The C-terminus of ephrin-B1 regulates metalloproteinase secretion and invasion of cancer cells

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Accepted 28 April 2007
Journal of Cell Science 120, 2179-2189 Published by The Company of Biologists 2007
doi:10.1242/jcs.008607

Summary

Interaction of the Eph family of receptor protein tyrosine kinases and their ligands, ephrin family members, induces bi-directional signaling via cell-cell contacts. High expression of B-type ephrin is associated with high invasion potential of tumors, however, the mechanism by which ephrin-B promotes cancer cell invasion is poorly understood. We show that interaction of ephrin-B1 with the Eph receptor B2 (EphB2) significantly enhances processing of the extracellular domain of ephrin-B1, which is regulated by the C-terminus. Matrix metalloproteinase-8 (MMP-8) is the key protease that cleaves ephrin-B1, and the C-terminus of ephrin-B1 regulates activation of the extracellular release of MMP-8 without requirement of de novo protein synthesis. One possible mechanism by which ephrin-B1 regulates the exocytosis of MMP-8 is the activation of Arf1 GTPase, a critical regulator of membrane trafficking. In support of this hypothesis, activation of ephrin-B1 increased GTP-bound Arf1, and the secretion of MMP-8 was reduced by expression of a dominant-negative mutant of Arf1. Expression of ephrin-B1 promoted the invasion of cancer cells in vivo, which required the C-terminus of ephrin-B1. Our results suggest a novel function of the C-terminus of ephrin-B1 in activating MMP-8 secretion, which promotes the invasion of cancer cells.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/13/2179/DC1

Key words: Eph, Ephrin, Metalloproteinase, Secretion

Introduction

The members of the Eph receptor family can be classified into two groups based on their sequence similarity and their preferential binding to ligands tethered to the cell surface either by a glycosylphosphatidyl inositol anchor (ephrin-A) or a transmembrane domain (ephrin-B) (Murai and Pasquale, 2003; Blits-Huizinga et al., 2004; Poliakov et al., 2004). The interaction of Eph receptor B2 (EphB2) protein tyrosine kinases and their ephrin-B ligands induces bi-directional signaling via the resultant cell-cell contacts. Ephrin-B has an intracellular domain, which includes sites for tyrosine phosphorylation via Src family kinases and a docking site for proteins with a PDZ domain (Lin et al., 1999; Cowan and Henkemeyer, 2001; Bong et al., 2004). These sites give ephrin-B ligands at least two ways of being involved in intracellular signaling. Although investigation of the functions of Eph receptors and ephrins have focused on the development of the vascular and nervous systems, the roles of Eph-ephrin pathways in epithelial cells and cancers have also attracted interest (Batlle et al., 2002; Klein, 2004; Batlle et al., 2005; Tanaka et al., 2005; Holmberg et al., 2006). Overexpression of B-type ephrin in cancer cells is reported to correlate with high invasion and high vascularity of tumors (Meyer et al., 2005; Castellvi et al., 2006; Nakada et al., 2006), and elevated expression of ephrin-B1 is observed in poorly differentiated invasive tumor cells and other tumors with poor clinical prognosis (Kataoka et al., 2002; Varelias et al., 2002). However, whether ephrin-B actually modifies tumor invasion in vivo has not been established, and the mechanism by which ephrin-B is involved in the invasion of cancer cells is unknown.

Some ephrins are cleaved by a protease. When ephrin-A5 binds with EphA3, their complex creates a recognition motif for a disintegrin and metalloproteinase (ADAM)10 membrane metalloproteinase, which allows effective cleavage of the extracellular domain of ephrin-A5 (Hattori et al., 2000; Janes et al., 2005). Protease-mediated cleavage also occurs for B-type ephrins; a rhomboid transmembrane serine protease RHBDL2 cleaves the transmembrane domain of ephrin-B3 (Pascall and Brown, 2004), and ephrin-B1 and ephrin-B2 are processed within the transmembrane region by the presenilin 1 (PS1)-γ-secretase system to release an intracellular peptide (Georgakopoulos et al., 2006; Tomita et al., 2006).

During the process of screening peptides secreted from pancreas cancer cell lines, we found evidence that the extracellular portion (ectodomain) of ephrin-B1 is secreted. This finding led us to study the mechanism of ephrin-B1 shedding within the ectodomain in cancer cells. In the present study, we show that the C-terminus of ephrin-B1 regulates the exocytosis of MMP-8, a key protease of ephrin-B1 cleavage, in response to the interaction with its receptor EphB2. An accumulating number of reports have shown that increased secretion of metalloproteinases often depends on their transcriptional activation (Chinni et al., 2006; Raymond et al., 2006; Reuben and Cheung, 2006). However, increase of MMP-8 secretion

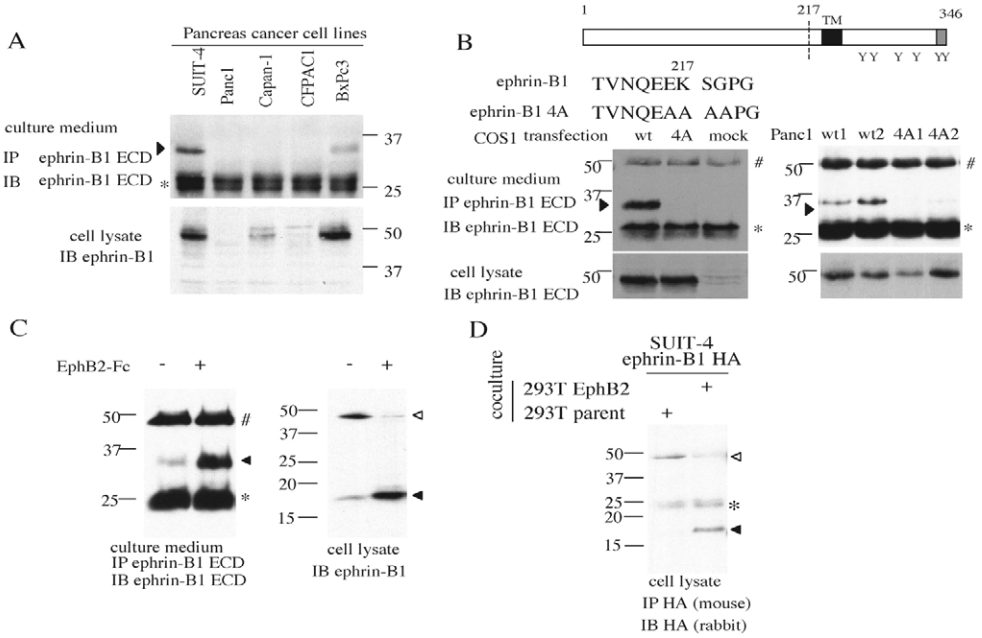

Fig. 1. The ectodomain of ephrin-B1 is secreted into the culture medium of human pancreas cancer cells. (A) Various pancreas cancer cell lines were cultured in medium containing 0.5% FBS. After 6 hours, conditioned medium was collected and subjected to immunoprecipitation (IP) and immunoblotting (IB) with polyclonal antibodies against the extracellular domain of ephrin-B1 (ephrin-B1 ECD). The immunoprecipitated 35 kDa ephrin-B1 fragment is indicated by an arrowhead. The expression of ephrin-B1 in each cell lysate was confirmed by immunoblotting (bottom). (B) A diagram of ephrin-B1 is shown at the top. TM, transmembrane domain; Y, tyrosine phosphorylation sites. Dotted line indicates the cleavage site of ephrin-B1, and the PDZ domain-binding motif is indicated as a gray box at the C-terminus. Four aa residues around the cleavage site were changed to alanine (ephrin-B1 4A) destroying the MMP-8 cleavage site. The conditioned medium of COS-1 cells transfected with wild-type (wt) or mutant ephrin-B1 (4A), or independent PANC-1 clones stably expressing ephrin-B1 were collected and subjected to immunoprecipitation and immunoblotting as described in A. (C) SUIT-4 cells were either treated with EphB2-Fc (4 μg/ml) for 2 hours (+) or left untreated (–). The ephrin-B1 fragment in the medium was detected as in A (left) or the cell lysates were subjected to immunoblotting with anti ephrin-B1 C18 (right panel). Open and filled arrowheads indicate uncleaved ephrin-B1 and its processed fragment (p17), respectively. (D) SUIT-4 cells were transiently transfected with ephrin-B1 tagged with HA at the C-terminus. Transfected SUIT-4 cells were overlayed on a monolayer of parent HEK293T cells or HEK293T cells stably expressing EphB2 for 2 hours. Cell lysates were prepared from co-cultured cells to detect HA-tagged p17 fragment derived from exogenously expressed ephrin-B1 in SUIT-4 cells by immunoprecipitation. # and * indicate the IgG heavy chain and light chain, respectively. Open and filled arrowheads indicate uncleaved ephrin-B1 and its processed fragment (p17), respectively.

induced by stimulation of ephrin-B1 did not depend on the elevation of MMP-8 expression level, but rather it was suggested to depend on the intracellular signaling mediated by ephrin-B1.

MMP-8, also known as neutrophil collagenase, is not only expressed in neutrophils, but it is also expressed in wide variety of cells, including chondrocytes, endothelial cells, synovial fibroblast and various cancer cells (Siller-Lopez et al., 2000; Stadlemann et al., 2003; Lint and Libert, 2006). MMP-8 cleaves all three α-chains of type I, II and III collagen and also a wide range of non-collagenous substrates, and plays important roles in inflammation and in cancer progression (Lint and Libert, 2006). Like other secretory proteins, proenzymes of soluble-type MMPs are secreted after the process of vesicle transport from Golgi to the plasma membrane, and then, extracellularly activated by removal of the propeptide domain (Sternlicht and Werb, 2001). In neutrophils, MMP-8 is stored in specific granules after being transported from Golgi, and released following activation by inflammatory mediators (Sternlicht and Werb, 2001), however, whether it is also the case in various cancer cells has yet to be fully investigated. Our analysis of the interaction between MMP-8 activity and EphB-ephrin-B1 signal transduction revealed a novel function of the C-terminus of ephrin-B1 in the secretion of MMP-8, which leads to the cleavage of ephrin-B1 and involves the negative regulation of the EphB-ephrin-B1 complex. Moreover, regulation of this MMP family member through the ephrin-B1 C-terminus may contribute to the highly invasive phenotype of ephrin-B1-expressing cancer cells by degradation of the extracellular matrix.

Results

The Ephrin-B1 ectodomain is secreted into the culture medium of pancreatic cancer cell lines

During screening of peptides secreted by the pancreatic cancer cell line SUIT-4, we identified,using MALDI-MS/MS analysis, three different peptides derived from ephrin-B1 sharing a common N-terminus. Analysis of these peptides indicated that ephrin-B1 undergoes limited cleavage between aa residues 217 and 218. Immunoprecipitation with antibodies against the extracellular domain of ephrin-B1 revealed a 35 kDa band from the conditioned media of two out of five pancreatic cancer cell lines. The 35 kDa fragment was also detected in COS-1 and PANC-1 cells when ephrin-B1 was expressed (Fig. 1A,B). These results indicate that the 35 kDa ectodomain fragment is released by cleavage of ephrin-B1 in these cell lines. The concentration of serum in the medium did not affect the cleavage of ephrin-B1 (data not shown). The cleavage site in ephrin-B1 estimated by MALDI-MS/MS was confirmed by the observation that substitution of the four amino acids (aa) at positions 216-219 (ephrin-B1$^{216\text{-}219}$) by alanine (ephrin-B1 4A), blocked the production of the 35 kDa ectodomain fragment in COS-1 and PANC-1 cells (Fig. 1B).

Cleavage of ephrin-B1 ectodomain is enhanced by interaction with its receptor EphB2, which is regulated by the C-terminus of ephrin-B1

Next, we examined whether the interaction of ephrin-B1 with its receptor EphB2 modifies the cleavage of ephrin-B1.

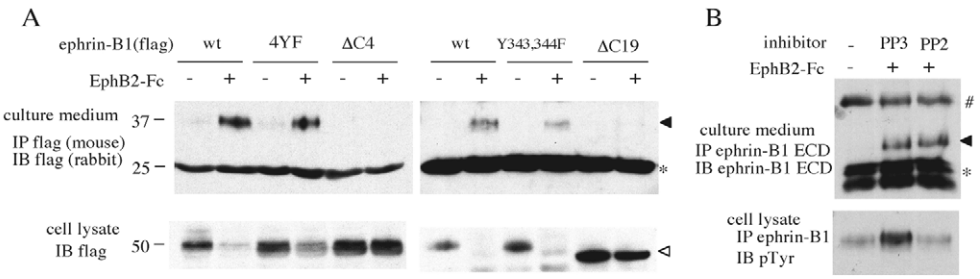

Fig. 2. Activation of ephrin-B1 cleavage requires its C-terminus. (A) Wild-type and various mutants of ephrin-B1 tagged with Flag at the N-terminus were expressed in Capan-1 cells by retrovirus-mediated gene transfer. The cells were treated with EphB2-Fc (2 μg/ml) for 1.5 hours (+) or left untreated (–) and conditioned medium was assayed for the presence of ephrin-B1 fragments by immunoprecipitation (IP) and immunoblotting (IB) with anti-flag antibody. The filled arrowhead indicates the ephrin-B1 ectodomain fragment. (Bottom) Expression of wild-type or mutated ephrin-B1 in cell lysates after treatment with EphB2-Fc. The open arrowhead indicates ephrin-B1 ΔC19. (B) SUIT-4 cells were treated with EphB2-Fc and PP2 or control PP3or left untreated, as indicated. Conditioned medium was collected after 2 hours and subjected to immunoprecipitation and immunoblotting. In the bottom panel, suppression of tyrosine phosphorylation of ephrin-B1 by PP2 treatment is shown. # and * indicate the IgG heavy chain and light chain, respectively.

Incubation of SUIT-4 cells with purified EphB2-Fc, a fusion protein of the extracellular domain of EphB2 with the Fc fragment of mouse IgG2b, significantly increased the amount of the 35 kDa fragment of ephrin-B1 in the conditioned medium (Fig. 1C, left). In EphB2-Fc-treated cell extract, a cellular fragment of ephrin-B1, produced by ectodomain shedding, was detected by immunoblotting with an antibody reacting to the C-terminal of ephrin-B1, as a band at 17 kDa (p17; Fig. 1C, right). The accumulation of p17 was also detected in ephrin-B1-expressing cells after contact with cells expressing EphB2. When ephrin-B1-expressing cells were overlayed on EphB2-expressing cells and co-cultured, significant reduction of uncleaved ephrin-B1 together with production of the p17 fragment was observed (Fig. 1D). This result indicates that cleavage of ephrin-B1 is also enhanced when ephrin-B1-expressing cells contact with heterologous cells expressing the EphB2.

In order to examine the processing mechanism of the ephrin-B1 ectodomain, several mutants of ephrin-B1 were analyzed. The cleavage of ephrin-B1 was also increased by EphB2-Fc treatment of Capan-1 cells expressing wild-type ephrin-B1 (Fig. 2A). However, expression of ephrin-B1 lacking the C-terminus (ΔC4 and ΔC19; the MMP-8 cleavage site at aa 217-218 is intact in these mutants), did not produce the 35 kDa ectodomain fragment in the culture medium upon treatment with EphB2-Fc (Fig. 2A). However, mutation of any of the four tyrosine residues in the cytoplasmic region (4YF) and tyrosines located at the C-terminus of ephrin-B1 (Y343, 344F) did not affect ephrin-B1 cleavage (Fig. 2A). In contrast to the significant reduction of full length wild-type or YF mutants of ephrin-B1 in cell lysates after EphB2-Fc treatment, level of C-terminally truncated ephrin-B1 mutants remained almost unchanged (Fig. 2A, bottom panels). We also observed similar results using PANC-1 cells, and confirmed that expression of ΔC mutants were localized on cell membrane (data not shown). In addition, treatment with PP2, an inhibitor of Src family kinases, significantly blocked tyrosine phosphorylation of ephrin-B1, but it did not affect cleavage of ephrin-B1 (Fig. 2B). Thus, the C-terminus of ephrin-B1, but not tyrosine phosphorylation of ephrin-B1, is required for induction of the proteolysis of ephrin-B1.

The Ephrin-B1 ectodomain is processed by MMP-8

The cleavage of ephrin-B1 was inhibited in PANC-1 cells expressing ephrin-B1, by incubation with the pan-matrix metalloproteinase (MMP) inhibitor GM6001, but not by inhibitors of other proteases including a cysteine protease, a serine protease, an aspartic protease or calpain (Fig. 3A). The amount of cleaved ephrin-B1 ectodomain was increased by treatment of DCI (3,4-dichloroisocoumarin), a serine protease inhibitor and TPCK (N^{α}-tosyl-phe chloromethyl ketone), a chymotrypsin inhibitor of unknown function. As the inhibition of ephrin-B1 cleavage by GM6001 was also confirmed in SUIT-4 cells (data not shown), we further attempted to identify the metalloproteinase responsible. When SUIT-4 cells were treated with natural MMP inhibitors, TIMPs, at low concentration (100 nM), cleavage of ephrin-B1 was most effectively inhibited by TIMP-1 compared with TIMP-2 and TIMP-3 (Fig. 3B). TIMP-3, which is known to inhibit tumor necrosis factor-α converting enzyme (TACE) did not inhibit the cleavage of ephrin-B1 at all at concentrations from 5-250 nM (data not shown), whereas it completely inhibited the cleavage of TNF-α expressed in THP-1 cells at 100 nM (Fig. 3B, right panel). Among inhibitors of several MMPs expressed in SUIT-4 cells, including MMP-1, MMP-2 or 3, MMP-8 and MMP-9, only the inhibitor of MMP-8 blocked the cleavage of ephrin-B1 (Fig. 3C), and this effect was also seen in COS-1 cells and PANC-1 cells expressing ephrin-B1 (data not shown).

In order to examine whether MMP-8 cleaves ephrin-B1, ephrin-B1-Fc fusion protein, which consists of the entire extracellular region of ephrin-B1 and the Fc fragment of mouse IgG2b, was incubated with purified activated MMPs in vitro. Incubation of ephrin-B1-Fc with activated MMP-8 produced two fragments of ephrin-B1-Fc corresponding to the predicted sizes (Fig. 3D). However, other membrane-type metalloproteinases such as MT1-MMP and ADAM10, which are also expressed in SUIT-4 cells, did not cleave ephrin-B1-Fc in vitro (Fig. 3D).

Expression of MMP-8 protein was detected at approximately the same levels in all of the cell lines we examined (Fig. 4A, left), although it was detected at high level when the cells were replated and decreased after the cells reached confluence (Fig. 4A, right). When expression of

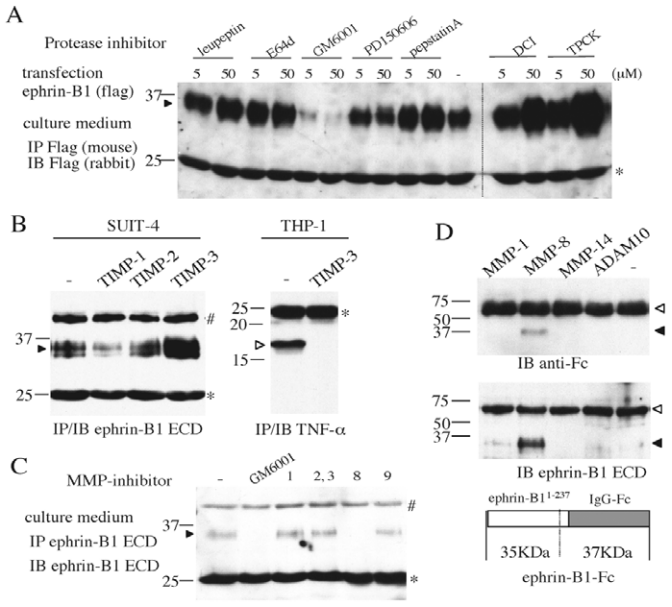

Fig. 3. Screening of a protease that cleaves the extracellular domain of ephrin-B1. (A) Panc-1 cells transiently expressing Flag-tagged ephrin-B1 were incubated with various protease inhibitors for 4 hours in medium containing 0.5% FBS: leupeptin and E64d (Loxistatin), a cystein protease inhibitor; GM6001, a pan-MMP inhibitor; PD150606, a calpain inhibitor; pepstatin A, an aspartic protease inhibitor; DCI (3,4-dichloroisocoumarin), a serine protease inhibitor; TPCK (N^{α}-tosyl-phe chloromethyl ketone), a chymotrypsin inhibitor. The cleavage of ephrin-B1 ectodomain was examined as described in Fig. 2A. (B) Left: SUIT-4 cells were incubated with EphB2-Fc (2 μg/ml) together with or without TIMPs (100 nM for each) as indicated for 2 hours. The processed ephrin-B1 fragment was detected in the culture medium. Right: THP-1 cells were treated with PMA (10 ng/ml) together with or without TIMP-3 (100 nM) for 6 hours. Open arrowhead indicates the processed fragment of TNF-α in the medium detected by immunoprecipitation with anti TNF-α antibody. (C) SUIT-4 cells were treated with various MMP inhibitors as indicated (1 μM for MMP-8 inhibitor and 5 μM for others) for 4 hours. Ephrin-B1 fragment was detected in the medium. (D) Purified ephrin-B1-Fc protein was incubated with activated MMP in vitro for 1 hour at 37°C, separated by SDS-PAGE, and immunoblotted with anti-Fc mouse IgG or anti-ephrin-B1. Bottom: a schematic representation of ephrin-B1-Fc with the MMP-8 cleavage site indicated by a dotted line. Open and filled arrowheads indicate uncleaved ephrin-B1-Fc and the processed fragments, respectively.

MMP-8 was reduced in SUIT-4 cells by treatment of cells with siRNA, the amount of processed ephrin-B1 ectodomain in the culture medium was decreased (Fig. 4B). By contrast, overexpression of activated MMP-8 cDNA, in which the propeptide domain was removed, in SUIT-4 cells evidently increased the cleavage of ephrin-B1 (Fig. 4C).

As some MMPs and their substrates physically associate directly or indirectly and make a protein complex, interaction between MMP-8 and ephrin-B1 was examined (Sawicki et al., 2005; Yu et al., 2007). Ephrin-B1 was coprecipitated with MMP-8 from extracts of L ephrin-B1 cells by MMP-8-specific antibodies, but not by control normal rabbit IgG1, indicating that MMP-8 forms a complex with ephrin-B1 (Fig. 4D). However, association of MMP-8 with the receptor EphB2 was not detected (data not shown). These results also suggest that MMP-8 is the key metalloproteinase that cleaves the ephrin-B1 ectodomain.

Stimulation of ephrin-B1 activates secretion of MMP-8, which is regulated by the C-terminus of ephrin-B1

To understand the mechanism of activation of ephrin-B1 cleavage by EphB2, we next examined whether it is accompanied by an increase in MMP-8 expression. The level of intracellular expression of MMP-8 mRNA was not affected by treatment with EphB2-Fc or contact of ephrin-B1-expressing cells with EphB2-expressing cells for 4 hours or longer (Fig. 5A). Moreover, the amount of processed ephrin-B1 ectodomain produced in the medium was not altered by the addition of cyclohexamide or actinomysin D, inhibitors of de novo synthesis of mRNA and proteins, respectively (Fig. 5B). These results indicate that activation of ephrin-B1 cleavage by EphB2 does not depend on the increased amount of MMP-8. In addition, when the de novo synthesis of MMP-8 was blocked, the amount of intracellular MMP-8 protein decreased slightly after 4 hours or longer treatment of ephrin-B1-expressing cells with EphB2-Fc (Fig. 5C), which suggests that stimulation of ephrin-B1 activates extracellular release of MMP-8 protein from the cytoplasm. Actually, the amount of MMP-8 protein in the culture medium was remarkably elevated after incubation of the cells with EphB2-Fc as detected by immunoprecipitation (Fig. 5D).

In order to show directly that stimulation of ephrin-B1 increased exocytosis of MMP-8, which was already synthesized and present in the cytoplasm, SUIT-4 cells were pulse-labeled with [^{35}S]methionine. When pulse-labeled cells were treated with EphB2-Fc, a higher amount of labeled MMP-8 protein was detected in the medium compared to treatment of cells with Fc (Fig. 5E, left). However, secretion of labeled MMP-7, which was examined as a control, was not altered by EphB2-Fc treatment (Fig. 5E, right). In addition, the secretion of [^{35}S]methionine-labeled MMP-8 was also increased by EphB2-Fc treatment of Capan-1 cells expressing wild-type ephrin-B1, but it was not found in the media of cells expressing ephrin-B1 lacking the C-terminus (ΔC4 and ΔC19) (Fig. 5F, left). Consistently, the total amount of MMP-8 protein in the culture medium from cells expressing wild-type ephrin-B1 was higher than in the medium from ΔC4 ephrin-B1-expressing cells (Fig. 5F, right). These results suggest that stimulation of ephrin-B1 by EphB2 upregulates the process of MMP-8 exocytosis, and the C-terminus of ephrin-B1 regulates this event.

Stimulation of ephrin-B1 by EphB2 induces activation of Arf1 GTPase

To further confirm that activation of MMP-8 secretion is involved in the elevated ephrin-B1 cleavage in response to stimulation with EphB2, the cells were treated with brefeldin A, an inhibitor of membrane trafficking through the Golgi, which blocks the secretion of proteins (Tamaki and Yamashina, 2002). EphB2-stimulated cleavage of ephrin-B1 was apparently reduced by brefeldin A treatment (Fig. 6A). As a mode of action of brefeldin A is to inhibit activation of ADP ribosylation factor 1 (Arf1), a ras family GTPase, by blocking of the exchange reaction from Arf1-GDP to Arf1-GTP (Niu et al., 2005; Zeeh et al., 2006), we further examined the activity of Arf1 in ephrin-

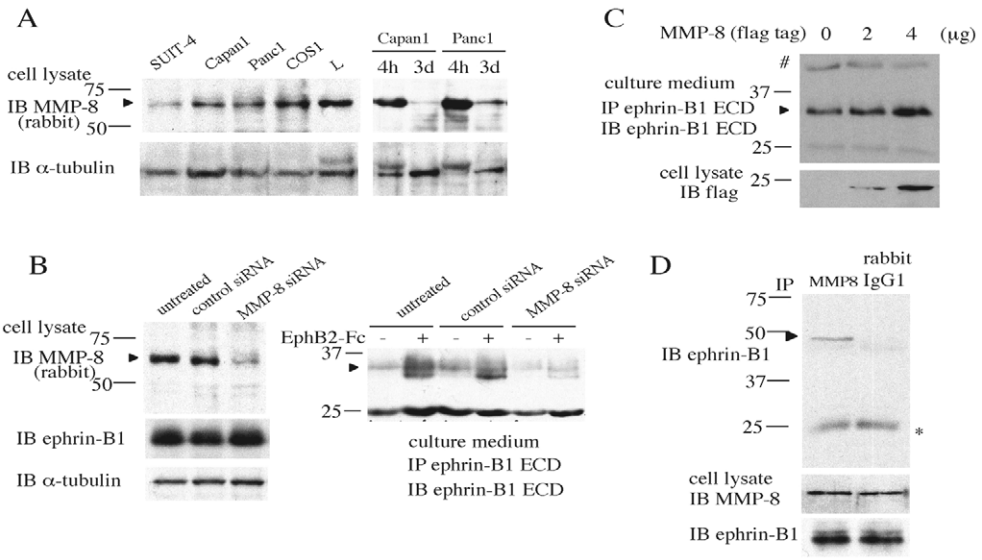

Fig. 4. MMP-8 is the key protease of ephrin-B1 cleavage. (A) Expression of MMP-8 in cell lysates. Left: The indicated cells were seeded on plates not to reach confluence. Cell lysates were prepared on the day after plating. Right: cell lysates were prepared 4 hours (4h) or 3 days (d3) after being plated on dishes. The cells were confluent on day 3 after plating. (B) SUIT-4 cells treated with either MMP-8 siRNA or control scrambled siRNA (control), or left untreated. The cells were detached 48 hours later, replated on new plates and further incubated for 24 hours. Left: Cellular levels of MMP-8 were analyzed 72 hours after treatment of SUIT-4 cells with siRNAs. Right: the culture medium was replaced with one fresh medium or medium containing EphB2-Fc (2 g/ml) and incubated for 2 hours to detect ephrin-B1 ectodomain in the medium. (C) SUIT-4 cells were transiently transfected with the indicated volume of a plasmid encoding Flag-tagged activated MMP-8 cDNA. After 48 hours of transfection, the medium was replaced and the cells were further incubated for 6 hours to detect processed ephrin-B1 ectodomain in the medium. The expression of transfected MMP-8 in each cell lysate was confirmed by immunoblotting with anti-Flag antibody (bottom). (D) The lysate of L ephrin-B1 cells was immunoprecipitated with anti-MMP-8 polyclonal antibody or control rabbit IgG1, and subjected to immunoblotting with anti-ephrin-B1 C18 antibody. HRP-conjugated anti-rabbit IgG (TrueBlot) was used as the secondary antibody for immunoblotting to avoid cross reaction with denatured rabbit IgG heavy chain of the antibody used for immunoprecipitation. The arrowhead indicates coprecipitated ephrin-B1. The asterisk indicates the IgG light chain.

B1-expressing cancer cells. To examine the amount of activated, GTP-bound Arf1, a pull-down approach was performed with an adaptor protein GGA3, which specifically binds to GTP-bound Arfs (Dell'Angelica et al., 2000). As a control experiment, we confirmed that constitutively activated Arf1 (Q71L), but not a dominant negative mutant of Arf1 (T31N) was coprecipitated with GST-tagged GGA3 (Fig. 6B, right panel). Incubation of SUIT-4 cells with EphB2-Fc increased the amount of Arf1-GTP coprecipitated with GST-tagged GGA3 (Fig. 6B). Similar results were also observed in PANC-1 cells expressing ephrin-B1, whereas treatment with EphB2-Fc did not affect activation of Arf1 in parent PANC-1 cells, which express ephrin-B1 in trace amounts (Fig. 6B). The amount of Arf1-GTP was also increased by EphB2-Fc treatment of Capan-1 cells expressing wild-type ephrin-B1 or ephrin-B1 4YF mutant, but not in cells expressing ΔC4 ephrin-B1 (Fig. 6C). These results suggest that stimulation of ephrin-B1 by EphB2 activates Arf1, and the signaling mediated by the C-terminus of ephrin-B1 is involved in this pathway. Furthermore, treatment of cells with brefeldin A, or overexpression of dominant negative mutant of Arf1, Arf1 T31N, decreased the secretion of MMP-8 in Capan-1 cells, indicating that Arf1 regulates the secretion of MMP-8 (Fig. 6D).

The C-terminus of ephrin-B1 is involved in the invasion by cancer cells

To investigate the biological implication of metalloproteinase activation caused by ephrin-B1-mediated signaling, we examined changes in the invasiveness of ephrin-B1-expressing cells with or without stimulation by EphB2 using an in vitro cell invasion assay. The invasion of collagen by Capan-1 cells was promoted by expression of ephrin-B1 and treatment of the cells with EphB2-Fc, and this invasion was inhibited by the addition of an MMP-8 inhibitor (Fig. 7A). On the other hand, the invasion by Capan-1 cells expressing ΔC4 ephrin-B1, or by parent Capan-1 cells was not significantly promoted by EphB2-Fc treatment (Fig. 7A).

We further examined whether expression of ephrin-B1 actually promotes cancer cell invasion in vivo using PANC-1 cells as a model system to study peritoneal dissemination. PANC-1 cells stably expressing wild-type ephrin-B1 (PANC-1 ephrin-B1) or ΔC4 ephrin-B1 (PANC-1 ΔC4) were established to compare their invasiveness with that of parent PANC-1 cells. Expression of wild-type or mutated ephrin-B1 did not affect BrdU incorporation into cells grown under normal two-dimensional cell culture conditions (data not shown). When these cells were injected intraperitoneally into nude mice, PANC-1 ephrin-B1 cells formed many tumor nodules in the mesenteric sheets and also in the peritoneal cavity, including the rectouterine region. By contrast, in mice injected with parent PANC-1 cells or PANC-1 ΔC4 cells, such tumors in mesentery sheets were fewer and smaller, and the total tumor volume involving the rectouterine region was much less (Fig. 7B,C, Table 1). The cells composing the mesenteric sheets express cognate receptors for ephrin-B1, EphB2 and

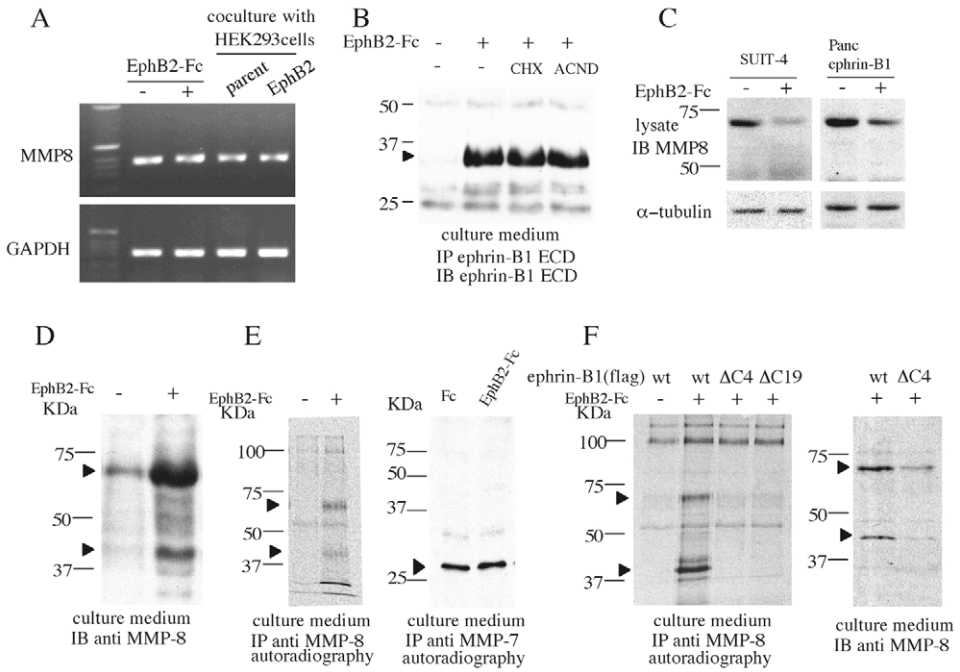

Fig. 5. Stimulation of ephrin-B1 with EphB2 increases MMP-8 secretion. (A) SUIT-4 cells were left untreated or were treated with EphB2-Fc or co-cultured with either parent or EphB2-expressing HEK293 cells for 4 hours. Cellular levels of MMP-8 were analyzed by RT-PCR using GAPDH as a control. (B) SUIT-4 cells were incubated with or without EphB2-Fc for 2 hours in the presence or absence of cyclohexamide (CHX, 100 μg/ml) or actinomycin D (ACND, 5 μg/ml) to detect the ephrin-B1 ectodomain in the medium. (C) SUIT-4 cells or PANC-1 cells stably expressing ephrin-B1 were treated with actinomycin D (5 μg/ml) together with or without EphB2-Fc (2 μg/ml) for 4 hours. Cell lysates were prepared and intracellular expression levels of MMP-8 were analyzed by western blotting using α-tubulin as a loading control. (D) Conditioned medium of SUIT-4 cells were collected after the cells were treated with EphB2-Fc or left untreated for 4 hours in serum-free medium. Proteins secreted in the medium were precipitated with trichloroacetic acid (10%), resuspended in sample buffer, and subjected to immunoblotting with anti MMP-8 polyclonal antibody. Arrowheads indicate MMP-8 protein (proenzyme and activated). (E) SUIT-4 cells were metabolically labeled with [^{35}S]methionine, then treated with EphB2-Fc or control Fc for 2 hours. The amount of labeled MMP-8 (left panel) or MMP-7 (right panel) in the medium was evaluated through immunoprecipitation from the conditioned medium followed by SDS-PAGE and autoradiography. Arrowhead indicates MMP-8 (proenzyme and activated; left) or MMP-7 (right) in the medium. (F) Wild-type and mutants of ephrin-B1 were expressed in Capan-1 cells as in Fig. 2A, and [^{35}S]methionine-labeled MMP-8 was detected in the medium (left panel). Right: The total amount of MMP-8 in the conditioned medium of Capan-1 cells was evaluated using the trichloroacetic acid (TCA) precipitation procedure, followed by immunoblotting with anti MMP-8 antibody as in D.

EphB4 (see Fig. S1 in supplementary material). These results indicate that ephrin-B1 actually promotes cancer cell invasion, which requires the C-terminus of ephrin-B1. EphB2 and EphB4 expressed in cells of the mesenteric sheet might act as interaction partners for ephrin-B1 present on PANC-1 cells.

Discussion

The interaction of Eph family receptor protein tyrosine kinases with their ligands, ephrin family proteins, induces bi-directional signaling. In this study, we showed for the first time that ephrin-B1 regulates the activation and release of a metalloproteinase. We observed that binding of EphB2 to ephrin-B1 promotes secretion of MMP-8 without increasing the expression level of MMP-8. Activation of several molecules, such as Erk, p38 and PI 3-kinase or Akt cause transcriptional activation of metalloproteinases (Chinni et al., 2006; Raymond et al., 2006; Reuben and Cheung, 2006). In our study, however, activation of ephrin-B1 by EphB2 binding did not alter the phosphorylation levels of Erk-1, 2, p38 or Akt (data not shown), as also reported by others (Huynh-Do et al., 2002). Our observation that ephrin-B1-induced secretion of MMP-8 was sensitive to brefeldin A, which blocks the membrane trafficking of coated vesicles at the Glogi/trans-Golgi network suggests that ephrin-B1 signaling resulted in increased transport of MMP-8 from the cytoplasm to the cell surface (Tamaki and Yamashina, 2002).

Regulation of the secretion of MMP-8 enables ephrin-B1 signaling to play an important role in regulating MMP-8 activity. Like other soluble MMPs, MMP-8 is proteolytically activated extracellularly by removal of its propeptide domain, and physiologically relevant level of MMP protease activity requires efficient release of the protease to the cellular surface (Sternlicht and Werb, 2001). Although the molecular mechanism of the MMP-8 secretory pathway is not well understood, our data indicate that signaling mediated by the carboxyl-terminal region of ephrin-B1 is involved. Notably, removal of the C-terminus of ephrin-B1 resulted in significant reduction of MMP-8 secretion and cleavage of the extracellular domain of ephrin-B1. In addition, the C-terminus of ephrin-B1 regulates the signal leading to activation of Arf1, a critical regulator of membrane traffic in the secretory pathway and one target of brefeldin A (Tamaki and Yamashina, 2002; Donaldson

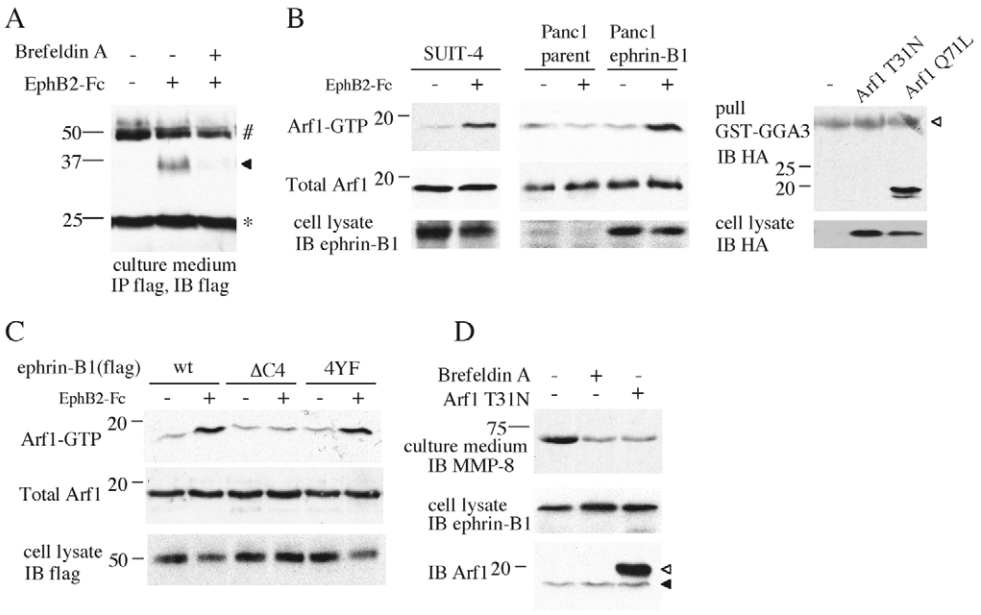

Fig. 6. Stimulation of ephrin-B1 with EphB2 activates Arf1. (A) Flag-tagged ephrin-B1 was expressed in Capan-1 cells. The cells were treated with or without EphB2-Fc (2 μg/ml) and brefeldin A (10 μg/ml) as indicated for 1.5 hours, and conditioned medium was assayed for the 35 kDa ectodomain of ephrin-B1. (B,C) The activity of Arf1 was analyzed in the indicated cells. (C) Wild-type or mutant ephrin-B1 was expressed in Capan-1 cells as in Fig. 2A. The cells were incubated with EphB2-Fc (4 μg/ml) for 20 minutes before being lysed. Arf1-GTP was pulled down with GST-GGA3 bound to glutathione-Sepharose. As controls, lysates of COS-1 cells transiently transfected with plasmids encoding Arf1 T31N or Arf1 Q71L, HA tagged at the C-terminus were analyzed (B, right panel). Open arrowhead indicates cross-reacted GST-GGA3 used for the pull-down assay. (D) Suppression of Arf1 activation decreased the MMP-8 secretion. Capan-1 cells stably expressing ephrin-B1 were used. In the right lane, Arf1 T31N was also transiently expressed in the cells by retrovirus mediated gene transfer. All cells were treated with EphB2-Fc together with (middle lane) or without brefeldin A for 4 hours, and the conditioned medium was subjected to TCA precipitation to detect MMP-8 through immunoblotting. The filled arrowhead indicates endogenous Arf1, and the open arrowhead indicates HA-tagged Arf1 T31N (bottom).

et al., 2005). Arf1 GTPase regulates the membrane association of coat proteins involved in intracellular membrane trafficking, which is critical for the vesicle transport of secretory proteins at the Golgi (Donaldson et al., 2005). Actually we observed that treatment of cells with brefeldin A, or expression of Arf1 T31N inhibited the secretion of MMP-8. Therefore, as one possibility, secretion of MMP-8 is upregulated by activation of Arf1 GTPase through ephrin-B1 signaling, although the molecular mechanisms connecting ephrin-B1 and Arf1 are still not well understood. As ephrin-B1 has a docking site for the PDZ domain at the C-terminus, some protein containing PDZ domains may be involved in this pathway. As a consequence, the increase of secreted MMP-8 may trigger the degradation of extracellular matrix and the cleavage of ephrin-B1 as a possible feedback mechanism (Fig. 8). In our preliminary observations, Arf1 activation occurs as fast as 10 minutes after stimulation with EphB2-Fc, and some increase of MMP-8 in the culture medium was detected from 0.5 hours after addition of EphB2-Fc. By contrast, the cleavage of ephrin-B1 was observed at 1 hour, but not at 10 minutes after stimulation with EphB2-Fc, by immunoblotting of a cleaved C-terminal fragment of ephrin-B1, p17, in cell lysates (data not shown). Although we cannot determine the precise time point of MMP-8 secretion because of the limitation of the antibody's sensitivity, these observations are compatible with the model that ephrin-B1 reverse signaling induces Arf1 activation, which leads to MMP-8 release and ephrin-B1 cleavage. As extracellular activation of MMPs can be triggered by activation of other MMPs, there is the possibility that ephrin-B1-mediated signaling may synergistically promote activity of several MMPs. In addition, Arf1 GTPase may involve the intracellular transport of not only MMP-8, but also several other MMPs. Whether metalloproteinases other than MMP-8 are also upregulated by EphB2 stimulation of ephrin-B1 should also be investigated.

We show that the 35 kDa ephrin-B1 fragment in the culture medium of SUIT-4 cells was generated by cleavage at aa 218. From the experiments using natural inhibitors of metalloproteinases, TIMP-1 but not TIMP-2 and TIMP-3 effectively blocked the cleavage of ephrin-B1. Among these TIMPs, TIMP-3 most effectively inhibits the function of ADAM family metalloproteinase, including ADAM-17 (TACE), ADAM-10 and ADAM-TS4 (Amour et al., 2000; Brew et al., 2000; Hashimoto et al., 2001). In addition, membrane-type metalloproteinases (MT-MMPs) are preferentially inhibited by TIMP-2 and TIMP-3, but not by TIMP-1 (Brew et al., 2000). Our observation that cleavage of ephrin-B1 was most effectively inhibited by TIMP-1, but not by TIMP-2 or TIMP-3 suggests that it is unlikely that those members of the ADAMs family are critically involved. However, we cannot exclude that TIMP-3-independent ADAM metalloproteinases may contribute to the processing of ephrin-B1. Together with the observation that ephrin-B1 cleavage was at least partially inhibited by RNA interference of MMP-8, and it was increased by overexpression of MMP-8, our results suggest that MMP-8 is the key metalloproteinase that cleaves ephrin-B1 ectodomain.

In addition to an extracellular 35 kDa peptide derived from the N-terminal of ephrin-B1, stimulation of ephrin-B1-expressing cells with EphB2-Fc resulted in the production of a 17 kDa intracellular fragment (p17) derived from the C-terminus of ephrin-B1. As detection of p17 was abolished in L cells expressing the ephrin-B1 4A mutant (data not shown), p17 was generated by cleavage of ephrin-B1 within the extracellular domain at aa 218. Ephrin-B1 and ephrin-B2 are cleaved within the transmembrane region by presenilin-dependent γ-secretase, which releases an approximately 12

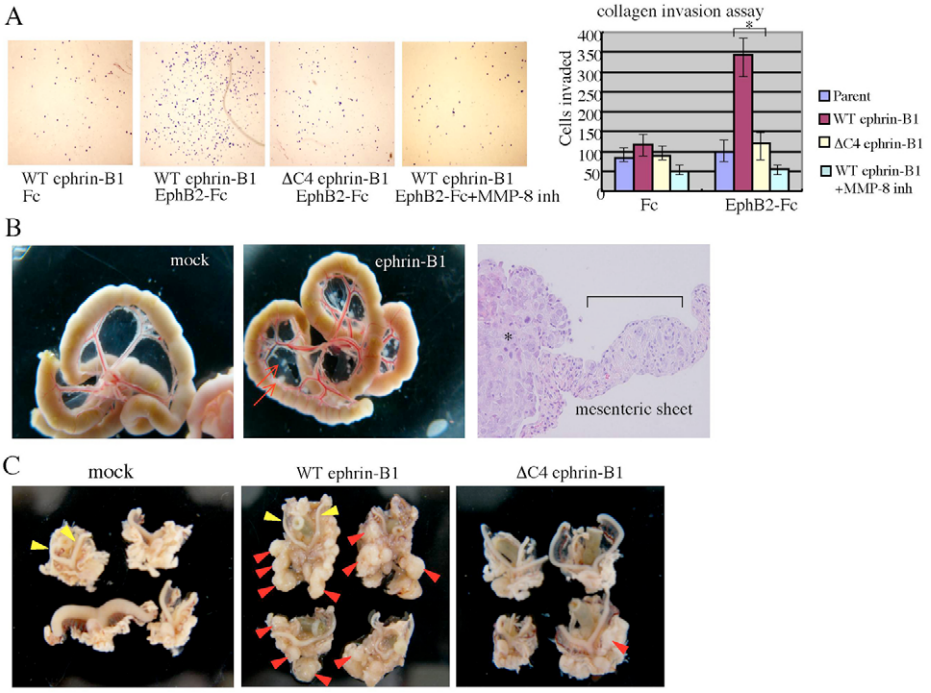

Fig. 7. The C-terminus of ephrin-B1 regulates the invasion of cancer cells. (A) Wild-type (WT) ephrin-B1 or ΔC4 ephrin-B1 mutant was expressed in Capan-1 cells. The cells were seeded onto a Transwell membrane coated with a collagen matrix (25 μg/ cm^2) in serum-free medium containing control Fc or EphB2-Fc (4 μg/ml) with or without addition of the MMP-8 inhibitor (1 μM). In the lower chamber, medium containing 5% FBS was added as a chemoattractant. After 8 hours incubation, the wells were harvested and cells that had invaded the collagen were counted. Representative fields are shown. (Right) The results from three independent experiments, each in duplicate, are shown as the mean ± s.d. **P*<0.01. (B) PANC-1 ephrin-B1 cells or PANC-1 cells transfected with a mock vector (mock) were injected intraperitoneally into nude mice. The representative appearance of intestinal loops 8 weeks after injection is shown. Arrows indicate disseminated tumor nodules in the mesentery. The right panel shows the histology of the tumors in the mesentery (×100). The asterisk indicates a tumor nodule. Microscopic invasion of cancer cells was observed in the mesenteric sheet (blanket). (C) Representative appearance of the tumors of panc1 cells expressing either mock vector, wild-type or ΔC4 ephrin-B1 in the rectouterine region was compared. Yellow and red arrowheads indicate uterine horns and tumor nodules, respectively.

kDa intracellular fragment (Georgakopoulos et al., 2006; Tomita et al., 2006). Although p17 may be further processed by γ-secretase and produce a small intracellular peptide, we did not detect such a product, possibly because of its rapid degradation or cell type-dependent differences in protease activity.

One possible function of ephrin-B1-mediated MMP-8 secretion is processing of ephrin-B1 and downregulation of EhpB2-stimulated ephrin-B1 intracellular signaling. Unlike wild-type ephrin-B1 protein, ΔC ephrin-B1 protein was not reduced after EphB2-Fc treatment, which seems to suggest that cleavage of ephrin-B1 contributes to the down regulation of ephrin-B1 after stimulation. However, recent reports show the trans-endocytosis of ephrin-B1 after engagement with EphB receptors, which regulates ephrin-B-mediated cell repulsion (Zimmer et al., 2003; Marston et al., 2003; Parker et al., 2004). We also observed that ephrin-B1 4A, which also triggers Arf1 activation and release of MMP-8, but is resistant to cleavage, was degraded and decreased in amount after stimulation with EphB2-Fc almost to the same degree with wild-type ephrin-B1 (data not shown). Because the C-terminus of ephrin-B1 may also modify its endocytosis, we cannot conclude at present that ephrin-B1 cleavage greatly affects the stability and turnover of the EphB-ephrin-B1 complex on the cell surface. Rather, the biological significance of ephrin-B1-mediated MMP-8 secretion is considered to be the promotion of the invasion potential of cancer cells via degradation of surrounding extracellular matrix. However, addition of the 35 kDa ephrin-B1 ectodomain prepared from conditioned medium of ephrin-B1 expressing COS-1 cells inhibited the motility of EphB2-expressing cells, similar to the effect of unclustered ephrin-B1-Fc, indicating the possible biological effect of secreted fragments on cell movement, whereas it did not affect cell proliferation (see Fig. S2 in supplementary material).

The potential significance of ephrin-B1 in cancer cell invasion is supported by our finding that ephrin-B1-mediated collagen invasion by Capan-1 cells was related to MMP-8. The inability of ΔC-ephrin-B1 to promote collagen invasion in the same assay revealed that signaling through C-terminus of ephrin-B1 affects the invasion ability of cancer cells. In addition, expression of ephrin-B1 in the pancreas cancer cells, PANC-1, promoted the dissemination of intraperitoneally injected cells into the mesentery and peritoneal cavity where they formed tumor nodules, demonstrating for the first time that ephrin-B1 actually promotes cancer cell invasion in vivo. Overexpression of B-type ephrins in cancer cells would affect cell-cell adhesion by their interaction with cell adhesion proteins such as claudin (Tanaka et al., 2005). Ephrin-B1-mediated intracellular signaling also results in aberrant

Table 1. Mesenterial dissemination after intraperitoneal inoculation of cancer cells

	Number of nodules*		
Cell line	0-10	10-30	30+
Mock	16	2	0
WT ephrin-B1	0	4	17
ΔC4 ephrin-B1	12	4	3

Data are shown as the number of mice with tumors in the mesentery.
*Number of tumor nodules larger than 2 mm in the mesentery per body.

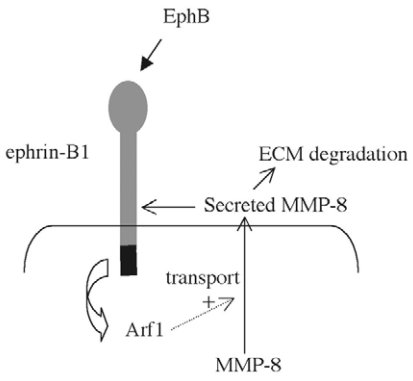

Fig. 8. Diagram showing the possible mechanism of ephrin-B1-mediated stimulation of MMP-8 secretion and cell invasion. When ephrin-B1 is stimulated by EphB receptors, Arf1 GTPase is activated through signaling mediated by the C-terminus ephrin-B1, which may stimulate the transport of MMP-8 for extracellular release. The increase of secreted MMP-8 triggers the degradation of extracellular matrix (ECM) and cleavage of ephrin-B1.

activation of RhoA and Rac1 (Tanaka et al., 2003; Tanaka et al., 2004; Lee et al., 2005). Together, events such as these would result in increased cell motility. These findings in conjunction with the ephrin-B1 induction of MMP-8 secretion, indicate that ephrin-B1 overexpression would result in an enhanced potential for invasion of surrounding tissues. For example, both MMP-8 and ephrin-B1 are frequently expressed in ovarian cancers, and their expression correlates with tumor grade and a poor prognosis (Castellvi et al., 2006; Varelias et al., 2002). Ephrin-B1 could also be involved in invasion of cancer cells circulating in the blood into sheets of endothelial cells which express EphB receptors and play a role in extravasation and metastasis. The inhibition of a specific cellular signal originating in ephrin-B1 stimulation may be a good candidate for regulating tumor invasion.

Materials and Methods

Plasmids, antibodies and reagents

Plasmids encoding full-length cDNAs of human ephrin-B1 and the Fc fusion protein construct of EphB2 and ephrin-B1 have been described previously (Tanaka et al., 2004). Fc fusion proteins were purified from the culture medium of COS-1 cells transfected with plasmids encoding EphB2-Fc or ephrin-B1-Fc using a protein A Sepharose column as described previously (Tanaka et al., 2004). Mutants of ephrin-B1 lacking the cytoplasmic tail (ΔC4 and ΔC19, truncation of four or 19 aa residues at the C-terminus, respectively) were generated using PCR-based techniques. Alanine substitution of four aa in the extracellular domain (aa 216-219) of ephrin-B1, ephrin-B1 4A, was performed using the Altered Sites Mutagenesis System (Promega). Generation of Ephrin-B1 with mutations of four tyrosine residues in the cytoplasmic domain (Y313, 317, 324, 329) and ephrin-B1 4YF, have been described previously (Tanaka et al., 2005). For making flag-tagged ephrin-B1, a DNA fragment encoding the Flag tag was inserted 3′ to the signal peptide of ephrin-B1 (aa 1-24). To generate a plasmid encoding human activated MMP-8, cDNA corresponding to nucleotides 336-824 of the reported sequence (GenBank accession number BC074988) was amplified with RT-PCR from a cDNA template derived from U937 cells. The amplified MMP-8 cDNA was tagged with the signal peptide and Flag at the 5′ terminus, and cloned into pcDNA3. GST-GGA3 was generated by cloning of PCR-amplified cDNA corresponding to aa 1-313 of human GGA3 short isoform (GenBank accession number AF219139) into pGEX4T2 (Amersham Pharmacia). The plasmids encoding wild-type Arf1 and Arf1 T31N bearing the HA epitope at the C-terminus were donated from J. S. Bonifacino (National Institute of Child Health and Human Development, NIH, Bethesda, MA). To generate the recombinant retrovirus, cDNAs were subcloned into pDON-AI vector (Takara). Monoclonal and polyclonal antibodies that recognize the Flag tag were purchased from Sigma and Affinity Bioreagents (Affinity Bioreagents, Golden, CO), respectively. Antibodies that recognize the HA tag were from InvivoGen (InvivoGen, San Diego, CA; monoclonal antibody) and Santa Cruz Biotechnology Inc. (polyclonal antibody). Rabbit polyclonal antibody that recognizes ephrin-B1 (C18) was purchased from Santa Cruz Biotechnology, Inc. The goat polyclonal antibody against ephrin-B1, which reacts with the entire extracellular domain, was purchased from R&D Systems. The polyclonal antibody against tyrosine-phosphorylated ephrin-B1 (ephrin-B1 pY317, aa residues 314-321) was raised in rabbits and affinity-purified as described previously (Tanaka et al., 2005). EphB2 and EphB4 polyclonal antibodies were from R&D Systems. Polyclonal and mouse monoclonal antibodies for MMP-8 were purchased from Chemicon and Daiichi Fine Chemical (Takaoka, Japan), respectively. The monoclonal antibody for phosphotyrosine (4G10) and Arf1 was from Upstate Biotechnology and Affinity Bioreagents, respectively. TrueBlot anti-rabbit IgG secondary antibody was purchased from eBioscience (San Diego, CA). Cyclohexamide and actinomycin D were purchased from Sigma. Purified TIMP-1, and 2 were purchased from Calbiochem, and TIMP-3 was from Sigma. The protease inhibitors shown in Fig. 3 and purified MMP-1 (proenzyme), MMP-8 (proenzyme), MT1-MMP (catalytic domain, aa 89-265) and ADAM10 (mature active ectodomain, aa 19-673) were purchased from Calbiochem. The MMP-8 inhibitor is (3R)-(+)-[2-(4-methoxybenzenesulfonyl)-1,2,3,4-tetrahydroisoquinoline-3-hydroxamate]. Src family kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) and the structural analog 4-amino-7-phenylpyrazolo[3,4-d]pyrimidine (PP3) were purchased from Calbiochem.

Cell culture, transfection and retrovirus infection

SUIT-4 (Kawano et al., 2004) and the other pancreas carcinoma cell lines were cultured in RPMI1640 supplemented with 10% fetal bovine serum. Mice fibroblast L cells and COS-1 cells were cultured in DMEM with 10% fetal bovine serum. For transient expression assays, COS-1 cells and SUIT-4 cells were transfected with plasmid DNA using Lipofectamine 2000 reagent (Invitrogen). Recombinant retroviral plasmid, pDON-AI was cotransfected with pCL-10A1 retrovirus packaging vector (IMGENEX) into 293gp cells to allow the production of retroviral particles. Capan-1 cells were infected with retroviruses for transient expression of ephrin-B1 or Arf1 mutants, and used for experiments 48 hours after infection. For some experiments, Capan-1 cells stably expressing wild-type ephrin-B1 were established after retrovirus infection through the selection in medium containing G418 (600 μg/ml). L cells stably expressing ephrin-B1 or EphB2 were established as described and cultured in medium containing hygromycin B at a concentration of 400 μg/ml (Tanaka et al., 2005). PANC-1 cells stably expressing ephrin-B1 were established through selection in medium containing puromycin at a concentration of 2 μg/ml for 2-3 weeks. Well isolated colonies were characterized further.

In vitro siRNA treatment

Stealth siRNA (Invitrogen) of MMP-8 was synthesized as follows. Sense: 5′-AAGGCAUGAGCAAGGAUUCCAUUGG-3′; antisense: 5′-CCAAUGGAAUCC-UUGCUCAUGCCUU-3′. The control siRNA (scramble II duplex: 5′-GCG-CGCUUUGUAGGAUUCGdTdT-3′) was purchased from Dharmacon. SiRNAs were incorporated into cells using Lipofectamine™ 2000 according to the manufacturer's instructions (Invitrogen). Assays were performed 72 hours post treatment.

Peptidomic analysis of secretory proteins

SUIT-4 cells were cultured in serum-free RPMI1640 medium and the conditioned medium was collected. Cleared supernatant was loaded onto a SepPak C18 cartridge (Waters) for peptide extraction. Peptides bound to the cartridge were eluted with 0.1% trifluoroacetic acid (TFA)/60% acetonitrile (ACN) and lyophilized. The resultant sample was reconstituted with the same solvent and applied to an HPLC gel filtration column (Pharmacia). Fractions containing peptides with a molecular mass below 8,000 Da were subjected to reductive alkylation as described previously (Sasaki et al., 2002) and desalted with an Empore disk cartridge (3M). The desalted material was separated with a 75 mm × 100 mm C18 column (LC Packings, Sunnyvale, CA) before matrix assisted laser desorption ionisation (MALDI)-MS/MS analysis using an Ultimate HPLC pump and gradient programmer (LC Packings). The solvent system was 5% acetonitrile (ACN) (solvent A) and 95% ACN (solvent B); both contained 0.1% TFA. A linear gradient from 5% B to 60% B over 50 minutes was used. Eluates were spotted at 20-second intervals using Probot (LC Packings) on a MALDI target plate. Mass spectra were obtained in reflector mode on a (MALDI-TOF/TOF 4700 mass spectrometer (Applied Biosystems). Ion signals above S/N 25 observed in the MSMS spectra were selected for MSMS ion search against human entries in the NCBI nr database using the Mascot (Matrix Science) search algorithm with no enzyme specification, with the mass tolerance of precursor ions and product ions set at 100 ppm and 0.25 Da, respectively.

Immunoprecipitation and immunoblotting

Cell lysates were prepared with protease inhibitors in PLC buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 100 mM NaF, 1 mM Na_3VO_4 and 1% Triton X-100]. To precipitate the proteins, 1 μg of

monoclonal or affinity purified polyclonal antibody was incubated with 500 μg of cell lysate for 2 hours at 4°C, and then precipitated with protein G agarose for 1 hr at 4°C. Immunoprecipitates were extensively washed with PLC buffer, separated by SDS-PAGE, and immunoblotted. In some experiments, TrueBlot anti-rabbit IgG (eBioscience), which does not react to the denatured rabbit IgG, was used as the secondary antibody of the immunoblotting. For detection of MMP-8, rabbit polyclonal antibody was basically used in this study, however, almost the same results were obtained by using mouse monoclonal antibody.

RT-PCR

Total RNA was prepared from cultured cells by Isogen (Nippon Gene) according to the manufacturer's instructions and treated with DNase I. cDNA was synthesized from 2 μg of total RNA, and polymerase chain reactions (PCR) were performed in a 25 μl reaction volume at an annealing temperature of 55°C. The linear area of the PCR for each reaction was defined; 15 cycles for GAPDH and 25 cycles for MMP-8. Specific primers for MMP-8 and GAPDH have been described previously (Wahlgren et al., 2001; Woo et al., 2003); the expected PCR products were 352 bp and 300 bp, respectively. PCR products were subjected to electrophoresis on 2% agarose gels, and DNA was visualized by ethidium bromide staining.

Metabolic labeling

Cells cultured in 60 mm diameter dishes were preincubated in methionine-free DMEM (Sigma) for 1 hour, then cultured in 1.5 ml of methionine-free medium containing 0.15 mCi of [^{35}S]methionine (Amersham) for a further 4 hours. The cells were rinsed extensively and incubated in medium supplemented with EphB2-Fc or control Fc at 4 μg/ml for 2 hours. MMP-8 in the conditioned medium was purified by immunoprecipitation using an anti-MMP-8 polyclonal antibody and separated on SDS-PAGE. The gel was dried and subjected to autoradiography. The results were visualized with a Bio Imaging Analyzer (BAS1000; Fuji).

In vitro cleavage of ephrin-B1

Purified MMP-1 and MMP-8 were activated prior to use by treatment with 2 mM *p*-aminophenylmercuric acetate for 90 minutes at 37°C and dialyzed against 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 5 mM $CaCl_2$ and 50 μM $ZnCl_2$. Activated enzymes (10 nM, each) were incubated with ephrin-B1-Fc (2 μM) in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 5 mM $CaCl_2$, 50 μM $ZnCl_2$ and 0.05% SDS at 37°C for 1 hour, separated by SDS-PAGE, and immunoblotted with anti-Fc of mouse IgG.

Arf1-GTP pull-down assay

To assess the amount of activated Arf1-GTP in cells, we performed a pull-down assay by using a GST-GGA3 construct (Dell's Angelica et al., 2000). Briefly, cells were left untreated or treated with EphB2-Fc (4 μg/ml) for 20 minutes. Cell lysates were prepared in lysis buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 100 mM NaF, 1 mM Na_3VO_4 and 1% Triton X-100], and then incubated with gluthathione-Sepharose beads containing a GST-GGA3 fusion protein for 45 minutes at 4°C. Precipitates were washed four times in the same buffer, and the precipitated Arf1 was detected by immunoblotting.

In vivo tumor invasion assay

The animal experimental protocols were approved by the Committee for Ethics of Animal Experimentation, and the experiments were conducted in accordance with the guidelines for Animal Experiments of the National Cancer Center. Peritoneal dissemination of tumors was tested by intraperitoneal injection of 1×10^7 PANC-1 cells suspended in 0.3 ml of RPMI1640 medium into 6-week-old BALB/c nude mice (CLEA Japan, Inc.). The mice were sacrificed 8 weeks after injection, and peritoneal dissemination was evaluated. To examine expression of Eph receptors in mesentery-derived cells, the mesenteric sheets were cut along the streak of arteries as described previously (Akedo et al., 1986). The cells were collected from dissected sheets by incubating at 37°C for about 20 minutes in 0.25% trypsin in PBS.

We thank D. B. Alexander (Department of Molecular Toxicology, Nagoya City University) for useful discussions and critical reading of this manuscript. We thank J. S. Bonifacino (National Institute of Child Health and Human Development, NIH) for donating Arf1 plasmids. This work was supported by the Program for the Promotion of Fundamental Studies in Health Science of the Organization for Pharmaceutical Safety and Research of Japan.

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