Proteolysis of EphA2 Converts It from a Tumor Suppressor to an Oncoprotein

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Abstract

Eph receptor tyrosine kinases are considered candidate therapeutic targets in cancer, but they can exert opposing effects on cell growth. In the presence of its ligands, Eph receptor EphA2 suppresses signaling by other growth factor receptors, including ErbB, whereas ligand-independent activation of EphA2 augments ErbB signaling. To deploy EphA2-targeting drugs effectively in tumors, the anti-oncogenic ligand-dependent activation state of EphA2 must be discriminated from its oncogenic ligand-independent state. Because the molecular basis for the latter is little understood, we investigated how the activation state of EphA2 can be switched in tumor tissue. We found that ligand-binding domain of EphA2 is cleaved frequently by the membrane metalloproteinase MT1-MMP, a powerful modulator of the pericellular environment in tumor cells. EphA2 immunostaining revealed a significant loss of the N-terminal portion of EphA2 in areas of tumor tissue that expressed MT1-MMP. Moreover, EphA2 phosphorylation patterns that signify ligand-independent activation were observed specifically in these areas of tumor tissue. Mechanistic experiments revealed that processing of EphA2 by MT1-MMP promoted ErbB signaling, anchorage-independent growth, and cell migration. Conversely, expression of a proteolysis-resistant mutant of EphA2 prevented tumorigenesis and metastasis of human tumor xenografts in mice. Overall, our results showed how the proteolytic state of EphA2 in tumors determines its effector function and influences its status as a candidate biomarker for targeted therapy.

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Introduction

Genes encoding receptor tyrosine kinases (RTK) and related signaling proteins are frequently mutated in human cancer (1), generating aberrantly activated products promoting tumor formation. Therefore, RTK-mediated signaling pathways have been studied extensively to develop effective molecular targeted therapies (2). Among RTKs expressed in cancer cells, erythropoietin-producing hepatocellular receptor-2 (EphA2), a member of the mammalian Eph receptor kinase family, is considered an attractive target due to its frequent overexpression in diverse tumor types (3, 4). Development of various EphA2-targeting therapies, including antibodies, siRNAs, immunotherapy, virus vectors, and chemotherapeutic agents with EphA2 kinase-inhibiting activity have been evaluated clinically (5).

However, current anti-EphA2 therapeutic approaches do not account for known EphA2-related tumor suppressor activities (9, 10). In normal tissues, EphA2 is expressed in epithelial cells. Membrane-bound ephrin A members (A1, A2, A3, A4, and A5) are expressed in neighboring cells. They bind EphA2, eliciting ligand-dependent signals (11), and are also involved in downstream maintenance of epithelial cell morphology and function (Supplementary Fig. S1A; ref. 12). Upon ligand binding, EphA2 is autophosphorylated at cytoplasmic tyrosine residues and then recruits p120Ras-GAP. p120Ras-GAP subsequently downregulates Ras-GTP that has been activated by ErbB-receptor signals (13–15). EphA2 autophosphorylation also suppresses activated Akt and downstream signaling by recruiting phosphatases that dephosphorylate Akt at serine and threonine residues (13). Thus, stimulation of EphA2 by ligands can effectively inhibit Ras/Erk1/2 activation and the PI3K/Akt oncogenic signaling pathways that are activated by stimulation of ErbB- and other growth factor receptors (15, 16).

Furthermore, EphA2 also contributes to tumor development (13). EphA2 is overexpressed in various tumor cell lines, such as fibrosarcoma, breast cancer, and ovarian cancer (Supplementary Fig. S1A), and high EphA2 levels correlate with increased malignancy and poor clinical prognosis (9). Moreover, exogenous EphA2 expression in cancer cells can promote tumor proliferation, motility, angiogenesis, invasion, and metastasis (13). In addition, expression of EphA2 alone in human mammary epithelial-derived MCF10A cells is sufficient to confer tumorigenicity...
in mice (17). High EphA2 expression levels have also been reported to confer resistance to trastuzumab in HER2-positive breast tumor patients (17).

A recent report on ligand-independent EphA2 activation (18) proposed a mechanism for the paradoxical pro-oncogenic activity of EphA2 (Supplementary Fig. S2A). In the absence of ligands, auto-tyrosine phosphorylation of the receptor EphA2 cannot be induced and suppression of Ras and Akt is abrogated. In this study, stimulation of ErbB-receptors efficiently activates the PK3/Akt pathway; activated Akt can phosphorylate EphA2 at the cytoplasmic S597 (19), which then recruits RhoG-GEF, Ephexin-4, and downstream promigratory signaling partners, including RhoG, ELMO-2, DOCK-4, and Rac1 (18). Thus, in the absence of stimulation by Eph ligands, EphA2 provides a positive onco-genic signal (Supplementary Fig. S2A).

It seems likely that the availability of EphA2 ligands would be reduced during tumor progression. However, EphA2 ligands, such as ephrin A1, are expressed abundantly in many tumor types, including breast cancer and melanoma (20, 21). Alternatively, EphA2 receptors become insensitive to ligands during tumor progression and therefore no longer suppress oncogenic signaling pathways. Therefore, we aimed to determine the mechanism by which EphA2 signaling is switched from ligand-dependent to ligand-independent state in cancer cells.

Recently, Sugiyama and colleagues reported processing of EphA2 by MT1-MMP at Y385-I and T395-I and observed increased cell motility (24). We also independently studied EphA2 processing and found that MT1-MMP cleaves additional sites of EphA2 besides Y385-I and T395-I. On the basis of these studies, we hypothesized that EphA2 processing could involve elimination of the N-terminal ligand-binding domain. In this study, we demonstrated that EphA2 cleavage by MT1-MMP indeed suppresses ligand-dependent signaling, but promotes ligand-independent signaling. Expression of an EphA2 version that had been engineered to exhibit resistance to proteolysis strongly prevented tumor cell growth and metastasis of xenografted human tumors.

Materials and Methods

Detailed methods are described in the Supplementary Data.

Cancer cells

Human cancer HT1080 and A431 cell lines were directly obtained from the ATCC. The SCC61 was obtained from W. Yarbrough (Vanderbilt University, Nashville, TN). These cells were passaged fewer than 6 months after purchase or giving for all the experiments. Cells were cultured in DMEM or RPMI-1640 supplemented with 10% FCS, 2 mmol/L l-glutamine, 1% penicillin–streptomycin, and 0.4 μg/mL hydrocortisone, at 37°C and under 5% CO₂ in culture dishes or plates coated with poly-HEMA as an ultra-hydrophilic polymer (Sigma-Aldrich).

Knockdown of MT1-MMP or EphA2 mRNAs in carcinoma cells

For transient knockdown, siRNAs targeting MT1-MMP (#1: 5'-ggauggacagcaggaauuu-3', #2: 5'-ggauggacagguccacuu-3') or EphA2 (#1: 5'-ggauggacagguccacuu-3', #2: 5'-ggauggacagguccagauu-3') were designed and prepared by B-Bridge International, Inc., and transfected into cells using Lipofectamine RNAiMAX (Invitrogen). For stable knockdown of MT1-MMP, shRNA entry and destination vectors were generated according to the manufacturer’s instructions, using a BLOCK-it U6 entry vector and the Gateway system (Invitrogen; see Supplementary Information).

Construction of EphA2 expression vectors

MT1-MMP constructs were prepared as previously described (25). A cDNA sequence corresponding to the open reading frame (ORF) of human EphA2 was amplified by PCR. Expression constructs for C-terminal myc or EphA2 cDNA were purchased from Open Biosystems (Thermo Fisher Scientific). A deletion EphA2-mutant cDNA, lacking amino acids 982–1593 in the ORF of the EphA2 construct, was prepared (see Supplementary Information).

Cell growth, migration, and invasion assays

Cell growth assays were performed using 3-dimensional (3D) collagen gel and anchorage-independent growth conditions. Cell migration and invasion assays were performed using a Transwell chamber (see Supplementary Information).

RhoG-GTPase pull-down assay

A RhoG-GTPase pull-down assay was performed as previously described (see Supplementary Information; ref. 18).

EphA2 phosphorylation by sEphrin-A1-Fc

sEphrin-A1-Fc–induced EphA2 phosphorylation at Y residues was examined in A431 cells after they had been incubated for 2 days in DMEM containing 10% FCS. A431 cells (1 × 10⁶ cells/60-mm dish) were serum-starved for 48 hours and were then treated with sEphrin-A1-Fc for 10 minutes. EphA2 was immunoprecipitated from 500 μg cell lysate using an anti-EphA2 (C-terminal) polyclonal antibody (pAb). Phospho-EphA2 was detected using an anti-phospho-Y mAb (PY-20), and EphA2 was detected using an anti-EphA2 pAb (C-20).

Immunofluorescence microscopy

We obtained written informed consent from all patients and the protocol was approved by the Institutional Review Board of Fukusuka University Hospital (Fukuoka, Japan) for Clinical Study (05-05-3, 16 April 2008). Ovarian adenocarcinoma (OAC) and ovarian adenoma tissue samples were surgically obtained at Fukusuka University Hospital (Fukuoka, Japan), snap-frozen, and embedded in OCT compound, and sections (8-μm thick) were prepared. Frozen tumor and normal tissue arrays containing gastric, ovarian, cervical, colon, and breast carcinoma specimens were purchased from BioChain. Specimens were fixed with 4% (w/v) paraformaldehyde and permeabilized using 0.1% (w/v) Triton X-100 in PBS for 10 minutes. After blocking, sections were immunofluorescently stained with anti-mouse EphA2 N-terminus or anti-MT1-MMP monoclonal antibody (mAb) and anti-EphA2 C-terminus, anti-phospho-EphA2 (EphA2-p-S597), or anti-phospho-EphA2 (EphA2-p-Y394) pAb. Alexa 488– and Alexa 568–conjugated IgG (Molecular Probes) were used as secondary antibodies. Images of cells were captured using an IX-81 (Olympus). Detailed experimental procedures were described previously (26).

Tumor growth and metastasis assays in vivo

Cell tumorigenicity was examined in 6-week-old male BALB/c nude mice. Briefly, 5 × 10⁶ cells of each transfectant type in the
exponential growth phase were suspended in 1 mL growth medium, and 200 μL of the suspension was injected s.c. into the dorsal side of the mice. The implanted tumor volume was measured weekly using a caliper, and the formula \[ V = \frac{(LW^2)}{6} \] was applied, where \( V \), volume (mm\(^3\)); \( L \), biggest diameter (mm); \( W \), smallest diameter (mm), was applied. The mice were euthanized at 4-weeks post-tumor inoculation.

A metastasis assay was performed in 6-week-old male BALB/c nude mice (CLEA). The cells (1 \times 10^6) were injected via the lateral tail veins of the mice (CLEA). Sixty days postinjection, the mice were sacrificed, the lungs were extirpated, and the spherical A431 colonies were counted.

Statistical analysis
Quantitative data are presented as mean ± SD and were analyzed by Student t test, with the exception of the survival curves, which were analyzed using the log-rank nonparametric test. \( P \) values < 0.05 were considered statistically significant.

Antibodies and reagents
Anti-FLAG mouse mAb was purchased from Sigma, and anti-c-myc mAb was from Roche Applied Science; anti-EphA2 (N-terminal) mAb was from R&D Systems; anti-EphA2 (C-terminal) pAb and anti-phospho-Erk1/2 (p-T202/p-Y204) mAb, anti-Erk pAb, anti-EphA2 mAb were from Santa Cruz Biotechnology; anti-Akt mAb and phospho-Akt (p-S473) mAbs were from Cell Signaling Technology; anti-phospho-EphA2 (p-S897) pAb and anti-phospho-EphA2 (p-Y594) pAbs were from Cell Applications; anti-phospho-Y mAb (4G10) and anti-actin mAbs were from Millipore. Horseradish peroxidase-linked antibodies were bought from GE Healthcare. The hydroxamate synthetic matrix metalloproteinase inhibitor, BB94, was provided by Dr. Peter D. Brown (British Biotech Pharmaceuticals Ltd.).

Results
MT1-MMP cleaves the ectodomain of EphA2
Possible mechanism for converting EphA2 into a ligand-insensitive form would be via proteolytic cleavage of the ligand-binding domain of EphA2 by cell surface proteases, as suggested by our recent finding of EphA2 association with MT1-MMP (27). MT1-MMP cleaved off about half of the associated membrane proteins (27), strongly suggesting conversion of EphA2 into a ligand-insensitive form. Moreover, MT1-MMP has potent proinvasive and growth-promoting activities due to proteolytic processing of multiple membrane proteins, including EphA2 involved in cancer progression (27, 28). During the preparation of our manuscript, Sugiyama and colleagues reported that MT1-MMP-processing of EphA2 resulted in increased cell migration (24). Although Sugiyama did not study the effect on receptor function, our results support the idea that MT1-MMP generates ligand-insensitive EphA2.

We previously observed association of MT1-MMP with EphA2 (27); in the current study, we tested whether MT1-MMP cleaves EphA2. C-terminally myc-tagged EphA2 (EphA2-myc) and wild-type MT1-MMP (MT1-WT) were stably expressed in HT1080 cells, and cell lysates were analyzed by Western blotting. Anti-myc mAb detected 130- and 100-ka bands, which correspond to glycosylated (mature) and unglycosylated (immature) forms (Fig. 1A, bottom). Anti-MT1-MMP mAb detected 130- and 100-ka bands, which correspond to glycosylated (mature) and unglycosylated (immature) forms (Fig. 1A, bottom). Anti-MT1-MMP mAb detected 130- and 100-ka bands, which correspond to glycosylated (mature) and unglycosylated (immature) forms (Fig. 1A, bottom). Anti-MT1-MMP mAb detected 130- and 100-ka bands, which correspond to glycosylated (mature) and unglycosylated (immature) forms (Fig. 1A, bottom). Anti-MT1-MMP mAb detected 130- and 100-ka bands, which correspond to glycosylated (mature) and unglycosylated (immature) forms (Fig. 1A, bottom). Anti-MT1-MMP mAb detected 130- and 100-ka bands, which correspond to glycosylated (mature) and unglycosylated (immature) forms (Fig. 1A, bottom).

Figure 1.
MT1-MMP cleaves off the ligand-binding domain of EphA2. A, C-terminally myc-tagged EphA2 (EphA2-myc) was stably expressed in HT1080 cells. MT1-MMP was transiently overexpressed in EphA2-myc-expressing HT1080 cells, treated with or without TIMP-2 or BB94. Arrows indicate endogenous and exogenous MT1-MMP, respectively. Intact and immature EphA2 are indicated by an arrow and asterisk, respectively. Arrows show proteolytic fragments. B, analysis of the processing event by endogenous EphA2 and MT1-MMP. A431 cells were transfected with siRNA against LacZ (control) or MT1-MMP (siMT1-KD). C, cell surface EphA2 and E-cadherin expression in transfected A431 cells. The processed 65- and 60-kDa EphA2 fragments were weakly detected (data not shown) due to lack of 12 of the 17 lysine residues necessary for biotinylation.
increased (Fig. 1A) as compared with cells expressing EphA2-myc alone (Fig. 1A), but were not detected with an anti-EphA2 mAb that recognizes the N-terminal domain (data not shown), indicating that these are processed C-terminal EphA2 fragments. Treatment of cells with the MMP inhibitors TIMP-2 and the synthetic inhibitor BB94 abrogated production of the two fragments (Fig. 1A). MT1-MMP is a member of the MT-MMP sub-group within the MMP protease family and is composed of 6 members (MT1–6-MMPs). We also assessed whether these MT-MMPs could cleave EphA2 by expressing them as a C-myc–tagged form (Supplementary Fig. S1B and S1C). Of these, MT1-MMP cleaved EphA2 most efficiently and is the most frequently expressed in tumors (29, 30), and is presumably the major EphA2-cleaving enzyme in tumors.

Sugiyama and colleagues reported that MT1-MMP cleaves EphA2 at Y385, T395, and T435 in COS1 cells expressing recombinant EphA2 and MT1-MMP. Therefore, to confirm EphA2 cleavage site(s) in HT1080 cells, the 65- and 60-kDa EphA2 fragments purified from HT1080 cells were subjected to N-terminal amino acid sequencing (Supplementary Fig. S1D). This yielded 432-val-S-N-Q and 435-Q-T-E-P-P sequences, respectively (Supplementary Fig. S1E). Unexpectedly, the expected sites identified here were not identical to those of Sugiyama and colleagues. To further assess whether EphA2 is cleaved directly by MT1-MMP at the identified sites, we prepared a synthetic 20-mer polypeptide spanning residues 422–441 of EphA2 and digested this with a recombinant MT1-MMP catalytic fragment. The peptide was cleaved at S-427F and S-432V (Supplementary Fig. S3A and S3B). The latter site was identical to that seen for the 65-kDa fragment and the flanking amino acid sequences corresponded to the reported consensus for MMP cleavage sequences. In contrast, the EphA2 fragment cleaved at S-427F could not be detected in the purified fragment, probably due to the efficient cleavage at the downstream S-432V. MT1-MMP did not cleave the I-335DS site in vitro, although the cleavage of EphA2 at the site is dependent on MT1-MMP in vivo. It is possible that cleavage at S-432V could be followed by further N-terminal processing to 435Asn-Gln-Thr via aminopeptidases. 435N is a potential N-glycosylation site; glycosylated N residues are known to be resistant to aminopeptidases.

Furthermore, MT1-MMP–mediated EphA2 cleavage was also observed in A431 cells expressing high EphA2 and MT1-MMP levels (Fig. 1B). Two endogenous EphA2 fragments (65 and 60 kDa) were detected (Fig. 1B) at low levels in cells in which MT1-MMP was knocked down using three unique 19-mer siRNA duplexes (Fig. 1B). Intact EphA2 levels in the knockdown cells (siMT1-KD) were also increased compared with those in control cells (Fig. 1B and 1C). To measure cell-surface EphA2 levels, cell-surface proteins were labeled with Sulfo-LC-biotin and pulled down using streptavidin-conjugated beads, followed by Western blotting (Fig. 1C). Cell-surface EphA2 levels were increased in the siMT1-KD cells and in control cells treated with the metalloproteinase inhibitor TIMP-2. In contrast, there was no effect on E-cadherin levels, which served as a negative control (Fig. 1B and 1C).

Lack of N-terminal EphA2 correlates with MT1-MMP expression in ovarian tissue

Because MT1-MMP cleaves EphA2 in vitro, we next examined how such processing correlated with MT1-MMP expression in clinical specimens. We observed that OAC, an aggressive form of cancer, expresses high MT1-MMP levels (29) and also expresses EphA2 (6, 31, 32). The OAC tumor grade is described in Supplementary Fig. S5A. First, we examined the localization of N- versus C-terminal portions of EphA2 by using region-specific antibodies. Immunofluorescent staining of normal ovarian tissue exhibited colocalization of both N- and C-terminal portions of EphA2 (Fig. 2A), indicating that EphA2 expressed in this tissue likely retains the N-terminal ligand-binding domain. However, levels of the N-terminal signals of EphA2 were markedly lower than levels of the C-terminal signals in OAC (Fig. 2B), indicating that the N-terminal portion of EphA2 is likely cleaved off in the tumor.

MT1-MMP was then immunostained in ovarian tissues; the majority of MT1-MMP colocalized with the C-terminal portion of EphA2 (Fig. 2C and Supplementary Fig. S1F). In contrast, most of the N-terminal portion of EphA2 was observed specifically within the tissue lacking MT1-MMP (Fig. 2D and Supplementary Fig. S1G). Hematoxylin and eosin (H&E)-stained images are shown in Fig. 2E. These data were consistent with a model in which MT1-MMP cleaves off the EphA2 N-terminal in OAC. MT1-MMP was not detected in normal ovarian and adenoma tissue, as we previously reported (33).

To confirm this processing, we analyzed tumor tissue lysates derived from four aggressive OAC and three ovarian adenoma samples by Western blotting (Fig. 2F and Supplementary S1H). Both EphA2 and MT1-MMP were expressed at high levels in all aggressive OACs. The intact form of EphA2 was detected faintly, mostly in processed forms. The major 60-kDa fragment corresponded to that observed in Fig. 2F and Supplementary Fig. S1H. EphA2 and MT1-MMP expression in ovarian adenomas was very low compared with that in OACs (Fig. 2F and Supplementary Fig. S1H).

MT1-MMP regulates the sensitivity of EphA2-expressing cells to ligands

Because MT1-MMP expression in tumor tissue correlated with the loss of the EphA2 N-terminal, we examined the effect of MT1-MMP on EphA2-mediated signals (Fig. 3), using A431 cells that stably expressed two sets of shRNA from siRNA against either MT1-MMP (shMT1-KD1) or LacZ (control; see Supplementary Information) to avoid possible off-target effects. A431 cells and shMT1-KD1 cells were cultured in the presence of EGF to activate the Ras/MAPK and PI3K/Akt pathways, and analyzed phosphorylated Erk1/2 and Akt levels by Western blotting (Figs. 3A–C).
These cells were further treated with a soluble form of ephrin A1 (Ephrin-A1-Fc) to examine the sensitivity of the cells to EphA2 ligands. Phosphorylated Erk1/2 and Akt levels were not affected in the control cells, but were decreased significantly in shMT1-KD1 cells (Figs. 3A–C). We then analyzed cell-associated Ephrin-A1-Fc, total EphA2 expression, and Erk1/2 activity after treatment of cells with Ephrin-A1-Fc for 10 to 240 minutes. Cell-associated Ephrin-A1-Fc, EphA2, and Erk1/2 activities were clearly increased in shMT1-KD1 cells compared with those in control cells after 10 minutes, although the signal was lost by 240 minutes, due to internalization of active EphA2 (Supplementary Fig. S4).

Thus, MT1-MMP abrogates the ability of the cell to respond to EphA2 ligands, because of cleavage of the ligand-binding domain. Similar results were obtained with shMT1-KD1 and shMT1-KD2 cells derived from head and neck squamous cell carcinoma (HNSCC) SCC61 cells (Fig. 3D–G).

S897 phosphorylated, but not Y594-phosphorylated EphA2, colocalizes with MT1-MMP in OAC.

To examine the role of MT1-MMP in ligand-dependent and ligand-independent EphA2-mediated signaling in vivo, IHC analysis was performed on clinical specimens. The OAC tumor grade is described in Supplementary Fig. S5A. We analyzed the phosphorylation of Y594 and S897 using site-specific phospho-pAbs (EphA2-p-Y594 and EphA2-p-S897) and compared it with the expression pattern of MT1-MMP (Fig. 4A–D and Supplementary Fig. S5A and S5B).

MT1-MMP is usually expressed at high levels at the invasive front of carcinoma tissues (29, 33). Statistically significant EphA2-p-S897 expression was detected in approximately 78% of the area in which MT1-MMP was expressed, while EphA2-p-Y594 signals were almost entirely absent (Fig. 4B and E and Supplementary Fig. S5B). In normal ovarian tissue, neither MT1-MMP nor EphA2-p-S897 was significantly detected in most regions (Fig. 4C and Supplementary S5B). However, signals for EphA2-p-Y594 were detected in the same area, indicating that ligand-dependent autophosphorylation predominates in normal tissue (Fig. 4D and Supplementary S5B).

Furthermore, in other types of tumor tissues, including HNSCC, gastric carcinomas, and colon carcinomas, MT1-MMP colocalized well with EphA2-p-S897, but not with EphA2-p-Y594 (Supplementary Fig. S5C–S5F).

MT1-MMP stimulates RhoG activity through ligand-independent activation of EphA2

Ligand-independent EphA2 signaling induces RhoG activation, resulting in enhanced cancer cell motility and invasion (18). Because MT1-MMP cleaves off the ligand-binding domain of EphA2 in A431 cells, we hypothesized that MT1-MMP regulates RhoG activation in cooperation with EGF stimulation. We pulled down active RhoG (RhoG-GTP) from cell lysates using ELMO-2, an effector of activated RhoG, and analyzed by Western blotting (Fig. 5A). Without EGF stimulation, RhoG-GTP was almost undetectable in both control and shMT1-KD1 cells, whereas EGF stimulation resulted in a marked increase in RhoG-GTP levels in both groups (Fig. 5). We then tested the effect of Ephrin-A1-Fc on RhoG activation; it suppressed the EGF-induced increase of RhoG-GTP specifically in shMT1-KD1 and SCC61 cells, but not in control cells (Fig. 5A–C). Thus, MT1-MMP abolishes the EphA2 response to its ligands and in turn promotes activation of RhoG by EphA2 in a ligand-independent manner upon EGF stimulation. To further analyze the physiologic consequences of MT1-MMP on EphA2-mediated signaling, we analyzed the effect of Ephrin-A1-Fc on A431 cell proliferation. Cells were 3D-cultured in collagen gels in the presence of EGF for 7 days, harvested, and counted (Fig. 5D). With Ephrin-A1-Fc, the growth of shMT1-KD1 cells, but not of control cells, was suppressed. To test the effect of Ephrin-A1-Fc on anchorage-independent growth, we used plates coated with an ultra-hydrophilic polymer, which prevented cell adhesion. With EGF, cells did not undergo apoptosis and formed spheres after a 7-day culture (Fig. 5E). Treatment with Ephrin-A1-Fc did not prevent sphere formation, but reduced its volume significantly (Fig. 5E and 5F).

Because Rho-G-GTP regulates actin reorganization during cell migration, we also analyzed the effect of ephrin A1 on A431 cell motility using Transwell chambers. Addition of Ephrin-A1-Fc decreased mobility of control and shMT1-KD1 cells, but that of shMT1-KD1 cells more so (Fig. 5G and 5H). Therefore, MT1-MMP augments activation of RhoG, cell growth, sphere formation, and cell migration by diminishing the cellular response to Ephrin-A1-Fc.

An uncleavable EphA2 mutant acts as a suppressor of tumor growth and metastasis of A431 cells in vivo

We hypothesized that, if MT1-MMP–mediated EphA2 processing is a major regulator of ligand-independent signaling and tumor cell proliferation and motility, blocking this processing should suppress in vivo tumor aggressiveness. Thus, we generated a mutant EphA2 that cannot be cleaved by MT1-MMP, due to a small deletion in the protease-sensitive stem region, but that retains the ligand-binding domain (ucEphA2-CF; Fig. 6A). A431 cells stably expressing the empty vector (Mock), the wild-type EphA2 (EphA2-CF), or uCEphA2-CF were prepared (Fig. 6B). Two cleaved EphA2 fragments derived from the endogenous EphA2 were detected faintly in all cells by an anti-EphA2 pAb recognizing the C terminus (Fig. 6B, #1 and #2). In contrast, anti-FLAG mAb detected only FLAG-tagged EphA2 and their processed fragments. No processed uCEphA2-CF was detected (Fig. 6B, #3). Recombinant EphA2-CF and uCEphA2-CF proteins were observed immunohistochemically in A431 cell membranes with an anti-EphA2 (C-ter) pAb and an anti-FLAG mAb (Supplementary Fig. S6A) at

Figure 3.
Effect of MT1-MMP on the ligand-dependent and ligand-independent activation of EphA2. A, effect of MT1-MMP on ephrin A1–mediated suppression of Erk1/2 and Akt phosphorylation. A431 cells stably expressing shRNA against LacZ (Control) or against MT1-MMP (shMT1-KD1) were cultured in the presence of EGF. B and C, phospho-Erk (B) and phospho-Akt (C) signal intensities were measured by densitometry and analyzed using ImageJ software. The phosphorylated to total protein ratio is shown (error bars, mean ± SD; n = 5). D, MT1-MMP detection in SCC61 cells expressing control shRNA (Control) or either of two different MT1-MMP shRNA sequences (shMT1-KD1 and 2). E, effect of ephrin A1 on Erk1/2 and Akt signaling in SCC61 cells in the presence of EGF. F, phospho-Erk1/2 bands were analyzed with ImageJ. The active to total protein ratio is indicated (error bars, mean ± SD; n = 3). G, intensities of bands for phospho-Akt were measured using ImageJ software.
Figure 4.
MT1-MMP colocalizes with EphA2-p-S897, but not EphA2-p-Y594, in ovarian adenocarcinoma. A, representative OAC (ID. #7) section immunologically stained with anti-MT1-MMP mAb (red) and anti-EphA2-p-S897 pAb (green). Bar, 50 μm. B, the OAC (ID. #7) section was analyzed similarly for MT1-MMP (red) and EphA2-p-Y594 (green). C and D, similar immunofluorescent staining was conducted in normal ovarian tissue. E, quantitative analysis of EphA2-p-S897 or EphA2-p-Y594 in MT1-MMP-positive or MT1-MMP-negative OAC tissues. Phosphorylation of EphA2 was analyzed using imaging software “Zen.” At least three different MT1-MMP-positive or MT1-MMP-negative areas in OAC were calculated. (Error bars, mean ± SE; n = 7).
Figure 5.
Effect of MT1-MMP on EphA2 mediated RhoG activation and cell regulation. A, effect of MT1-MMP on RhoG activation mediated by ligand-independent activation of EphA2. Cells were additionally treated with Ephrin-A1-Fc. B, intensities of the pulled-down RhoG were measured by densitometry and analyzed with ImageJ software. The ratio of the measured value to that of the control cells, not treated with Ephrin-A1-Fc, is presented (error bars, mean ± SD; n = 5). C, activation of RhoG in SCC61. SCC61 cells were analyzed as presented in A. Two independent knockdown cell lines (shMT1-KD1 and shMT1-KD2) were used. D, effect of MT1-MMP and ephrin A1 on cell growth after incubation for 7 days in collagen gel. After the cells were recovered from the gel, the cell number was counted (error bars, mean ± SD; n = 4). E, growth and survival of the same set of A431 cells were tested in non-adherent culture conditions for 7-day incubation in the presence of EGF. Cells that survived in culture formed spheroids. Bar, 100 μm. F, number of cells and average volume of the spheroids were measured (error bars, mean ± SD; n = 4). G, effect of MT1-MMP and ephrin A1 on cell migration. Bar, 50 μm. H, the number of cells that migrated through the filter was counted (error bars, mean ± SD; n = 4).
equivalent levels in 10% FCS-containing growth medium (Fig. 6B). However, it should be noted that endogenous EphA2 expression levels were significantly higher in ucEphA2-expressing cells than in serum-starved mock cells (Fig. 6C), due to an unknown mechanism. Treatment of ucEphA2-CF–expressing cells with Ephrin-A1-Fc induced EphA2 autophosphorylation at Y594.

Figure 6.
Suppression of orthotropic carcinoma cell growth and lung metastasis by an uncleavable EphA2 mutant. A, schematic representation of C-terminally FLAG-tagged wild-type EphA2 (EphA2-CF) and an uncleavable EphA2 mutant (ucEphA2-CF). nt, nucleotide number; SP, signal peptide; EBD, ephrin-binding; Stem, fibronectin type III repeat; TM, transmembrane domain; Cytoplasmic, cytoplasmic domain; F, FLAG peptide. B, Western blotting of EphA2 fragments (65- and 60-kDa) using anti-EphA2 C-ter pAb (#1, short exposure; #2, long exposure) and anti-FLAG mAb (#3). Total cell lysate (TCL) was collected from the indicated transfectant cells cultured in 10% FCS-containing medium. C, EphA2 p-Y detection in A431-transfected cells (Mock, EphA2-CF, and ucEphA2-CF) under serum-starved conditions. D, A431 cells (5 × 10^6 cells) transfected with mock (red), EphA2-CF– (blue), or ucEphA2-CF-expression plasmids were inoculated into nude mice s.c. E, tumor size was monitored for 28 days and expressed as the mean ± SE (n = 10). F, mice were sacrificed after 28 days and tumor volume was measured. G, transfectant cells (1 × 10^6 cells) were injected into the tail veins of 7-week-old mice and lung metastasis was analyzed after 60 days. Macroscopic observation of murine lungs. H, number of tumor nodules observed on the surface of the lung was counted using a stereoscopic microscope (error bars, mean ± SD; n = 5).
indicating that ucEphA2-CF retains the ability to bind ligands and activate the intracellular signaling pathway (Fig. 6C, middle). Consistent with enhanced ligand-dependent signaling, ucEphA2-CF–expressing A431 cells exhibited a more classic epithelial morphology in culture than the control (Supplementary Fig. S6B).

To evaluate the effect of the uncleavable mutant on orthotopic s.c. injection, mock, ucEphA2-CF, or EphA2-CF1 cells (5 \times 10^6 cells) were implanted s.c. in nude mice (n = 10) and the tumor size and weight monitored. Mock and EphA2-CF1 cells formed larger tumors than did ucEphA2-CF cells (Fig. 6D).

Significant suppression of tumor growth (44%–49% volume and 55%–68% weight inhibition at 28 days postinjection) was observed with ucEphA2-CF1 cells (Fig. 6D–F). To further analyze the effect of the uncleavable mutant on metastasis, these transfectant cells (1 \times 10^6 cells) were injected into the tail vein of nude mice (n = 5) and metastatic colonies in the lungs were analyzed after 60 days by macroscopic observation (Fig. 6G) and counting the number of nodules (Fig. 6H). ucEphA2-CF1 expression markedly suppressed lung metastasis compared to the mock or wild-type EphA2 cells (67%–76% metastasis inhibition at 60 days postinjection). Taken together, our results indicated that MT1-MMP promotes aggressive tumor cell behavior through EphA2 processing.

Discussion

EphA2 is a key factor in the suppression or enhancement of ErbB-receptor-mediated signals, whose opposing actions are switched by the availability of EphA2 ligands (Supplementary Fig. S2A and S2B). A lack of EphA2 ligands in tissues is a possible mechanism for shifting the behavior of EphA2 from that of a suppressor of the ErbB-receptor to that of a tumor promoter. Here, we demonstrated a new mechanism that can activate EphA2 ligand independently. We found that (i) MT1-MMP converts EphA2 into a ligand-insensitive form by proteolytic removal of the ligand-binding domain; (ii) the phosphorylation pattern of EphA2 representing ligand-independent signaling was specific to the tumor region; (iii) MT1-MMP–mediated EphA2 processing specifically induced activation of ligand-independent EphA2 signaling in cancer cells; and (iv) conversely, expression of an MT1-MMP–uncleavable mutant EphA2 rescues EphA2 ligand-dependent signaling. Therefore, MT1-MMP–mediated EphA2 proteolysis is not necessarily an immediate trigger for receptor degradation, and the truncated receptor presumably has sufficient time to act as a ligand-free receptor at the cell surface. We propose a model for conversion of EphA2 to a ligand-insensitive form during tumor development (Fig. 7).

EphA2 is considered a promising molecular target for cancer therapy and multiple approaches to developing therapeutics for this target have been attempted (5). Thus far, a siRNA-based approach (6) and a multitargeted tyrosine kinase inhibitor, dasatinib, which can inhibit EphA2 kinase activity, have been evaluated clinically (7, 8). However, these approaches target either all EphA2 activities (siRNA) or only the antioncogenic tyrosine kinase activity of EphA2 (dasatinib).

Dasatinib is an orally active inhibitor developed to target multiple kinases from the Bcr-Abl and Src families (34, 35), and kinase activity of EphA2 (dasatinib).

On the basis of our results, we propose that MT1-MMP–cleaved EphA2 is the oncogenic form present in tumors, whereas intact EphA2 is an antioncogenic form that is extant when ligands are present (Fig. 7). Indeed, forced expression of an uncleavable EphA2 mutant in A431 cells prevented MT1-MMP–mediated EphA2 processing (Fig. 6A), promoting potent tumor suppressor activity, induced by ligand-dependent EphA2 phosphorylation, which caused a decrease in tumor growth and metastatic activities. Concurrently, it promoted morphologic conversion of cells to
a normal epithelial cell appearance (Supplementary Fig. S6B). These data were consistent with the presence of abundant EphA2 ligands in the tumor microenvironment (22, 41) and indicated that suppression of MT1-MMP-mediated EphA2 cleavage facilitates reversion of invasive cells to a noninvasive epithelial state.

EphA2 may have multiple protease-sensitive sites downstream of the ligand-binding domain. Although we could not detect the Sugiyama's sites in our study, there is a possibility that MT1-MMP cleaved these sites as well. Indeed, we detected two major EphA2 N-terminal fragments (30 and 35 kDa) in the culture medium of A431 cells, and the cleavage was sensitive to an MMP inhibitor (Supplementary Fig. S7A), suggesting that EphA2 is cleaved at two major sites. Sugiyama's cleavage sites are expected to generate the 30-kDa fragment, whereas the 35-kDa fragment is likely derived from the sites identified in our study (Supplementary Fig. S7B). Sensitivity of each cleavage site is affected by modification of EphA2 and the surrounding cell surface conditions. We think, in our assay conditions, EphA2 is cleaved at Sugiyama's sites and subsequently at our sites. Both Sugiyama's sites and our sites are located downstream of the ligand-binding domain and the ucEphA2 mutant that was used in our study lacks all these sites.

In summary, we demonstrated that proteolytic regulation of EphA2 by MT1-MMP is a likely mechanism underlying conversion of the tumor suppressor EphA2 into an oncogenic signal transducer (Fig. 7). The cleaved EphA2 fragment transduces ligand-independent EphA2 oncogenic signals and may be a better target for cancer therapy than intact EphA2. Our findings conclude that the paradoxical actions of EphA2 in tumor progression provide crucial information for reconsidering current strategies for anti-EphA2 tumor therapy development that target oncogenic form of EphA2 and tumor-suppressor form.

Disclosure of Potential Conflicts of Interest
N. Koshikawa reports receiving commercial research grant from DAIICHI SANKYO COMPANY. H. Taniguchi is project associate professor in Donations. No potential conflicts of interest were disclosed by the other authors.

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