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EphA8-ephrinA5 Signaling and Clathrin-Mediated Endocytosis Is Regulated by Tiam-1, a Rac-Specific Guanine Nucleotide Exchange Factor

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Recent studies indicate that endocytosis of Eph-ephrin complexes may be one of the mechanisms by which a high affinity cell-cell adhesion is converted to a repulsive interaction. In this study, we show that EphA8 undergoes clathrin-mediated endocytosis upon treatment with ephrin-A5, and that EphA8 is associated tightly with Tiam-1, a Rac-specific guanine nucleotide exchange factor. Analysis of EphA8 deletion mutants revealed that a juxtamembrane region in EphA8 is critically involved in endocytosis of EphA8-ephrinA5 complexes. An EphA8 mutant lacking this juxtamembrane portion was defective for endocytosis with ephrinA5, and also displayed a weak association with Tiam-1. Expression of an endocytosis-defective version of EphA8 resulted in a low level of Rac activity in response to ephrin-A5 stimulation. More importantly, down-regulation of Tiam-1 resulted in inefficient endocytosis of EphA8-ephrinA5 complexes. These results suggest that Tiam-1 plays a role in clathrin-dependent endocytosis of EphA8-ephrinA5 complexes.

INTRODUCTION

Eph receptor tyrosine kinases (RTKs) and their ligands, ephrins, play important roles during embryonic development by triggering intracellular signaling pathways that regulate diverse cell behaviors such as axon guidance, cell adhesion, and cell migration (Flanagan and Vanderhaeghen, 1998; Kullander and Klein, 2002; O'Leary and Wilkinson, 1999; Pasquale, 2005; Wilkinson, 2001). An important feature of Eph/ephrin signaling is that Ephs and ephrins induce bidirectional signaling between two opposing cells that is contact-dependent because both molecules are membrane-attached (Bruckner and Klein, 1998; Holland et al., 1996; Knoll and Drescher, 2002). In this system, the signal is transduced into receptor-expressing cells (forward signaling) and also into ligand-expressing cells (reverse signaling). Another important feature of Eph/ephrin signaling is that, in general, Eph-ephrin interactions trigger cell-cell repulsion (O'Leary and Wilkinson, 1999; Pasquale, 2005; Wilkinson, 2000). The ability of an adhesive Eph receptor/ephrin ligand interaction to generate a repellent response is paradoxical. An elegant solution to this paradox may be that the ligand-receptor complex is removed from the cell surface, thereby converting adhesive events into repulsive events. Two possible mechanisms for removal of the ligand-receptor complex have been reported: proteolytic shedding of the ephrin ectodomain following Eph engagement (Hattori et al., 2000; Janes et al., 2005) and rapid endocytosis of Eph-ephrin complexes (Marston et al., 2003; Zimmer et al., 2003).

Endocytosis of Eph-ephrin complexes has been strongly implicated as a mechanism for switching between cell-cell adhesion and repulsion upon cell-cell contact. For example, when presented in soluble form, both EphB2 and ephrinB1 are endocytosed after binding to their respective partners and when EphB2–expressing cells encounter ephrinB1-expressing cells in culture (Zimmer et al., 2003). Similar results were observed in cells expressing EphB4 and ephrinB2, and both EphB4-ephrinB2 internalization and cell retraction were shown to be dependent on Rac signaling within receptor-expressing cells (Marston et al., 2003). In addition, synaptojanin 1, a phosphatidylinositol 5′-phosphatase, was shown to be essential for clathrin-mediated endocytosis of EphB2 after ephrinB2 treatment (Irie et al., 2005). Although Eph-ephrin internalization is clearly linked to repulsion, it is not clear whether internalization plays a direct role in the choice between repulsion and adhesion.

In the current study, we show that soluble ephrin-A5 is rapidly internalized by the EphA8 receptor at the cell surface and that this process occurs via clathrin-mediated endocytosis. Identification of an endocytosis-defective version of EphA8 led us to find that Tiam-1 may play a role in clathrin-dependent endocytosis of EphA8-ephrinA5 complexes. These results suggest that the EphA8 mutant lacking the critical domain for endocytosis may have a dominant negative effect on endocytosis of other Eph receptors by blocking Rac signaling implicated in actin polymerization.

MATERIALS AND METHODS

Expression plasmids

Mouse wild-type (WT) and kinase-inactive (KD) EphA8 cDNAs tagged with a nine-amino-acid hemagglutinin (HA) epitope

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(YPYDVPDYA) at their COOH termini have been described elsewhere (Choi and Park, 1999). To construct EphA8-E10 (with a deletion of aa 588 to 643 of mouse EphA8), we amplified a 281-bp PCR product using primers matching nucleotides (nt) 1570 to 1590 (5′-gtgtcaggcctcaaaccagg-3′) and nt 1823 to 1840 (5′-cctgactcccctgtccgttctggtaat-3′) of the EphA8 cDNA as well as a 260-bp PCR product using primers matching nt 2006 to 2024 (5′-acggacaggggagtcaggagaagtgtg-3′) and nt 2236 to 2255 (5′-gtcctgaggaaggcatctag-3′) of the EphA8 cDNA. The resulting two partially complementary PCR fragments were annealed and used as a template in another PCR performed with primers matching nt 1570 to 1590 and nt 2236 to 2255 of the EphA8 cDNA. Next, the 521-bp PCR product was digested with NsiI/XbaI and subcloned into the corresponding region of WT EphA8-HA DNA. The same procedures were used to generate EphA8-E9, except that different primers were used. A 531-bp PCR product was amplified using primers matching nt 1232 to 1250 (5′-tcccgcagcagacaagcct-3′) and nt 1755 to 1772 (5′-ggtgggggtgctcttcttgcagatgaga-3′) of EphA8 cDNA, and a 164-bp PCR product was amplified using primers matching nt 1841 to 1858 (5′-gcaagaagagcacccccacctgtcttct-3′) and nt 1977 to 1994 (5′-atcttctcaatgtggatc-3′) of EphA8 cDNA. To construct EphA4-E10 (with a deletion of aa 592 to 630 of mouse EphA4), a 311-bp sequence was amplified with primers matching nt 1501 to 1520 (5′-tccggacagctgccaggaac-3′) and nt 1781 to 1802 (5′-tccaaattcttgattcaaatgtttctcttca-3′), and a 870-bp PCR product was amplified using primers matching nt 1920 to 1937 (5′-ttgaatcaagaatttggagaggtctgc-3′) and nt 2761 to 2780 (5′-gcccactgatactacagcag-3′) of EphA4 cDNA. The two partially complementary PCR fragments were annealed and used as the templates in another PCR performed with primers matching nt 1501 to 1520 and nt 2761 to 2780 of the EphA4 cDNA. Next, the 1163-bp PCR product was digested with EcoRV/EcoRI and subcloned into the corresponding region of WT EphA4-HA DNA. To construct EphB4-E11 (with a deletion of aa 586 to 623 of mouse EphB4), we amplified a 306-bp PCR product using primers matching nucleotides (nt) 1978 to 1955 (5′-tcagaaaaccgagctgag-3′) and nt 2257 to 2271 (5′-gccgaactcaccgtgcccgatgagata-3′) of the EphB4 cDNA as well as a 279-bp PCR product using primers matching nt 2389 to 2406 (5′-gggcacggtgagttcggcgaggtgtgc-3′) and nt 2638 to 2655 (5′-tgtgaactgcccgtcgtt-3′) of the EphB4 cDNA. The resulting two partially complementary PCR fragments were annealed and used as a template in another PCR performed with primers matching nt 1978 to 1955 and nt 2638 to 2655 of the EphB4 cDNA. Next, the 564-bp PCR product was digested with ScaI/EcoRV and subcloned into the corresponding region of WT EphB4-HA DNA.

Endocytosis assay

HEK293 cells (3×10^5) were seeded on fibronectin-coated glass coverslips in 35 mm dishes and cultured for 16-24 h. Growth medium was replaced with 1 ml starvation medium (DMEM + 0.5% fetal bovine serum) per dishes. Two hours later, cells were stimulated with 1 μg/ml ephrinA5-Fc or control Fc clustered with goat-anti human IgG for 30 min at 37°C or 4°C. Cells were gently washed three times with phosphate-buffered saline, fixed with 4% paraformaldehyde/2% sucrose in PBS for 10 min on ice and rinsed three times for 5 min with PBS. To strip unincorporated ligand from the cell surface, cells were treated with ice-cold acid solution (0.2 M acetic acid, 0.5 M NaCl) for 10 min on ice prior to fixing. After washing three times with PBS, cells were blocked with 3% (w/v) BSA, 5% (v/v) horse serum, PBS in the presence or absence of 0.1% (v/v) Triton X-100 for 30 min at room temperature. Surface bound or total ephrin-A5-Fc or control Fc was detected using anti goat-human Fc conjugated with TRITC at 1:500 dilution for 1 hr, and rinsed three times for 5 min with TBST (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100). For immunofluorescence staining, cells were blocked with TBST containing 0.3% bovine serum albumin for 30 min at room temperature, and then incubated with primary antibodies (rabbit anti-EphA8, 1:1000; anti-EphA4, 1:500; anti-EphB4, 1:500; anti-CHC 1:1000; anti-EEA1, 1:500) for overnight at 4°C on gentle rocker. Next day, after three times washing with TBST, cells were incubated with the appropriate secondary antibodies (TRITC-conjugated goat anti-rabbit, 1:500, or FITC-conjugated goat anti-mouse, 1:500). Cells were washed three times in TBST and mounted with Vectashield (Vector Laboratory). Images were observed by confocal microscopy. Internalization was quantified by drawing an area under the cell membrane (within the cell but excluding cell membrane) of each cell on the confocal image, and pixel of internalized vesicles within the area were counted. Pixel density was calculated using NIH image software in a blinded manner. For fluorescent transferrin uptake experiments, cells were cultured on coverslips and incubated on ice for 20 min with Texas red-conjugated transferrin (50 μg/ml) and 1 μg/ml ephrinA5-Fc or control Fc. The cells were shifted to 37°C for 20 min. Uptake was stopped by placing the cells on ice and washing with ice-cold acid solution for 10 min at 4°C to remove any surface-bound transferrin. Eph receptors were detected with rabbit anti-Eph antibodies, followed by the appropriate secondary antibodies.

Co-immunoprecipitation and Western blot analyses

HEK293 cells were transfected with 2 μg each of HA-tagged wt EphA8, kd EphA8, EphA8-E10. Twenty-four hours after transfection, growth media was changed with 4 ml starvation media. Two hours after, cells were stimulated with 1 μg/ml ephrinA5-Fc or control Fc clustered as described above. Immunoprecipitation and Western blotting were performed essentially as described previously (Park and Yun, 2001).

Rac activity assay

Cells were serum-starved for 18 h in 0.5% FBS followed by stimulation with ephrin-A5 (1 μg/ml) for 3 min. Lysates were prepared and incubated with Pak-1 binding domain (PBD)-GST beads (Upstate Biotechnology) as described by manufacturer's protocol to pull-down GTP-bound Rac. Activated Rac1 or total Rac1 were detected by immunoblot using anti-Rac1 antibodies.

RNA interference

For the silencing of Tiam-1, a human-specific SMARTpool was designed by Dharmacon. Human Tiam-1-specific small interfering RNAs (siRNAs) were also purchased from Dharmacon. For every experiment performed, non-targeting, RNA-induced silencing complex–free siRNAs were used as a non-silencing control (Dharmacon). Three micrograms of double-stranded siRNAs were introduced into 2×10^6 HEK293 cells with Nucleofactor II (Amaxa) set at program Q-01, using a Cell Line Nucleofator Kit V (Amaxa) according to the manufacturer's instructions.

Antibodies

A polyclonal rabbit antibody specific for the JM domain of EphA8 was described previously (Choi and Park, 1999). Monoclonal anti-CHC (Clathrin Heavy Chain) was purchased from Affinity BioReagents. Monoclonal anti-EEA1 and anti-Rac1 was purchased from BD Biosciences. Polyclonal anti-HA antibodies were purchased from Zymed. Polyclonal anti-actin antibodies were purchased form Sigma-Aldrich. Polyclonal anti-EphA4,

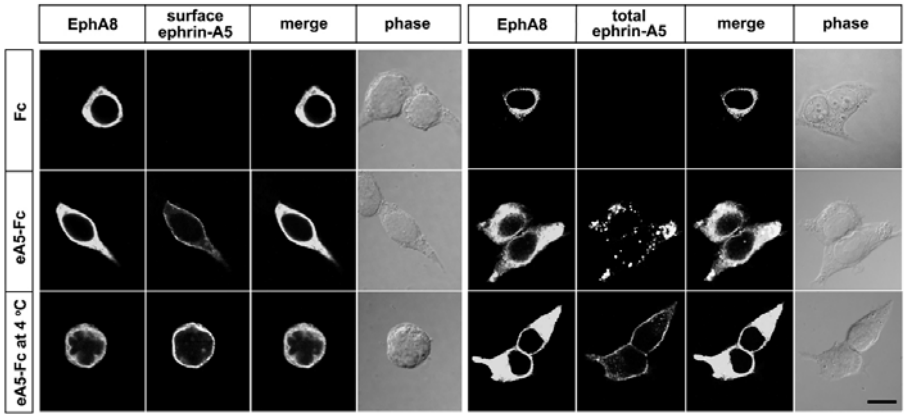

Fig. 1. Endocytosis of ephrinA5-Fc into EphA8-expressing 293 cells. Cells transiently transfected with wild type EphA8 were stimulated with either control Fc protein (panels in the first row) or with preclustered ephrinA5-Fc (panels in the second and third rows). After 30 min, cells were fixed and ephrinA5-Fc was immunolabelled on the cell surface using TRITC-conjugated antibody directed against Fc (panels in the second column). EphA8 was stained with anti-EphA8 antibody followed by FITC-conjugated secondary antibody after permeabilization (panels in the first column). The total pool of ephrinA5-Fc was also visualized using TRITC-conjugated antibody directed against Fc after cell permeabilization (panels in the sixth column). To test whether inhibition of the endocytotic process impairs the internalization of ephrinA5-Fc, cells expressing EphA8 were incubated with preclustered ephrinA5-Fc on ice and then staining for surface and total ephrinA5-Fc proteins were performed as described above.

anti-EphB4 and anti-Tiam-1 antibodies were purchased from Santa Cruz Biotechnology. Goat anti-human IgG conjugated with TRITC, goat anti-rabbit IgG conjugated with TRITC and goat anti-mouse IgG conjugated with FITC were purchased from Chemicon. Goat anti-human IgG was purchased from Jackson Immuno Research Laboratories. Texas red-conjugated Transferrin was purchased from Molecular Probe.

RESULTS AND DISCUSSION

Ephrin-A5 is rapidly internalized by the EphA8 receptor at the cell surface

Previous studies indicated that endocytosis of Eph receptors upon binding of their cognate ligand is induced to elicit the rapid internalization of cell surface-bound ligand (Irie et al., 2005; Marston et al., 2003; Zimmer et al., 2003). To assess whether EphA8 is able to internalize ephrin-A5 upon their specific interaction on the cell surface, EphA8 was expressed in 293 cells by transient transfection and cells were treated with preclustered ephrinA5-Fc for 30 min. Expression of EphA8 was confirmed by immunostaining using anti-EphA8 antibody (Fig. 1, panels in the first and fifth columns). To visualize the specific binding of ephrinA5-Fc to EphA8 on the cell surface, cells were fixed and incubated with Fc-specific secondary antibody in the absence of detergents. As expected, the binding of ephrinA5-Fc was detectable only at the surface of cells expressing EphA8, whereas binding of Fc protein was not detectable (Fig. 1, panels in the second column). However, when EphA8-expressing cells were incubated with Fc-specific secondary antibody in the presence of detergents, abundant clusters of ephrinA5-Fc were detected at the intracellular vesicular structures, suggesting that the cell surface bound ephrinA5-Fc was internalized by the EphA8 receptor. We also observed that ephrinA5-Fc was detectable inside the cells at 5 min after the ligand was added, suggesting this endocytotic process takes place very rapidly (data not shown). It is known that low temperature, such as 4°C, blocks vesicular trafficking and internalization. Consistent with this, when EphA8-expressing cells were stimulated with preclustered ephrinA5-Fc at low temperature, ephrin-A5 was observed only at the cell surface irrespective of permeabilization condition (Fig. 1, panels in the third row). Taken together, these results indicate that ephrin-A5 binds specifically to EphA8 receptor on the cell surface, inducing a rapid internalization of EphA8-ephrinA5 complexes.

Endocytosis of EphA8-ephrinA5 complexes occurs via clathrin-dependent endocytotic mechanism

To investigate whether EphA8-mediated endocytosis of ephrin-A5 is also dependent on a clathrin-mediated mechanism, we analyzed co-localization of the internalized EphA8-ephrinA5 complex with transferrin, which is internalized via a clathrin-dependent mechanism. As shown in Fig. 2A, when cells expressing EphA8 were treated with a mixture containing both ephrinA5-Fc and transferrin for 30 min, internalized ephrin-A5 co-localized well with transferrin inside the cells, suggesting that ephrin-A5 is internalized by EphA8 via a clathrin-depedent mechanism (panels in the first row). Similar results were also observed in cells expressing EphA4 or EphB4 (Fig. 2A, panels in the second and third rows), strongly suggesting that Eph receptors utilize clathrin-dependent endocytosis as a universal mechanism in order to internalize their bound ligands.

To further confirm whether EphA8-mediated endocytosis of ephrin-A5 is dependent on a clathrin-mediated mechanism, we determined whether clathrin heavy chain is directly co-localized with the internalized ephrin-A5 in EphA8-expressing cells. As shown in Fig. 2B, the internalized ephrin-A5 was found together with clathrin inside the cells (panels in the first row). In addition, the internalized ephrin-A5 was detectable in the vesicles stained with EEA1, early endosomal antigen-1, which is a marker for early endosomes (Fig. 2B, panels in the second row). Taken together, these results strongly suggest that a clathrin-dependent mechanism mediates endocytosis of EphA8-ephrinA5 complexes upon their specific interactions.

A juxtamembrane region in EphA8 is critically involved in endocytosis of EphA8-ephrinA5 complexes

Next, we investigated which segment of EphA8 is responsible for internalization of the EphA8 receptor upon binding its cognate ligand. For this purpose, we used a series of EphA8 deletion mutants lacking specific intracytoplasmic regions as previously reported (Gu et al., 2005). Interestingly, we found that the EphA8 deletion mutant lacking the entire juxtamembrane region was poorly internalized in response to ephrin-A5 stimulation (data not shown). Since the juxtamembrane region of EphA8 is encoded by both exon 9 and exon 10, we generated smaller EphA8 deletion mutants lacking the specific region encoded by

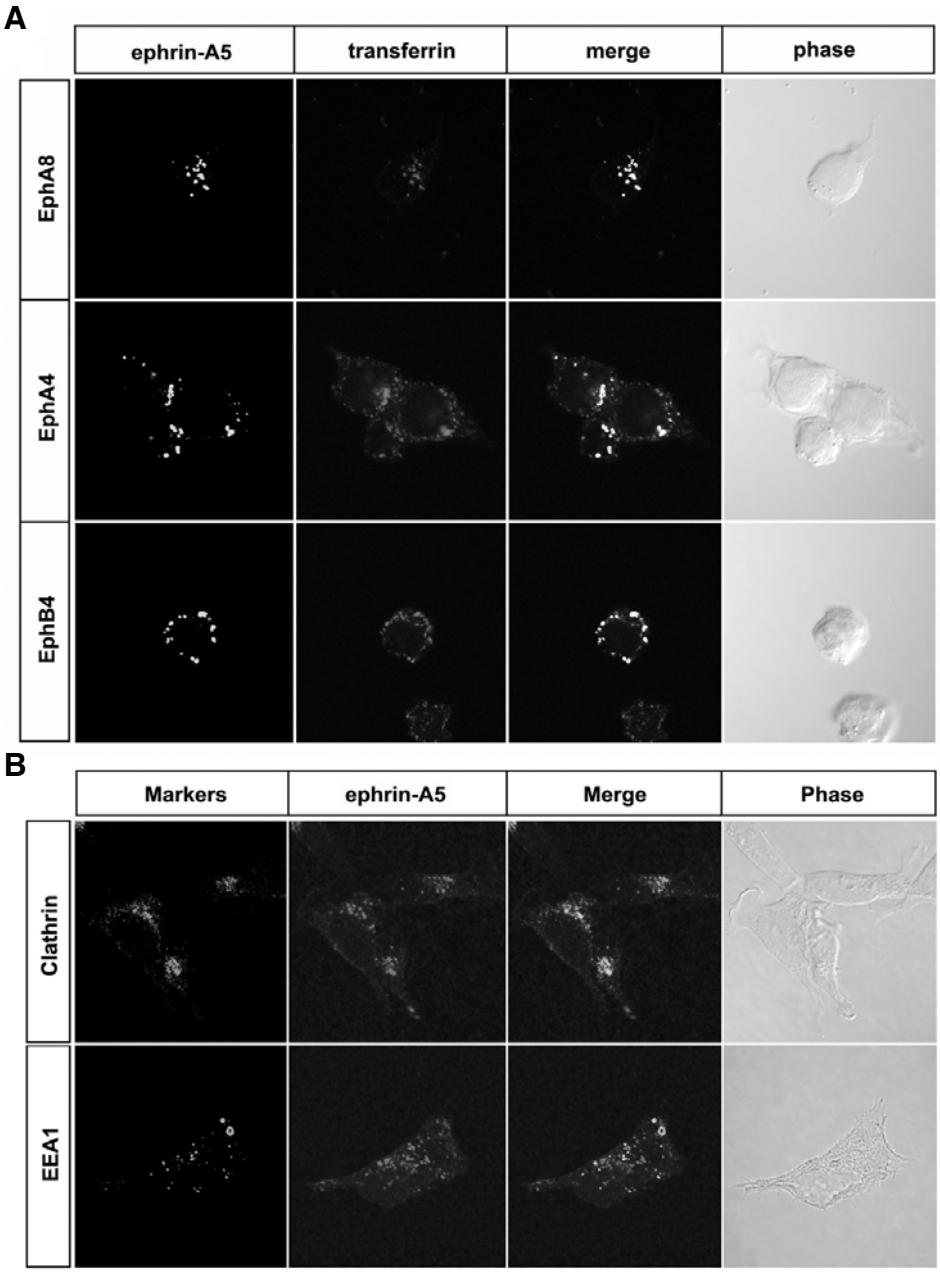

Fig. 2. Endocytosis of EphA8-ephrinA5 complexes is mediated via clathrin-dependent mechanism. (A) Cells transiently transfected with the indicated constructs were stimulated with preclustered ephrinA5-Fc in the presence of Texas Red-conjugated transferrin. After 20 min, cells were fixed and total ephrinA5-Fc was immunolabelled using FITC-conjugated antibody directed against Fc. (B) Cells stably expressing EphA8 were stimulated with preclustered ephrinA5-Fc for 15 min and then total ephrinA5-Fc was immunolabelled using TRITC-conjugated antibody directed against Fc. The same cells were also stained for clathrin and EEA1 using specific primary antibody followed by FITC-conjugated secondary antibody.

exon 9 or exon 10 (EphA8-E9 and EphA8-E10, respectively, see Fig. 3A). Interestingly, endocytosis of the EphA8-E10 mutant was drastically impaired in response to ephrin-A5 stimulation (Fig. 3B, first panel), whereas the EphA8-E9 mutant underwent normal endocytosis when stimulated with ephrin-A5 (Fig. 3B, second panel). These results suggest that the exon-10-encoded peptide motif is a crucial determinant of ligand-induced endocytosis of EphA8. The peptide motif required for ligand-induced endocytosis lies between residues 587 and 643 in EphA8 and is evolutionarily conserved in other Eph members (Fig. 3A).

To further illustrate the role of the exon-10-encoded peptide motif in EphA8, the corresponding exon-encoded regions were deleted from EphA4 and EphB4, respectively. For the EphA4-E10 deletion mutant, internalization of ephrin-A5 was not significantly altered relative to wild type EphA4 (Fig. 3B, third panel; Fig. 3C, bars 4 and 5). In contrast, in the absence of this conserved peptide motif, EphB4 was defective for ligand-induced endocytosis, suggesting that the endocytotic function of the exon10-encoded peptide motif in EphA8 is conserved in the EphB4 receptor (Fig. 3B, fourth panel; Fig. 3C, bars 6 and 7).

Association of Tiam-1 with EphA8 is significantly reduced in cells expressing EphA8-E10 mutant

Previous studies have shown that the internalization of Eph-ephrin complexes is dependent on actin polymerization, in which Rac signaling plays a crucial role (Irie et al., 2005; Marston et al., 2003). Consistent with these studies, we found that Tiam-1, a Rac-specific guanine nucleotide exchange factor, co-immunoprecipitates with EphA8 in response to ephrin-A5

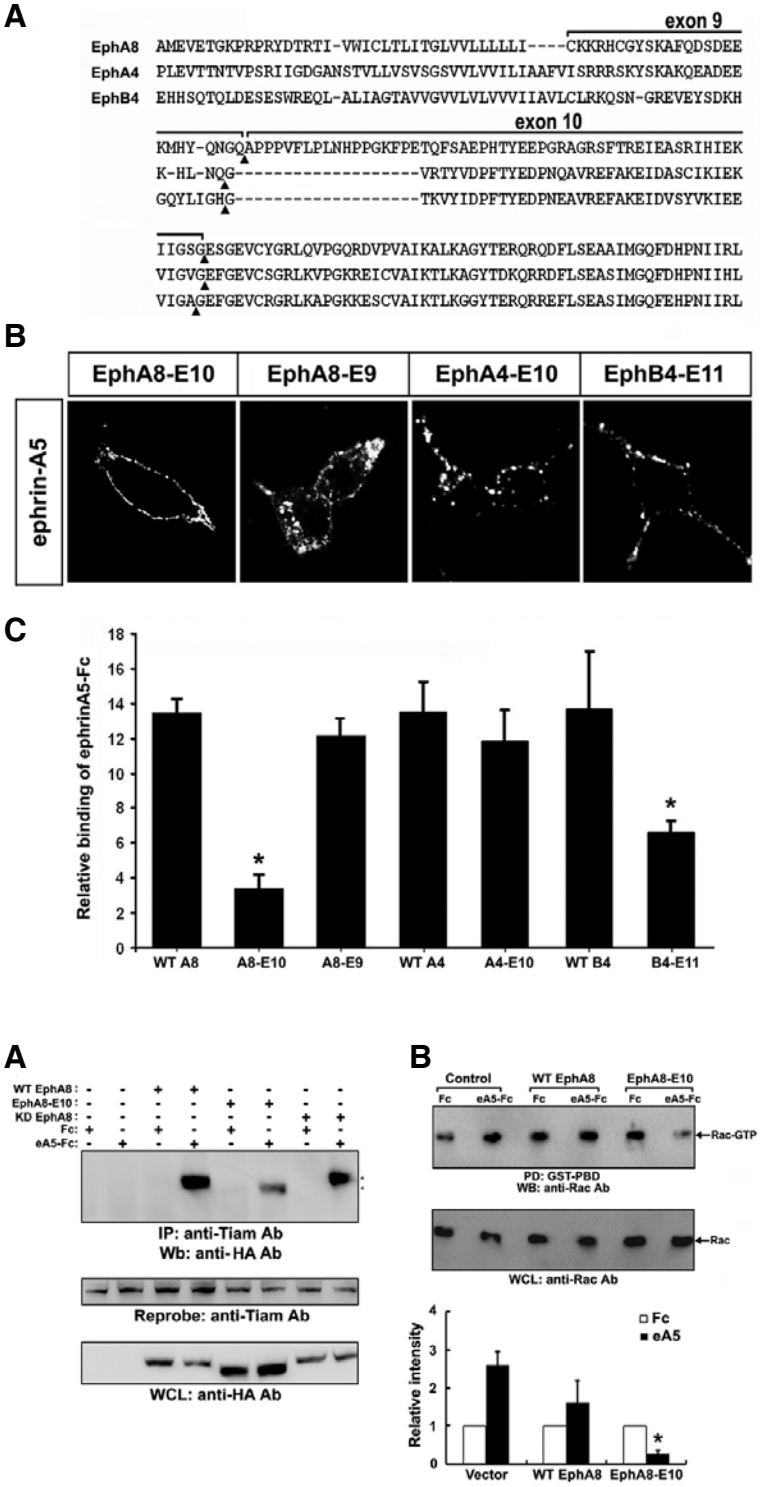

Fig. 3. The exon10-encoded peptide motif in EphA8 is important for endocytosis of EphA8-ephrinA5 complexes. (A) The juxtamembrane amino acid sequences of the indicated Eph family receptors were aligned using the Clu-stalW program. Gaps are represented by dashes. The arrow heads mark the location of amino acids deleted in EphA8-E10, EphA4-E10 and EphB4-E11. (B) Cells transiently transfected with the indicated constructs were stimulated with preclustered ephrinA5-Fc. After 30 min, cells were fixed and total ephrinA5-Fc was immunolabelled using TRITC-conjugated antibody directed against Fc. (C) The data in Fig. 3B were quantitated using ImageJ software. Data represent the mean ± SE for at least five independent experiments, with 30 cells counted for each condition. Relative bindings of ephrinA5-Fc under different conditions were compared with that in the wild type EphA8-transfected condition with acid washing (*$p < 0.001$, ANOVA).

Fig. 4. Rac signaling is reduced in cells expressing the EphA8-E10 mutant. (A) HEK293 cells were transfected with the indicated EphA8 constructs. Note that EphA8 proteins are tagged with an HA epitope at their COOH-terminus. Cell lysates were subjected to immunoprecipitation (IP) using anti-Tiam-1 antibody, and immune complexes were analyzed by Western blot (Wb) using anti-HA antibody (top panels). The blots were reprobed to determine the level of EphA8 proteins (middle panel). (B) Activity of Rac was measured by pull-down (PD) assay in each cell under control Fc or eprhinA5-Fc treated condition. The GTP-bound form of Rac was precipitated by GST-Pak-1-RBD and probed with anti-Rac1 Ab (top panel). Total levels of Rac were visualized by Western blot using anti-Rac1 antibody (middle panel). The ratio of GTP-Rac and total level of Rac was quantified and described as bar graph with SE (n = 3). Relative Rac activity under different conditions was compared with that in the vector-transfected condition (*$p < 0.01$, ANOVA). (C) Immunofluorescence of each cell by Fc-specfic antibody and Tiam-1 specific antibody was performed as described in the legend for Fig. 2B.

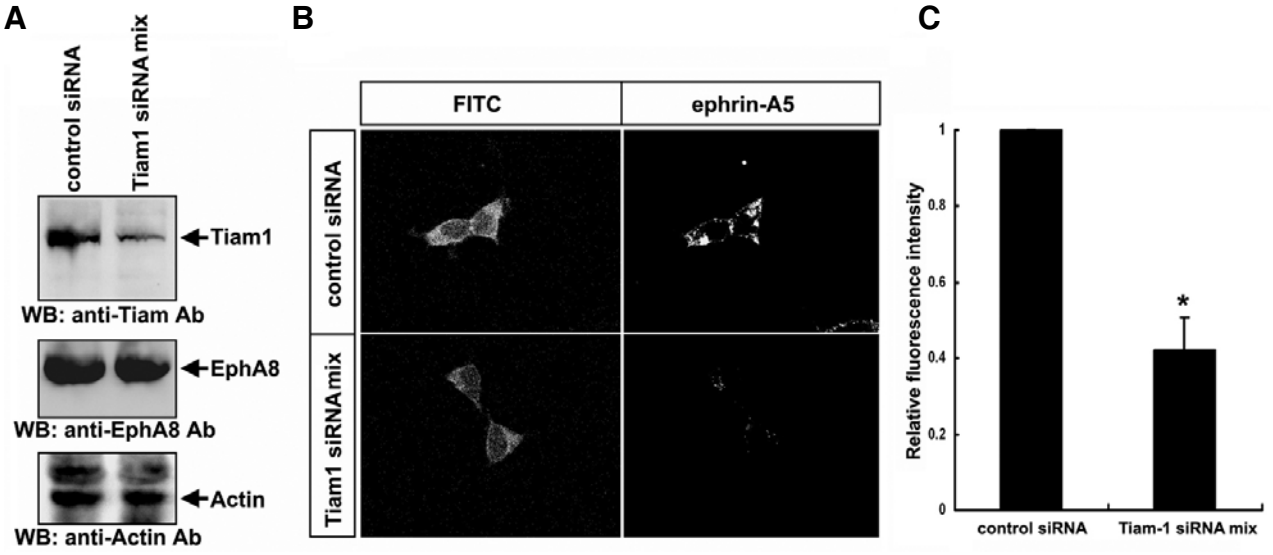

Fig. 5. Knock-down of Tiam-1 in EphA8-expressing cells. (A) EphA8-expressing 293 cells were transfected with control siRNA or SMART pool of human Tiam-1 siRNAs, and 72 h later, cells were lysed and probed by immunoblotting with the indicated antibodies. (B) In each transfection, FITC-labled control siRNA was included to visualize the siRNA-transfected cells. Immunofluorescence staining of ephrinA5-Fc was performed as described in the legend for Fig. 1. Only FITC-labeled cells were analyzed for internalization of ephrinA5-Fc. (C) The data in Fig. 5B were quantitated using ImageJ software. Data represent the mean ± SE for at least five independent experiments, with 30 cells counted for each condition (*$p < 0.001$, Student's *t* test).

stimulation (Fig. 4A, lane 4). Interestingly, the association of Tiam-1 with EphA8 was not dependent on the tyrosine kinase activity of EphA8 (Fig. 4A, lane 8). The association of Tiam-1 with the endocytosis-defective EphA8 mutant, EphA8-E10, was significantly reduced (lane 6), suggesting that the exon-10-encoded peptide motif in EphA8 contributes to its tight association with Tiam-1. We then assessed whether Rac activity is altered in accordance with the decreased association between Tiam-1 and EphA8-E10 in 293 cells. Rac activity was measured using a pull-down assay of Rac-GTP with a Pak-1 GST-RBD fusion protein as previously described (Sander et al., 1998). In control or wild type EphA8-expressing cells, Rac activity was elevated in response to ephrin-A5 stimulation (Fig. 4B, lanes 1-4). However, in EphA8-E10 expressing cells, lower Rac activity was observed in response to ephrin-A5 treatment. It is not clear why the endocytosis-defective EphA8 mutant causes a reduction of Rac activity in response to ephrin-A5 stimulation. One possibility is that EphA8-E10 associates with other Eph receptors more tightly in response to ephrin-A5 stimulation, thereby blocking the Rac signaling downstream of Eph receptors. To compare the localization of Tiam-1 in EphA8 and EphA8-E10 expressing cells in response to ephrinA5-Fc treatment, cells were labeled with Tiam-1 antibody and Fc-specific antibody. As expected, Tiam-1 was co-localized with ephrin-A5 in both cell surface and cytoplasm of the EphA8-expressing cells, suggesting that EphA8-ephrinA5 complexes are internalized together with Tiam-1 (Fig. 5C, panels in the second row). In contrast, in EphA8-E10 expressing cells, most Tiam-1 was localized to the cell surface, and co-stained with ephrinA5-Fc bound to EphA8-E10 mutant on the cell surface (Fig. 5C, panels in the third row). Although Tiam-1 is primarily localized to cell membrane in EphA8-E10 expressing cells, its interaction with Rac at the cell surface may be somehow impaired, thereby leading to low levels of Rac activity after ligand treatment. This possibility is currently under investigation in our laboratory. Taken together, our results strongly suggest that the function of Tiam-1 is disturbed in cells expressing the EphA8-E10 mutant.

Down-regulation of Tiam-1 results in inefficient endocytosis of EphA8-ephrinA5 complexes

To determine whether Tiam-1 plays a role in regulating endocytosis of the EphA8 receptor, we knocked down Tiam-1 endogenous expression in 293 cells. For down-regulation of Tiam-1, SMART pool siRNAs specific for human Tiam-1 were transfected into 293 cells stably expressing EphA8. Western blot analysis revealed that endogenous Tiam-1 was effectively down-regulated (Fig. 5A, first panel). Expression levels of EphA8 and actin were not altered by siRNA transfection (Fig. 5A, second and third panels). FITC-labeled control siRNA was included in each transfection to visualize the siRNA-transfected cells. siRNA transfected cells were stimulated with preclustered ephrinA5-Fc for 30 min to measure endocytotic behavior of EphA8-ephrinA5 complexes. These cells were then treated briefly with an acidic buffer to eliminate cell surface bound ephrin-A5, fixed under permeabilization conditions, and stained for ephrinA5-Fc using Fc-specific secondary antibody. This staining reveals the intracellular pool of EphA8-ephrinA5 complexes. As expected, EphA8-ephrinA5 complexes were effectively internalized in control siRNA transfected cells, but not in Tiam-1 siRNA-transfected cells (Figs. 5B and 5C). Taken together, these results indicate that Tiam-1 is critically involved in endocytosis of EphA8-ephrinA5 complexes, perhaps by regulating the Rac signaling downstream of the EphA8 receptor.

Here, we show that a juxtamembrane region in EphA8 contains the critical domain for endocytosis. This peptide region was shown to be important for the tight association of EphA8 with Tiam-1, a Rac-specific guanine nucleotide exchange factor. More importantly, Rac activity is drastically reduced in cells expressing the endocytosis-defective EphA8 mutant. Based on these observations, we postulate that the endocytosis-defective EphA8 mutant associates with other Eph receptors through heterodimerization or multimerization in response to ligand

stimulation. Although Tiam-1 is poorly associated with the endocytosis-defective EphA8 mutant, it may be tightly associated with other Eph receptors but its activity toward Rac may be somehow blocked. The EphA8 mutant lacking the critical domain for endocytosis is thus likely to play a dominant negative role in endocytosis of other Eph receptors by interfering with Rac signaling implicated in actin polymerization. Endocytosis of Eph and ephrin complexes is considered an important mechanism to destabilize the cell-cell contact since the outcome of high-affinity interactions between Eph receptors and ephrins is generally cell-cell repulsion. The endocytosis-defective EphA8 mutant we describe might be useful in further exploration of the importance of endocytosis of Eph receptors and ephrin ligands in the choice between attraction and repulsion *in vivo*.

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