Fc were immobilized on to anti-mouse Ig magnetic beads (Dynal) and incubated with pre-warmed transfected cells for the indicated times. The cells were then resuspended in buffer A, sonicated and fractionated into cytosolic and membrane fractions as described above (see the Subcellular fractionation subsection). Cytosolic and membrane fractions were separated by SDS/PAGE, blotted and the filters were hybridized with the indicated antibodies. For shedding experiments, serum-starved transfected HEK-293T cells were incubated for the indicated times with anti-mouse Ig cross-linked EphA2-Fc (1:5). The cells were then pelleted using a microcentrifuge and the incubation medium was separated by SDS/PAGE, blotted and hybridized with an anti-ephrin-A1 antibody.

EphA2 activation

Activation of EphA2 by ephrin-A1a transfectants or ephrin-A1a + ephrin-A1b co-transfectants was evaluated by inducing phosphorylation. HEK-293T cells were transfected with EphA2 or ephrin-A1a or co-transfected with ephrin-A1a + ephrin-A1b. Serum-starved (14 h) EphA2-transfected cells (2 × 10⁶) were mixed with the same number of either ephrin-A1 transfectants

Figure 1 Comparison of ephrin-A1a with ephrin-A1b and genomic organization of *EFNA1*

(A) Amino-acid sequence comparison of ephrin-A1a (a) with ephrin-A1b (b). Residues 131–152 in ephrin-A1a are missing in ephrin-A1b. (B) Genomic organization of *EFNA1*. The transcript corresponding to ephrin-A1a consists of five exons (upper panel). Exon 3 (arrow) is spliced out in the transcript encoding ephrin-A1b. The sequences at the exon–intron junctions are shown in the lower panel. (C) Relative expression levels of ephrin-A1a- and ephrin-A1b-encoding transcripts were evaluated by PCR performed on cDNA generated from different tissues. An ethidium bromide-stained 2 % agarose gel is shown. St, DNA standard; B, brain; P, pancreas; K, kidney; L, lung; C, colon. The heavier band represents ephrin-A1a (a) and the lighter band represents ephrin-A1b (b).

or ephrin-A1a + ephrin-A1b co-transfectants in a total volume of 200 μ l of PBS at 37 °C. After 10 or 20 min incubation, cells were lysed in lysis buffer (50 mM Tris/HCl, pH 7.5/150 mM NaCl/1 % Nonidet P40) and the insoluble fraction was pelleted and discarded. EphA2 was immunoprecipitated from the lysates using an anti-EphA2 antiserum, separated by SDS/PAGE and blotted. Tyrosine phosphorylation was detected with an anti-phosphotyrosine-specific antibody (4g10), and equal loading of immunoprecipitated EphA2 was detected after stripping the blot and hybridizing with an anti-EphA2 antiserum.

RESULTS

Identification of an ephrin-A1 spliced variant

Previously, we detected ephrin-A1 mRNA expression in tonsil cells by Northern-blot analysis (results not shown). In the course of studying the expression of ephrin-A1 in tonsils, an ephrin-A1 spliced variant that has not been described so far was identified (see the Experimental section). Sequence analysis of this variant revealed that the nucleotide sequence corresponding to amino acid residues 131–152 in the ephrin-A1 sequence deposited previously in GenBank® (accession no. NP_004419) [20] was deleted. A BLAST search was performed with this sequence, and five ESTs were identified harbouring the same deletion (GenBank® accession nos. BI754211, BI545974, BI772038, BG256240 and AL833641). These EST clones were identified

in brain, hippocampus, pooled lung and spleen, adenocarcinoma and human endometrium carcinoma cell line cDNA libraries. One of these ESTs (accession no. BI754211) was obtained from HGMP Resource Centre UK and was fully sequenced. Sequence analysis revealed that this clone encoded an ephrin-A1 isoform with a deletion of amino acids 131–152 in the previously published ephrin-A1 sequence (accession no. NP_004419). The sequence included the GPI-signal sequence, which anchors it to the cell membrane. At the end of the present study, the originally described ephrin-A1 mRNA sequence [20,21] was curated by the NCBI (National Center for Biotechnology Information) staff and given the name ephrin-A1 transcript variant 1 (accession no. NM_004428). The corresponding protein was given the name ephrin-A1a. The new ephrin-A1 sequence we describe here is identical with ephrin-A1 transcript variant 2 (accession no. NM_182685), and the corresponding protein has been named ephrin-A1b.

A comparison of the two different ephrin-A1 sequences is shown in Figure 1(A). To determine the molecular basis of the two ephrin-A1 variants, we have analysed the genomic organization of the gene encoding ephrin-A1 (*EFNA1*). Using the ephrin-A1 sequence published previously (accession no. NM_004428) in a GenBank® search, a chromosome 1 clone (GenBank® accession no. AL713999) was identified. The sequence of this clone was used to generate the genomic organization. *EFNA1* consists of five exons: the 5'-untranslated region and the translation start codon are located in the first exon, whereas the GPI-linkage sequence,

Figure 2 Binding characteristics of the two ephrin-A1 isoforms

(A) Flow-cytometry analysis of ephrin-A1-transfected cells. Cells were stained with the control fusion protein CD19-Fc (bold line) or EphA2-Fc (dotted line). Ephrin-A1b transfectants showed weak binding, whereas ephrin-A1a transfectants showed strong binding of EphA2-Fc. (B) Flow-cytometric analysis of ephrin-A1-transfected cells. Cells were stained with an irrelevant control antibody (bold line) or an anti-ephrin-A1 antibody (dotted line). Lower staining intensity was observed for ephrin-A1b compared with ephrin-A1a. (C) Western-blot analysis of cell lysates from ephrin-A1-transfected cells. Equal amounts of total cell lysates were loaded on to the gel. The filter was hybridized with an anti-ephrin-A1 antibody. M, mock; a, ephrin-A1a; b, ephrin-A1b.

the stop codon and the 3'-untranslated region are located in the last exon (Figure 1B). The chromosomal organization shows that exon 3 is spliced out in the ephrin-A1b-encoding transcript, leading to the loss of 22 amino acids.

To analyse the relative expression of ephrin-A1a- and ephrin-A1b-encoding transcripts, specific primers were used to amplify ephrin-A1 from different tissues known to express ephrin-A1. Significant expression of both variants at similar levels could be observed in brain and kidney, whereas ephrin-A1a was the main transcript observed in pancreas and lungs (Figure 1C). A low level of expression was observed in the colon. The heavier band observed on a 2% agarose gel corresponds to the ephrin-A1a-amplified transcript, whereas the lighter band corresponds to the ephrin-A1b-amplified transcripts, as confirmed by isolation of the bands, cloning and sequencing.

Characterization of EphA2-Fc and anti-ephrin-A1 binding

Ephrin-A1a binds with high affinity to the EphA2 receptor [21]. To evaluate the effects of the deleted amino acids in ephrin-A1b on receptor binding, both ephrin-A1a and ephrin-A1b full-length cDNAs were cloned and expressed in HEK-293T cells. Binding studies were then performed. Cells were stained, 2 days after transfection, with either EphA2-Fc or an anti-ephrin-A1 antibody and subsequently analysed by flow cytometry (Figure 2). Interestingly, we detected weak binding of EphA2-Fc to the ephrin-A1b transfectants, whereas ephrin-A1a-transfected cells showed a strong binding (Figure 2A). No binding to cells was observed with the control fusion protein CD19-Fc. Similar results were obtained using COS cells transfected with ephrin-A1a or

Figure 3 Cell-membrane association of the two ephrin-A1 isoforms

(A) Transfection of cells and subcellular fractionation of lysates into membrane and cytosolic fractions (see the Experimental section). One-half of the membrane fractions and one-tenth of the cytosolic fractions were applied on the gel. Upper panel: anti-ephrin-A1 hybridization. The same blot was stripped and hybridized with anti-actin to validate the subcellular fractionation (lower panel). M, mock; a, ephrin-A1a; b, ephrin-A1b. (B) Cell surfaces were biotinylated and the biotinylated proteins were isolated using streptavidin-coated beads before gel separation. The blot was hybridized with an anti-ephrin-A1 antibody. Positions of molecular-mass standards are indicated.

ephrin-A1b constructs (results not shown). In addition, a binding difference was also observed for the cell-surface binding of an ephrin-A1-specific antibody to the different ephrin-A1 transfectants. The antibody bound more strongly to ephrin-A1a transfectants when compared with ephrin-A1b transfectants (Figure 2B).

To exclude the possibility that the difference in binding could be related to total protein expression, cell lysates of transfected cells were subjected to Western-blot analysis (Figure 2C). No significant difference in the expression levels of ephrin-A1a and ephrin-A1b was observed. This shows that the antibody recognizes ephrin-A1b under denaturing conditions, although it has lower affinity for membrane-bound native ephrin-A1b compared with ephrin-A1a. Taken together, the deletion of amino acids 131–152 in ephrin-A1 altered the receptor-binding capacity of the ephrin-A1 molecule.

Both ephrin-A1a and ephrin-A1b are membrane-bound molecules

The ephrin-A1-specific antibody and soluble EphA2-Fc bound weakly to ephrin-A1b transfectants as shown by flow-cytometric analysis. However, our results do not rule out the possibility that ephrin-A1b is not displayed on the cell surface. Two approaches were therefore used to evaluate the cell-surface expression of ephrin-A1 on transfected cells. First, ephrin-A1 expression in cytosolic and membrane fractions was evaluated after subcellular fractionation by ultracentrifugation of sonicated cells (Figure 3A). No differences in the expression of ephrin-A1a and ephrin-A1b were observed in the membrane or cytosolic fraction, indicating that ephrin-A1b, similar to ephrin-A1a, is present at the cell membrane. Hybridization with an anti-actin antibody showed expression of actin mainly in the cytosolic fraction, validating the subcellular fractionation (Figure 3A). In the second approach, the cell surfaces of transfected cells were biotinylated, cell lysates were prepared, and biotinylated cell-surface proteins were isolated using magnetic streptavidin beads and then analysed by immunoblotting using an anti-ephrin-A1-specific antibody. As shown in Figure 3(B), both the ephrin-A1 isoforms are biotinylated and therefore expressed at the cell surface. Repeatedly, in these

experiments, we also observed a heavier complex of ephrin-A1b, indicating that this variant may form tighter complexes in the membrane without stimulation by a receptor (Figure 3B). Finally, transfected cells were fixed and stained with the anti-ephrin-A1 antibody. A similar staining pattern, showing cell-surface association, was observed for both the ephrin isoforms (results not shown). To conclude, these experiments show that ephrin-A1b is expressed at the cell surface at similar levels compared with ephrin-A1a in transfectants.

Ephrin-A4 deletion mutant

A GenBank® search was performed to find out whether the corresponding ephrin-A1b isoforms also exist for other members of the ephrin-A family. To this end, we have not identified any other ephrin-A member with a similar deletion. Still, to investigate if the area deleted in ephrin-A1b could lead to loss of receptor binding in other ephrin-A variants, a deletion mutant of ephrin-A4 was generated. An ephrin-A4 construct was generated by PCR and it carried a deletion of 22 amino acids from 135 to 156 in ephrin-A4 (GenBank® accession no. CAA06992). When compared with the ephrin-A1 sequence (amino acids 131–152), eight amino acids are identical in this area; in particular, a conserved cysteine residue exists (Figure 4A). The ephrin-A4 deletion mutant and wild-type ephrin-A4 (ephrin-A4del and ephrin-A4wt respectively) were transfected into HEK-293T cells and stained with soluble EphA2-Fc as described above. Weak or no binding of EphA2-Fc to ephrin-A4del was observed, whereas the soluble receptor bound strongly to ephrin-A4wt (Figure 4B). Both the ephrin-A4 variants were expressed at similar levels in HEK-293T cells (Figure 4C). This result is similar to the EphA2-Fc-binding studies of ephrin-A1a and ephrin-A1b transfectants (Figure 2), and it further underlines the importance of the deleted region in receptor binding.

Intracellular signalling

To investigate whether ephrin-A1a and ephrin-A1b could interact with each other, precipitation studies were performed using EphA2-Fc. EphA2-Fc precipitated ephrin-A1a but not ephrin-A1b, whereas both were detected after precipitation with EphA2-Fc in co-transfectants (Figure 5A). This result indicates that ephrin-A1b can associate with ephrin-A1a, as shown by a characteristic three-band appearance after hybridization of Western blots with an ephrin-A1 antibody, compared with the characteristic two-band appearance of ephrin-A1a alone (Figure 5A). Thus, if ephrin-A1b associates with ephrin-A1a, it may interfere with the signalling capacity of ephrin-A1a.

Next, we investigated whether the ephrin-A1b isoform affected the ability of the ephrin-A1a isoform to activate EphA2 by induction of tyrosine phosphorylation. EphA2 transfectants were mixed with either ephrin-A1a transfectants or ephrin-A1a + ephrin-A1b co-transfectants at 37 °C. Time points chosen were 10 and 20 min. EphA2 was then immunoprecipitated and phosphorylation was visualized by hybridization with a phosphotyrosine-specific antibody. The results show that ephrin-A1 transfectants induced the phosphorylation of EphA2 and no quantitative differences in the phosphorylation induction could be observed between ephrin-A1a transfectants and ephrin-A1a + ephrin-A1b co-transfectants (Figure 5B). This indicates that ephrin-A1b does not seem to affect the ability of ephrin-A1a to activate EphA2.

Several studies have identified proteins that are tyrosine-phosphorylated in response to receptor engagement of ephrin-A ligands, and these signalling events are suggested to regulate cellular adhesion [13,14]. To study phosphorylation events, transfectants were serum-starved and incubated with EphA2-Fc immobilized on magnetic beads for different time points. Tyrosine

Figure 4 Binding characteristics of ephrin-A4wt and ephrin-A4del

(A) Comparison of the sequences of ephrin-A1a and ephrin-A4. Identical amino acids are marked with asterisks. (B) Flow-cytometric analysis of ephrin-A4-transfected cells. Cells were stained with CD19-Fc (bold line) or EphA2-Fc (dotted line). Very weak binding of EphA2-Fc to ephrin-A4del was observed. (C) Western-blot analysis of cell lysates from ephrin-A4-transfected cells. Equal amounts of proteins were loaded on to the gel. The filter was hybridized with an anti-ephrin-A4 antibody.

phosphorylation of cellular proteins was investigated after sub-cellular fractionation of cell lysates into cytosolic and membrane fractions. The time points chosen were 0, 15, 30 and 60 min.

In the cytosolic fraction, distinct phosphorylation and dephosphorylation of proteins appeared as visualized by hybridization with a phosphotyrosine-specific antibody (Figure 5C). A transient tyrosine phosphorylation induced by receptor binding was observed for a protein of approx. 85 kDa after 15 min stimulation, whereas dephosphorylation of at least three proteins of approx. 175, 70 and 55 kDa was observed after 30 min stimulation (Figure 5C). However, no distinct differences in the phosphorylation/dephosphorylation kinetics could be observed between cells expressing ephrin-A1a alone and cells co-expressing ephrin-A1a and ephrin-A1b (Figure 5C). Thus the complexes of ephrin-A1a and ephrin-A1b did not seem to alter the ephrin signalling capacity, compared with ephrin-A1a-transfected cells. No distinct phosphorylation or dephosphorylation of proteins during the chosen time points was seen in cells transfected with ephrin-A1b and incubated with immobilized EphA2-Fc (results not shown).

Figure 5 Intracellular tyrosine phosphorylation of ephrin-A1 transfectants after EphA2-Fc interaction

(A) Immunoprecipitation (IP) of ephrin-A1 with EphA2-Fc from transfected cell lysates. IP with EphA2-Fc was successful for ephrin-A1a (a) and ephrin-A1a + ephrin-A1b (b), but not for ephrin-A1b single transfectants. M, mock transfectants. Filters were hybridized with an anti-ephrin-A1 antibody. (B) Induction of phosphorylation of EphA2 after ephrin-A1 interaction. EphA2 transfectants were mixed with either ephrin-A1a transfectants (a) or ephrin-A1a + ephrin-A1b co-transfectants (a + b) after 10 and 20 min. The 0 min time point corresponds to EphA2 transfectants alone. Tyrosine phosphorylation of immunoprecipitated EphA2 was detected with the 4g10 antibody (upper panel). Equal loading of immunoprecipitated EphA2 was detected by using an anti-EphA2 antiserum (lower panel) on the same blot. (C) Detection of tyrosine-phosphorylated proteins using an anti-phosphotyrosine antibody (PY99) in the cytosolic fraction of ephrin-A transfectants after 0, 15, 30 and 60 min incubations with EphA2-Fc (upper panel). Arrows indicate induction of phosphorylation or dephosphorylation of proteins during the time course. Positions of molecular-mass standards are indicated. The filter was stripped and hybridized with an anti-actin antibody to confirm equal loading (lower panel).

Cleavage of ephrin-A1

Recently, it was shown that clustered EphA3 receptors activate the cleavage of ephrin-A2 from the cell membrane by a regulated mechanism involving the metalloproteinase ADAM10/Kuzbanian [15]. A conserved motif was deduced based on the proteins whose cleavage can be mediated by ADAM10. This motif is also conserved in both ephrin-A1a and ephrin-A1b (Figure 6A). Since ephrin-A1b might associate with ephrin-A1a, we investigated if ephrin-A1b affected the cleavage of ephrin-A1 from the cell surface after receptor interaction. Serum-starved ephrin-A1a or ephrin-A1b transfectants or ephrin-A1a + ephrin-A1b co-transfectants were incubated with clustered EphA2-Fc for the indicated times. The cells were pelleted, and the incubation medium was analysed by immunoblotting (Figure 6). The results show clearly that ephrin-A1a was cleaved from the cell surface and released to the medium after receptor interaction (Figures 6B and 6C). Maximum cleavage was observed after 45 min as described previously for ephrin-A2 (Figures 6B and 6C) [15]. The molecular mass of the cleaved product was approx. 20 kDa. Interestingly, co-expression of ephrin-A1b with ephrin-A1a clearly inhibited shedding (Figure 6). In the experiment presented in Figure 6(B), equal amounts of ephrin-A1a and ephrin-A1b plasmids were co-transfected, whereas Figure 6(C) includes plasmids transfected

Figure 6 Cleavage of ephrin-A1 after EphA2-Fc interaction

(A) Comparison of a conserved motif (underlined) in human ephrin-A1 and mouse and human ephrin-A2 whose cleavage can be mediated by ADAM10. (B) Ephrin-A1 transfectants were incubated with EphA2-Fc for the indicated times and the cells were pelleted; the resulting supernatants were gel-separated to evaluate the cleaved product (left panel). Cell lysates of an equal number of cells from the start point (0) were gel-separated to confirm the total ephrin-A1 expression level in the transfectants (right panel). Both filters were hybridized with an anti-ephrin-A1 antibody. a, ephrin-A1a; b, ephrin-A1b. Equal amounts of the different ephrin-A1 plasmids were transfected. (C) Comparison of different ratios of ephrin-A1a and ephrin-A1b plasmids transfected into cells. EphA2-Fc-induced cleavage was evaluated (left panel). Ratio of ephrin-A1a to ephrin-A1b: a + b, 1:1; a + 1/5b, 5:1. Cell lysates of equal number of cells from the start point (0) were gel-separated to confirm the total ephrin-A1 expression level in the transfectants (right panel). Both filters were hybridized with an anti-ephrin-A1 antibody.

with ephrin-A1a + ephrin-A1b in the ratio 5:1. Even at this ratio, ephrin-A1b affected shedding markedly (Figure 6C). To conclude, clustered EphA2-Fc induces shedding of ephrin-A1a, whereas the occurrence of ephrin-A1b inhibits shedding.

DISCUSSION

In the present study, we describe a distinct ephrin-A1 isoform (ephrin-A1b), lacking 22 amino acids from 131 to 152 in the originally published ephrin-A1 molecule (ephrin-A1a). The gene encoding ephrin-A1, *EFNA1*, consists of five exons. By an alternative splice mechanism, exon 3 is spliced out of the transcript encoding ephrin-A1b. The deleted amino acids lie outside both the predicted receptor-binding domain and the dimerization domain of ephrin molecules [22,23], but lies close to the area shown to be involved in cleavage by ADAM10 [15,23]. In contrast with ephrin-A1a, our studies demonstrate that the soluble EphA2 receptor binds weakly to ephrin-A1b, despite being expressed on the cell surface of transfectants. The deletion of the 22 amino acids may lead to a difference in the conformational structure of ephrin-A1b compared with ephrin-A1a, leading to the loss of receptor binding. The same effect was also obtained when a deletion in the corresponding amino acids in ephrin-A4 was made, indicating a general principle.

Ephrin-A1b mRNA was initially detected by PCR of tonsil cells. Several EST clones exist in GenBank®, and many of these clones were isolated from brain tissue cDNA libraries. In addition, we have detected expression of ephrin-A1b at similar levels to ephrin-A1a by PCR of mRNA from brain and kidney tissues. Detection of the expression at the protein level and in various cell types (e.g. in brain) warrants further study.

In the increasing literature on Eph receptors and ephrins, several other ephrin-A family-member splice variants have been described. There are two reports on secreted variants of ephrin-A4

and ephrin-A6 whose functions are still unknown [15,24]. Two forms of ephrin-A5 have been described, ephrin-A5 α and ephrin-A5 β , where β lacks 27 amino acids within a variable spacer region [25,26]. This deletion does not affect the receptor binding capacity of the ligand. In addition, an ephrin-A3 isoform has been described with a corresponding deletion seen in ephrin-A5 β also capable of receptor binding [27]. None of these deletions correspond to the 22 amino acids spliced out in ephrin-A1b. Concerning ephrin-B members, an alternatively spliced variant has been described for mouse ephrin-B2. A 93-nucleotide deletion corresponding to 31 amino acids is located in the same area as that described for ephrin-A1b. This deletion corresponds to the position of exon 3 in the ephrin-B2 gene [28]. To the best of our knowledge, no results have been described regarding the function of this variant.

Since ephrin-A1b shows altered receptor binding properties, a biological function for this variant is probably in co-operation with ephrin-A1a that binds EphA2. In this respect, co-precipitation studies show that both variants might interact at the cell surface. Furthermore, the presence of mRNAs of both variants in brain and kidney indicates that they may be expressed simultaneously in a cell.

Reverse signalling through ephrin-A ligands has been reported previously [13,14,29]. Ephrin-A molecules are targeted to lipid rafts, and clustering of ephrin-A molecules with EphA-Fc fusion proteins can recruit the Src family kinase Fyn into lipid rafts [13]. This is accompanied by redistribution of vinculin, activation of mitogen-activated protein kinase, tyrosine phosphorylation of a 120 or 80 kDa lipid raft protein and increased cell-substrate adhesion [13,14]. Events triggered by the clustering of ephrin-A1 with EphA2-Fc were compared between ephrin-A1a transfectants and ephrin-A1a + ephrin-A1b co-transfectants. Increased adhesion on extracellular matrix-coated surfaces after receptor interaction of ephrin-A-positive cells has been described previously [14,29]. We have observed no difference in the kinetics of adhesion or spreading capability to plates coated with fibronectin and EphA2-Fc (within 30–60 min) between ephrin-A1a transfectants and ephrin-A1 + ephrin-A1b co-transfectants (results not shown).

No difference in the general tyrosine phosphorylation pattern after receptor interaction was observed between ephrin-A1a transfectants and ephrin-A1 + ephrin-A1b co-transfectants. On the other hand, we observed both induced phosphorylation and dephosphorylation of proteins with molecular masses that have not been described previously. These proteins are in the process of being identified to understand better the signalling cascade initiated through receptor ligation of ephrin-A1. In addition, ephrin-A1b did not seem to affect the ability of ephrin-A1a to activate the phosphorylation of the EphA2 receptor.

Cleavage of ephrin-A2 after receptor interaction involving ADAM10 metalloproteinase has been shown previously [15]. The function of this may be dual: ephrin-A cleavage from the cell surface allows Eph receptor-bearing cells to change their response from adhesion to repulsion, and cleavage may affect both forward and reverse intracellular signalling [7,15]. Our results show that ephrin-A1a is cleaved off the cell surface after receptor interaction, whereas cleavage is inhibited in the presence of ephrin-A1b. This is observed even when the concentration of transfected ephrin-A1b plasmid was decreased to one-fifth of ephrin-A1a. We have not yet been able to identify the proteinase responsible for this in HEK-293T cells, but to our knowledge, ADAM10 is not expressed in HEK-293T cells. Thus it is probable that another ADAM family member associates with ephrin-A1 and is responsible for the cleavage in these cells. One explanation for the inhibition of shedding is that ephrin-A1b interacts with ephrin-A1a, thereby sterically hindering proteinase interaction with

ephrin-A1a. Another possibility is that ephrin-A1b may interfere with the activation of the proteinase responsible for the cleavage.

In conclusion, an ephrin-A1 isoform, ephrin-A1b, lacking 22 amino acids, shows altered receptor binding properties compared with ephrin-A1a and inhibits the shedding of ephrin-A1a. The mechanistic consequences remain to be studied, but the inhibition of cleavage of ephrin-A1a may have consequences for repulsive/adhesive outcomes of Eph-ephrin interaction on cells.

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