The reversion-inducing cysteine-rich protein with Kazal motifs (RECK) interacts with membrane type 1 matrix metalloproteinase and CD13/aminopeptidase N and modulates their endocytic pathways

The reversion-inducing cysteine-rich protein with Kazal motifs (RECK) is anchored to the cell surface via glycosylphosphatidylinositol. This molecule antagonizes the function of membrane type 1 matrix metalloproteinase (MT1-MMP) to promote proMMP-2 maturation. Here, we attempt to clarify the mechanism underlying RECK functions. First, we found that RECK forms a complex with MT1-MMP and inhibits its proteolytic activity. Notably, RECK increases the amount of MT1-MMP that associates with detergent-resistant membranes during sucrose gradient ultracentrifugation. Furthermore, perturbation of membrane cholesterol significantly affected the function of RECK in suppressing MT1-MMP function. These findings indicate that RECK possibly regulates MT1-MMP function by modulating its behavior on the cell surface as well as by enzymatic action; this prompted us to find another molecule whose behavior in detergent-resistant membranes is influenced by RECK. Subsequently, we found that RECK interacts with CD13/aminopeptidase N. Further, we found that RECK inhibits the proteolytic activity of CD13 in a cholesterol perturbation-sensitive manner. Finally, we examined whether RECK influences the behavior of MT1-MMP and CD13 during their internalization from the cell surface. In the absence of RECK, MT1-MMP and CD13 were internalized along with the markers of clathrin- or caveolea-dependent endocytosis. However, interestingly, in the presence of RECK these molecules were internalized preferentially with an endocytosis that is neither clathrin- nor caveolea-dependent, indicating that RECK modulates endocytic pathways of MT1-MMP and CD13. This modulation was correlated with the accelerated internalization and decay of MT1-MMP and CD13. This study unravels the novel function and target molecules of RECK.

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Modulation of Endocytic Pathway by RECK

roglobulin, which is a major plasma inhibitor of metalloproteases (8). As compared with soluble MMP inhibitors represented by tissue inhibitor of metalloproteinases (TIMPs) (8), the most distinguishing feature of RECK is its ability to covalently anchor to the membrane surface via a post-translational modification (glycosylphosphatidylinositol (GPI) anchor) that is conserved among species (1). In contrast to RECK-deficient mice, deletion of any TIMP genes had little impact on embryonic development (3, 8). In this work, we hypothesize that the membrane anchoring of RECK assigns unknown active roles to this molecule in addition to rendering it highly accessible to membrane-bound MMPs. In consonance, a soluble mutant of RECK that lacks the hydrophobic domain at the carboxyl-terminal failed to attenuate the proteolytic activity of MT1-MMP when introduced into HT1080 cells, suggesting that the membrane anchoring is required for RECK to exert its function to antagonize MT1-MMP.

MT1-MMP directly degrades various components of the extracellular matrix (9). CD44, ov-integrin, and syndecan-1 are also substrates of MT1-MMP; the shedding of these molecules significantly affects cell motility through various mechanisms (9). Furthermore, MT1-MMP directly processes proMMP-2 and proMMP-13 and brings about their activation (9, 10). TIMP-2 has been identified as its soluble inhibitor (8). Further, TIMP-2 facilitates the activation of proMMP-2 by MT1-MMP via ternary complex formation (11). Because RECK overexpression significantly suppressed MT1-MMP-dependent activation of proMMP-2 in cultured cells (3), RECK has been proposed to be an inhibitor of MT1-MMP; however, no direct evidence and mechanism have been provided.

Because of the membrane-tethering ability of MT1-MMP, it undergoes a unique mode of post-translational regulation that is initiated by internalization. Recent studies indicate that MT1-MMP internalization is controlled by a clathrin- or caveolae-dependent endocytic pathway or by a combination of these two pathways (12–17). These mechanisms contribute to the selective internalization of MT1-MMP from a specific compartment of the cell membrane and its sorting to endosomes for subsequent degradation or recycling (9, 10). This machinery appeared to be implicated in the control of proMMP-2 maturation and cell motility (14, 15). β1 integrin is co-localized with MT1-MMP on the surface of human endothelial cells. This interaction appeared to modulate the mechanism of MT1-MMP internalization when the cells were exposed to β1 integrin-dependent extracellular matrices (18). These findings indicate that cell surface molecules can participate in regulating MT1-MMP internalization and thereby influence cell behavior.

CD13/aminopeptidase N (APN) is another membrane protease whose gene transcription is up-regulated by the ras oncogene product (19). This molecule functions as a metal-dependent ectopeptidase that is involved in processing angiotensins, met-enkephalin, neurokinin A, somatostatin, monocyte chemotactic protein (MCP)-1, and macrophage inhibitory protein (MIP)-1, and it also acts as a receptor for viruses, including coronavirus 229E that, in turn, mediates infection by human cytomegalovirus (20, 21). Hence, this molecule is expected to play pivotal roles in tumor progression, angiogenesis, the cardiovascular system, immune systems, and in viral infection. The treatment of animals or cells using synthetic inhibitors of CD13 (e.g. bestatin, amastatin, and actinonin) or the anti-CD13 antibody resulted in abnormalities in various biological systems, i.e. cell proliferation and survival, blood pressure, cytokine levels, angiogenesis, and vasculogenesis (20–22). Although the mechanism is unclear, the anti-CD13 antibody suppresses type IV collagen degradation by tumor cells (HT1080 cells) and thereby inhibits their invasion (23). Unlike MT1-MMP, no endogenous inhibitor of CD13 has been discovered thus far.

Here, we report that RECK forms complex with MT1-MMP and CD13, competitively inhibits their proteolytic activities, and influences their behavior on the cell surface. In addition, this study demonstrates that RECK is internalized most likely via a recently identified novel endocytic pathway that involves the GPI-anchored protein enriched early endosomal compartments (GEECs) (24). Previous reports indicated that the internalization of MT1-MMP is clathrin- and/or caveolae-dependent (13, 16) and that of CD13 is caveolae-dependent (25). This study demonstrates that endocytic pathways for these molecules can be changed to one that is preferred by RECK.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HT1080 cells were transfected with the mammalian expression vector pCXN2neo, pCXN2neo containing human RECK (hRECK) cDNA (1), pLXSb, or pLXSb containing FLAG- or Myc-tagged hRECK cDNA (see below); they were selected for 10 days in the presence of 1.0 mg/ml G418 or 8 µg/ml blasticidin. The selected cells were immediately pooled or used for the following experiments within several passages. RECK−/− mouse embryonic fibroblasts (MEFs) were described previously (3). MT1-MMP−/− MEFs were prepared from MT1-MMP−/− mice (a gift from M. Seiki) at embryonic day 13.0, infected with retrovirus generated by transfecting EcoPak293 cells (Clontech) with pLXSb-MT1-MMP, and selected with 8 µg/ml blasticidin (26). pSG5-FLAG-MT1-MMP was presented by M. Seiki (15). Human umbilical vein endothelial cells (HUVECs) were obtained from CAMBREX (CC2517), maintained according to the provider’s protocol, transfected with pSG5-FLAG-MT1-MMP using FuGENE 6 (11814443001; Roche Applied Sciences), and sorted by using FACS Aria (BD Biosciences).

Construction of Plasmids—To generate Myc- or FLAG-tagged hRECK, a mutation (sense primer, 5′-CCCGCAGTGCCGGAGCCGGCCCTC′-3′) was induced in pLXSb-hRECK (27) to generate two novel restriction enzyme sites (Apal and BspEI) that facilitate the insertion of a DNA cassette encoding a FLAG (Pro-Thr-Met-Asp-Tyr-Lys-Asp-Asp-Lys) or a Myc (Glu-Gln-Lys-Ile-Ser-Glu-Glu-Asp-Leu-Leu-Ala) epitope between Gly77 and Asp29 in hRECK. The amino-terminal signal peptide of hRECK ends at Gly27 (1).

Immunoprecipitation and Immunoblotting—The cells were lysed in a solution containing 25 mM Hepes, pH 7.5, 0.15 M NaCl, 1% Nonidet P-40, 0.25% deoxycholate, 10% glycerol, 10 mM MgCl2, and 1 mM EDTA in the presence of protease inhib-
itor mixture (03969–21; Nacalai tesque). FLAG-tagged RECK was immunoprecipitated with rabbit anti-FLAG polyclonal antibody (F7425; Sigma), MT1-MMP with mouse anti-MT1-MMP antibody (1D8; a gift from M. Seiki) (28), and CD13 with rabbit anti-CD13 antibody (a gift from S. Roffler) (29). The immunoprecipitates were collected on protein G-agarose beads (45210; Pierce), washed five times with lysis buffer, and eluted. The cell lysates or immunoprecipitates were separated by SDS-PAGE and used for immunoblotting as described previously (1).

Production and Purification of Recombinant RECK—293F (Invitrogen) cells were transfected with pCXN2-hRECK His<sub>6</sub> and the culture supernatants were recovered as the protein source. Purification of the product was performed by using a Hi-trap chelating column (GE Healthcare) and FPLC (Bio-Rad). The purity of this material was >97%.

Kinetics of MT1-MMP Inhibition—To study the kinetics of MT1-MMP inhibition, 58 ng of recombinant soluble catalytic domain of MT1-MMP (S.E.-259; BIOMOL) was mixed with various concentrations of a labeled synthetic peptide Mca-Lys-Pro-Leu-Gly-Leu-dpa-Ala-Arg-NH<sub>2</sub> (0.75–6.75 nM; ES010; R&D Systems) and recombinant soluble RECK protein (0–13.3 nM) in 200 ml of assay buffer containing 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM CaCl<sub>2</sub>, 10 mM ZnCl<sub>2</sub>, 0.1% Brij-35, and 0.1% polyethylene glycol (PEG) 6000. The output signal (excitation wavelength, 325 nm; emission wavelength, 393 nm) was recorded for 2 min at 37 °C by using SPECTRAMax (Molecular Devices). The dissociation constant <i>K<sub>i</sub></i> was calculated as described previously (1).

Sucrose Gradient Centrifugation—Protein fractionation by sucrose gradient centrifugation was performed as described previously (30). The anti-clathrin (610499; BD Biosciences) and anti-caveolin-1 (610059; BD Biosciences) antibodies were used. The distribution of the proteins of interest to detergent-resistant membrane (DRM) was estimated by quantifying the intensity of the bands obtained after immunoblotting by using NIH image (ver. 1.61). In each transfectant, MBCD (C4767; Sigma)-treated cells were set to zero.

Gelatin Zymography—2 × 10<sup>4</sup> cells were plated on a 12-well type dish and incubated for 48 h before starting preparation of culture supernatants that were prepared by incubating the cells with Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum for 2.5 h and subsequently with 0.1% fetal calf serum for another 24 h. The culture supernatants from the final 24-h incubation were analyzed as described previously (1). The cell number after the final 24-h incubation was estimated by using the cell number-counting reagent SF (07553; Nacalai tesque) that was used for adjusting loading volume.

Mass Spectrometry—Mass spectrometric identification of proteins was performed by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as described previously (31).

Aminopeptidase Assay and Kinetics—Transfected cells (1 × 10<sup>4</sup> cells) were grown in a 96-well flat-bottomed microtiter plate for 24 h and successively treated with or without bestatin (B8385; Sigma) at 37 °C. After 6 h of incubation, alanine-4-methylcoumaryl-7-amide (Peptide Institute) (32) was added to the wells as the substrate at a final concentration of 0.2 mM. The cells were incubated for another 10 min, and the plate was then chilled at 0 °C for 10 min. The supernatant was collected, cleared by centrifugation, and subjected to fluorometric analysis of 7-amino-4-methylcoumarin (excitation wavelength, 360 nm; emission wavelength, 440 nm) (32). The nonspecific (basal) fluorescence level was determined by analyzing the sample in the presence of a saturating amount (5 μg/ml) of anti-CD13 blocking antibody (WM15; BD Biosciences), and this value was set to zero. Embryonic cells were prepared from E10.0 RECK null embryos or wild type littermates as described previously (3). Embryonic cells (1 × 10<sup>4</sup>) were incubated in a 96-well flat-bottomed microtiter plate for 24 h, and the CD13 peptidase activity was then measured. For the kinetics assay, 6 ng of recombinant soluble CD13/APN (2335-ZN; R&D Systems) was mixed with various concentrations of alanine-4-methylcoumaryl-7-amide (0.025–0.15 mM) and recombinant soluble RECK protein (0–12.5 nm) in 200 μl of assay buffer (10 mM Tris at pH 7.5, 150 mM NaCl, and 0.05% Triton X-100). The output signal (440 nm) was recorded for 15 min at 37 °C by using SPECTRAMax (Molecular Devices). The dissociation constant <i>K<sub>i</sub></i> was calculated as described previously (1).

ImmunoFluorescence Analysis—The cells that had been cotransfected with pSG5-FLAG-MT1-MMP and pLXSB-Myc-hRECK were grown on chronic acid-cleaned cover glasses, incubated for 30 min at 4 °C with Alexa Fluor 488 (Zenon kit; Molecular Probes)-labeled anti-FLAG (M2; Sigma), Alexa Fluor 555-labeled anti-Myc (R950-25; Invitrogen), or Alexa Fluor 647-labeled anti-CD13 (WM15; BD Biosciences) antibodies dissolved in growth medium and then washed extensively with ice-cold phosphate-buffered saline (PBS) to remove any unbound antibody. By replacing the medium with warm (37 °C) growth medium, the cells were allowed to resume internalization of the cell surface proteins coupled to the antibodies for 2 min. The cells were quickly placed at 4 °C to terminate the internalization process, fixed, and analyzed using a laser confocal fluorescence microscope (FV300; Olympus). For caveolin-1 staining, cells that had been transfected with pLXSB-FLAG-RECK were incubated for 30 min at 4 °C with Alexa Fluor 488 (Zenon kit; Molecular Probes)-labeled anti-FLAG antibody, washed, and incubated for 2 min at 37 °C in growth medium (Dulbecco’s modified Eagle’s medium with 10% fetal calf serum). The uninternalized fluorogenic antibody was removed using PBS and acidic wash buffer (50 mM glycine and 150 mM NaCl, pH 2.0). The cells were fixed at −20 °C with pre-chilled methanol and acetone for 5 min each and blocked with 2 mg/ml bovine serum albumin in PBS prior to incubation with anti-caveolin-1 (610059; BD Biosciences) and the secondary (anti-rabbit Cy5; Chemicon) antibody.

Specificity of Antibodies—The specificity of antibodies to Myc and FLAG was examined by incubating them with mock-transfected cells; there were barely detectable background signals. The specificity of the mouse monoclonal antibody to CD13 (WM15; BD Biosciences) was examined by comparing its staining pattern on cells to that obtained by using independently manufactured rabbit polyclonal antibody to CD13 (a gift from S. Roffler) (29); two patterns were almost indistinguishable. In addition, both antibodies specifically detected exogenously introduced CD13 in MDA-MB231 cells that were
known to have very low endogenous CD13 (33). The specificity of the antibody to caveolin-1 was examined by staining cells transfected with caveolin-1 siRNA as demonstrated in Fig. 5B.

RNA Interference—The siRNAs for RECK, dynamin-2, and caveolin-1 were obtained from Ambion (29149 for siRNA1; 29235 for siRNA2), Invitrogen (32451864), and Qiagen (SI00299642), respectively. For transfection, HT1080 cells (4 × 10⁴ cells) or HUVECs (5 × 10⁴) were seeded onto 6-well plates and transfected with 100 μM siRNA using Lipofectamine 2000 (Invitrogen) according to the instructions provided by the manufacturer.

Transferrin and Dextran Uptake—HT1080 cells that were cotransfected with pSG5-FLAG-MT1-MMP (15) and pLXSBS-Myc-hRECK were selected with 8 μg/ml blastcidin and incubated with the anti-FLAG (M2; Sigma) and anti-Myc (R950-25; Invitrogen) antibodies; they were then sorted and enriched using FACS Aria (BD Biosciences). The resultant cells were incubated with Alexa Fluor 488-conjugated transferrin (10 μg/ml) (Molecular Probes), TRITC-conjugated anti-FLAG antibody (10 μg/ml) (Molecular Probes), or Alexa Fluor 555-labeled anti-CD13 antibody (Zenon kit Z25005; Molecular Probes) for 30 min in growth medium on ice, washed, and incubated at 37 °C for 2 min in growth medium containing Alexa 647-conjugated dextran (D22914; Molecular Probes). The uninternalized fluorogenic reagents were removed using PBS and acidic wash buffer (50 mM glycine and 150 mM NaCl, pH 2.0). The cells were fixed with 4% paraformaldehyde and analyzed using a laser confocal fluorescence microscope (FV300; Olympus).

Biotinylation and Internalization of Cell Surface Protein—The cells were washed twice and incubated with ice-cold PBS, pH 8.0. The cell surface proteins were labeled by incubating the cells with 0.3 mg/ml of cell-impermeable NHS-SS-biotin (Pierce) (13). After incubation for 30 min, the free NHS-SS-biotin was quenched and washed out using 50 mM glycine and acidic wash buffer (50 mM glycine and 150 mM NaCl, pH 2.0). The cells were fixed with 4% paraformaldehyde and analyzed using a laser confocal fluorescence microscope (FV300; Olympus).

RESULTS

RECK Physically Interacts with MT1-MMP and Competitively Inhibits Its Proteolytic Activity—To understand the mechanism underlying the suppression of MT1-MMP activity by RECK, we first attempted to determine whether RECK physically interacts with MT1-MMP. FLAG-tagged RECK was first introduced into HT1080 (a human fibrosarcoma line) cells that express undetectable level of endogenous RECK (1), and it was then immunologically precipitated. The precipitate contained an active form (60 kDa) and a degraded form (44 kDa) of MT1-MMP (Fig. 1A). This finding suggests that RECK forms a complex with MT1-MMP while MT1-MMP is in an activated and a degraded form.

In parallel, we found that the introduction of RECK into HT1080 cells significantly attenuated the autocatalytic degradation of MT1-MMP (Fig. 1A). This finding motivated us to examine whether RECK directly suppresses the proteolytic activity of MT1-MMP. By using 293F cells, we synthesized a His-tagged soluble form of RECK lacking its carboxyl-terminal end that is required for membrane anchoring. Subsequently, the product was purified up to 97% purity by fast protein liquid chromatography. This product inhibited the proteolysis of a labeled synthetic peptide substrate (Mca-Lys-Pro-Leu-Gly-Leu-dpa-Ala-Arg-NH₂) by the recombinant soluble catalytic
done in shown. Quantification of MT1-MMP association with DRMs was performed as indicated genotype. A representative of three independent experiments is shown. From the results, the ratio of MT1-MMP population in fractions 4 and 5 to all the fractions was estimated by densitometry (right). The columns represent the mean ± S.E. *, p = 0.018; **, p = 0.006 by Student’s t test. C, the association of MT1-MMP with DRMs was compared among MEFs of the indicated genotype. A representative of three independent experiments is shown. Quantification of MT1-MMP association with DRMs was performed as done in panel B (right). *, p = 0.017. D, detection of the indicated proteins in MT1-MMP-/- MEFs transfected with pLXSB (vector) or pLXSB-MT1-MMP (MT1-MMP) (upper). The association of RECK with DRMs in these cells was assessed (lower). A representative of four independent experiments is shown. Quantification of RECK association with DRMs was performed as done in the above experiments (right). E, zymographic analysis of the culture supernatants derived from HT1080 cells transfected with pCXN2neo (−) or MT1-MMP had no effect on the association of RECK with this fraction (Fig. 2D), leading us to conclude that RECK conducts the association of MT1-MMP with DRMs.

The Antagonistic Function of RECK on MT1-MMP Is Sensitive to Cholesterol Perturbation—Next, we addressed whether MBCD treatment modulates the influence of RECK on MT1-MMP function. We observed that MBCD antagonized the function of RECK in inhibiting proMMP-2 maturation (Fig. 2E). The concentration of MBCD that we used in our experiment did not provide any evidence of lowered cell viability (data not shown). These findings suggest that the ability of RECK in functionally suppressing MT1-MMP is sensitive to cholesterol perturbation in the lipid bilayer.

RECK Promotes the Metallloprotease-dependent Processing of CD13 in DRMs—RECK increased the degree of the association of one of its targets, MT1-MMP, with DRMs. This feature might be instrumental for screening other proteins that interact with RECK. We have sought proteins that exhibited altered

domain of MT1-MMP in a competitive manner with the dissociation constant (K) < 19.3 nM (Fig. 1B). These findings support the previously proposed hypothesis that RECK is the endogenous inhibitor of MT1-MMP (3).

RECK Promotes the Association of MT1-MMP with DRMs—RECK is linked to the cell surface by a GPI anchor (1), whereas MT1-MMP is linked by a transmembrane domain. Given that they interact with each other, does this interaction influence their behavior on the cell membrane? To determine this, we generated HT1080 cells that express exogenous RECK, lysed the cells in 1% Triton X, and fractionated them on a sucrose density gradient in order to discriminate the proteins in DRMs from those in detergent-soluble membranes. Consistent with its possession of GPI moiety, in HT1080 cells the majority of RECK was associated with DRMs (Fig. 2A, fraction 4). In the same fraction, caveolin-1 was enriched, but clathrin was not. Cholesterol perturbation that was caused by the treatment of the cells with methyl-β-cyclodextrin (MBCD) at a non-cytotoxic concentration almost completely disrupted the association of RECK and caveolin-1 with DRMs (Fig. 2A).

The observation of a physical interaction between RECK and MT1-MMP prompted us to investigate whether the presence of RECK influenced the behavior of MT1-MMP in the same assay. In the absence of RECK, an average of 12% MT1-MMP was observed to be associated with DRMs. Interestingly, in the presence of RECK, the population of MT1-MMP that was observed to be associated with DRMs increased to an average of 36% (Fig. 2B). Cholesterol perturbation almost completely abolished the function of RECK in enhancing the association of MT1-MMP with DRMs (Fig. 2B, bottom). The comparison of MEFs that were derived from RECK+/+ and RECK−/− mice indicated that endogenous RECK significantly contributes to the association of MT1-MMP with DRMs (Fig. 2C). This justifies the use of HT1080 cells in our experiments, because the introduction of exogenous RECK in these cells exhibited an activity similar to that of endogenous RECK in MEFs. By contrast, the lack of MT1-MMP had no effect on the association of RECK with this fraction (Fig. 2D), leading us to conclude that RECK conducts the association of MT1-MMP with DRMs.

RECK Promotes the Association of MT1-MMP with DRMs—RECK was associated with DRMs (Fig. 2D) and treated with (+) or without (−) 5 mM MBCD. The molecular species corresponding to each band is indicated. A representative of four independent experiments is shown.
Modulation of Endocytic Pathway by RECK

A. silver staining of the sucrose gradient fraction 4 (see panel B) from HT1080 cells transfected with pCXN2neo (vector) or pCXN2neo-hRECK (RECK). The arrows indicate the bands that were identified as CD13/APN by mass spectrometry. B. immunoblotting of sucrose gradient fractions from HT1080 cells transfected with pCXN2neo (-) or pCXN2neo-hRECK (+) with the anti-CD13 antibody. Arrows indicate the degraded forms of CD13. C. RECK-transfected HT1080 cells treated with the indicated reagents (analogues: an inactive analogue of GM6001) were subjected to sucrose gradient ultracentrifugation. Fraction 4 was analyzed by immunoblotting with the anti-CD13 antibody. Arrowheads indicate degraded forms of CD13.

D. Western blot of the aminopeptidase activity of recombinant soluble CD13 (6 ng) was nullified to a large extent (Fig. 3A), suggesting that RECK promotes CD13 degradation in a metalloprotease-dependent manner. The depletion of MT1-MMP by RNA interference failed to impede the CD13-dependent processing of CD13 (data not shown), suggesting that the MT1-MMP activity is not solely responsible for promoting RECK-dependent CD13 processing.

FIGURE 3. Interaction of RECK and CD13. A, silver staining of the sucrose gradient fraction 4 (see panel B) from HT1080 cells transfected with pCXN2neo (vector) or pCXN2neo-hRECK (RECK). The arrows indicate the bands that were identified as CD13/APN by mass spectrometry. B, immunoblotting of sucrose gradient fractions from HT1080 cells transfected with pCXN2neo (-) or pCXN2neo-hRECK (+) with the anti-CD13 antibody. Arrows indicate the degraded forms of CD13. C, RECK-transfected HT1080 cells treated with the indicated reagents (analogues: an inactive analogue of GM6001) were subjected to sucrose gradient ultracentrifugation. Fraction 4 was analyzed by immunoblotting with the anti-CD13 antibody. Arrowheads indicate degraded forms of CD13. D, HT1080 cells transfected with pLXS8 (-) or pLXS8-FLAG-hRECK (+) were immunoprecipitated (IP) with antibodies to the indicated proteins, and the precipitates were analyzed by immunoblotting (IB) using antibodies to the indicated proteins. E, the aminopeptidase activity of HT1080 cells transfected with pCXN2neo (-) or pCXN2neo-hRECK (+) and treated with bestatin at a concentration of 0 (—), 10 (+), or 100(++) µg/ml, in the absence (—) or presence (+) of 10 mM MBCD was measured. The activity of the vector-transfected cells that had not been treated with MBCD was set to 100% (open column). The columns represent mean ± S.E. from four independent experiments. *p < 0.001; **p = 0.032 by Student’s t test. F, the effect of recombinant soluble RECK and bestatin on the aminopeptidase activity of recombinant soluble CD13 (6 ng) was assessed in relation to the open column (100%). The concentration of RECK (µM) or bestatin (µg/ml) used in the assay is indicated. The columns represent mean ± S.E. from four independent experiments.

behavior in the DRMs depending on the presence of RECK. DRMs that were derived from vector or RECK-transfected HT1080 cells were separated by one-dimensional SDS-PAGE and compared by silver staining. We noted the number of bands that specifically emerged in DRMs of RECK-transfected HT1080 cells (Fig. 3A). Two of these bands were identified as CD13/APN by mass spectrometry. However, the molecular sizes of the bands analyzed (110 and 120 kDa) were significantly smaller than the known size of intact CD13 (150 kDa) (Fig. 3A).

Mass spectrometry revealed that no sequence information corresponded to the amino-terminal region of CD13 (data not shown). Thus, we concluded that the 110- and 120-kDa bands represented degraded forms of CD13. Consequently, an immunoblot study of the analyzed sucrose gradient fractions revealed that smaller forms of CD13 specifically emerged in the DRMs of RECK-transfected cells (Fig. 3B). It should be noted that, unlike MT1-MMP, the intact CD13 was enriched in DRMs regardless of the RECK status; however, their degraded products appeared in DRMs specifically in the presence of RECK. The emergence of these degraded forms was almost completely disrupted by treatment with a synthetic metalloprotease inhibitor (Fig. 3C), suggesting that RECK promotes CD13 degradation in a metalloprotease-dependent manner. The depletion of MT1-MMP by RNA interference failed to impede the RECK-dependent processing of CD13 (data not shown), suggesting that the MT1-MMP activity is not solely responsible for promoting RECK-dependent CD13 processing.

RECK Physically Interacts with CD13 and Competitively Inhibits Its Proteolytic Activity—We further investigated the relationship between RECK and CD13. RECK was immunologically co-precipitated with CD13 and vice versa (Fig. 3D). Furthermore, we found that RECK-CD13 complex involved MT1-MMP (Fig. 3D). We also observed the physical interaction between MT1-MMP and intact CD13 that did not depend on the status of RECK (Fig. 3D), although the physiological significance of this interaction has not been further addressed in this work. Next, we addressed whether RECK can influence the aminopeptidase activity of CD13. Introduction of exogenous RECK reduced the cell surface aminopeptidase activity to a level comparable with that in cells that were treated with a non-cytotoxic concentration of bestatin, a specific aminopeptidase inhibitor (31) (Fig. 3E). Under these conditions, RECK and bestatin were observed to act in an additive manner (Fig. 3E). In the presence of MBCD, this inhibitory effect of RECK on the aminopeptidase activity was nullified to a large extent (Fig. 3E), suggesting that, like MT1-MMP, RECK inhibits the proteolytic activity of CD13 in a cholesterol perturbation-sensitive manner.

the mean ± S.E. from three independent experiments. G, a double reciprocal plot of the enzyme kinetics of recombinant soluble CD13 in the presence of various concentrations of recombinant soluble RECK. Circle, 0 nM; triangle, 12.5 nM; square, 18.8 nM soluble RECK. The concentration of fluorogenic substrates used in this assay ranges from 25 to 150 µM. H, the aminopeptidase activity in E10.0 whole embryos with the indicated genotype was measured. A comparison was made by setting the activity in the wild type embryo (open column) as 100%. The columns represent mean ± S.E. from four embryos of each genotype. *p = 0.002.
To determine whether the enzymatic relationship between RECK and CD13 is similar to that between RECK and MT1-MMP, we assessed the effect of recombinant soluble RECK on the aminopeptidase activity of recombinant soluble CD13. We observed that RECK inhibited the aminopeptidase activity of CD13 in a dose-dependent manner (Fig. 3F). Consistent with the result of the experiment using living cells (Fig. 3E), RECK and bestatin acted on CD13 in an additive manner (Fig. 3F). In the kinetic assay, the recombinant soluble RECK competitively inhibited the aminopeptidase activity of the recombinant soluble CD13 with a $K_i \approx 67.0$ nM (Fig. 3G). Further, the in vivo relevance of this function of RECK was demonstrated in cells derived from RECK-null embryos whose intrinsic aminopeptidase activity was significantly higher than that of the wild type (Fig. 3H). These findings indicate that RECK is an endogenous inhibitor of CD13.

**RECK Is Internalized Together with MT1-MMP and CD13 in a Cholesterol Perturbation-sensitive Manner**—RECK appeared to modulate the behavior of MT1-MMP and CD13 on the cell membrane as well as their proteolytic activity. This prompted us to examine whether RECK has any effect on the manner by which MT1-MMP and CD13 are internalized from the cell membrane. To this end, HT1080 cells were exogenously transduced with Myc-tagged RECK and FLAG-tagged MT1-MMP. These cells were then incubated with Alexa Fluor 555-labeled anti-Myc, Alexa Fluor 488-labeled anti-FLAG, and Alexa Fluor 647-labeled anti-CD13 antibodies at 4 °C (0 min) and warmed to 37 °C (2 min). Mono-color images for indicated molecules are presented individually (left three panels). Arrows in merged pictures (right two panels) indicate the signals containing all three colors (red, green, and blue signals merge to form a white signal). B, the same experiment was performed in the presence of 10 mM MBCD.

**FIGURE 4.** Colocalization of RECK with MT1-MMP and CD13 on the cell surface and during internalization. A, HT1080 cells transfected with pSG5-FLAG-MT1-MMP and pX58-Myc-hRECK were incubated with Alexa Fluor 555-labeled anti-Myc (Myc-RECK, red), Alexa Fluor 488-labeled anti-FLAG (FLAG-MT1-MMP, green), and Alexa Fluor 647-labeled anti-CD13 (blue) antibodies at 4 °C (0 min) and warmed to 37 °C (2 min). Mono-color images for indicated molecules are presented individually (left three panels). Arrows in merged pictures (right two panels) indicate the signals containing all three colors (red, green, and blue signals merge to form a white signal). B, the same experiment was performed in the presence of 10 mM MBCD.
ent endocytosis (data not shown). These findings suggest that the colocalization of RECK, MT1-MMP, and CD13 on the cell surface and during internalization is sensitive to cholesterol perturbation.

The Endocytic Pathway for RECK Is Similar to That Mediated by GEECs—To further characterize the endocytic pathway for RECK, FLAG-tagged RECK (F-RECK) was introduced into HT1080 cells, and the early phase of its de novo endocytosis was monitored in cells treated with appropriate fluorogenic anti-FLAG antibody at 4 °C and warmed to 37 °C to resume endocytosis. To observe colocalization of F-RECK with caveolin-1 during internalization, we first treated F-RECK-transfected cells with Alexa Fluor 488-labeled anti-FLAG antibody at 4 °C. After incubation at 37 °C, these cells were fixed and stained with the specific antibody to caveolin-1. The colocalization of F-RECK with transferrin was assessed by treating F-RECK-transfected cells simultaneously with TRITC-conjugated anti-FLAG antibody and Alexa Fluor 488-conjugated transferrin at 4 °C. After incubation at 37 °C, these cells were fixed and analyzed.

In HT1080 cells, RECK and caveolin-1 signals exhibited very low frequency of overlap during early endocytosis (Fig. 5A). Further, caveolin-1 depletion by RNA interference had virtually no effect on RECK internalization (Fig. 5B). Caveolin-1 depletion is known to be sufficient to impede caveola-dependent endocytosis (34). Next, the colocalization of RECK and transferrin signals in early endosomes was assessed; 95% of RECK signal was not overlapped with transferrin signal, but 5% was (Fig. 5C, left). RECK internalization was unaffected by blocking transferrin endocytosis caused by depleting dynamin-2 (Fig. 5C, middle and right) that is required for the budding of clathrin-coated pits (35). These findings suggest that the endocytic pathway by which the majority of RECK is internalized is distinct from both clathrin- and caveola-dependent pathways.

Mayor and co-workers (24) have recently identified a novel endocytic pathway by which multiple GPI-anchored proteins are internalized. This pathway is independent of both clathrin- and caveola-mediated endocytic pathways, and it is primarily characterized by the involvement of fluid phase uptake markers represented by dextran (24). We found that in HT1080 cells transfected with F-RECK and treated with TRITC-conjugated anti-FLAG antibody, Alexa Fluor 647-conjugated dextran, and Alexa Fluor 488-conjugated transferrin, a significant population of transferrin-free F-RECK signal was colocalized with dextran during early internalization (Fig. 5D, left and middle). Transferrin signal rarely overlapped with F-RECK-dextran double positive signals (Fig. 5D, right). Moreover, the colocalization of RECK and dextran was unaffected by the depletion of dynamin-2 (Fig. 5E). By contrast, dextran uptake was completely disrupted by treatment with Clostridium difficile Toxin-B (Fig. 5F), which irreversibly inactivates many Rho/Rac family GTPases (36). Transferrin uptake was unaffected by this treatment (Fig. 5F). Despite the disappearance of internalized dextran-positive particles, the net internalization of RECK in Toxin-B-treated cells was unaffected (Fig. 5F and see below).

As well as by the involvement of dextran, the above mentioned novel endocytic pathway advocated by Mayor and co-workers (24) was characterized by sensitivity to C. difficile

![characterization of the endocytic pathway for RECK](image)

**FIGURE 5.** Characterization of the endocytic pathway for RECK. **A,** HT1080 cells transfected with pLXSB-FLAG-hRECK were incubated with Alexa Fluor 488-labeled anti-FLAG antibody (red) prior to resuming endocytosis. After 2 min of incubation at 37 °C, unincorporated antibody was removed from the cells, which were then fixed and stained with the anti-caveolin-1 antibody (green). **B,** the same transfectants were transduced with caveolin-1 siRNA and analyzed in the same way as above. **C,** HT1080 cells transfected with pLXSB-FLAG-hRECK were incubated with TRITC-conjugated anti-FLAG antibody (red) and Alexa Fluor 488-transferrin (Ti, green) prior to resuming endocytosis. After 2 min of incubation at 37 °C, the cells were removed of unincorporated antibody and probe, fixed, and observed (left). HT1080 cells transfected with pLXSB-FLAG-hRECK and dynamin-2 siRNA (middle) or its control siRNA were stained in the same way. The number of TF-positive internalized particles/cell was counted in 50 cells and quantified as that of untreated set to 100% (right; columns, mean ± S.E.). **D,** HT1080 cells transfected with pLXSB-FLAG-hRECK were incubated with TRITC-conjugated anti-FLAG antibody (red), Alexa Fluor 647-dextran (Dex, green), and Alexa Fluor 488-transferrin (Ti, blue) prior to resuming endocytosis. After 2 min of incubation at 37 °C, the cells were fixed and observed (left). Arrows indicate the colocalization of FLAG-RECK and dextran (yellow). The dextran-positive population in total RECK-positive-TF-negative internalized particles (yellow) and that in total RECK-positive-TF-positive internalized particles (white) were quantified (right; columns, mean ± S.E. from random observation of 50 cells). **E,** HT1080 cells transfected with pLXSB-FLAG-hRECK and dynamin-2 siRNA or its control were analyzed in the same way as in panel D. Arrows indicate the colocalization of FLAG-RECK and dextran (yellow). The dextran-positive population in total RECK-positive internalized particles was quantified and compared (right; columns, mean ± S.E. from random observation of 50 cells each). **F,** HT1080 cells transfected with pLXSB-FLAG-hRECK were treated with 200 ng/ml C. difficile Toxin-B for 3 h and analyzed in the same way as in panel D. The net internalization of indicated molecules in Toxin-B-untreated (open) and treated (solid) cells was estimated by FACS and quantified as that of untreated set to 100%.

Toxin-B treatment or inactivation of Cdc42 and insensitivity to inactivation of RhoA or dynamin-2 activity. Further, it was reported by the same group that Toxin-B selectively blocked this novel endocytic pathway but the net internalization of the
tested GPI-anchored protein was unaffected (24), similar to the case of RECK (Fig. 5f). They designated endosomes that composed this newly identified pathway as GEECs (24, 37, 38). Our observation proposes that the endocytic pathway by which RECK is internalized is identical to the above mentioned pathway. However, in HT1080 cells, the overexpression of a dominant negative form of Cdc42 (Cdc42N17) failed to block dextran uptake and subsequently did not disrupt the dextran-RECK colocalization (data not shown). This discrepancy stems from the cell type-dependent variation in the role of Rho family proteins. Nonetheless, our characterization of the endocytic pathway for RECK revealed that it was overall similar to that mediated by GEECs.

Exogenous RECK Modulates the Route of MT1-MMP and CD13 Internalization—After characterizing the endocytic pathway used for RECK internalization, we finally addressed whether the route of MT1-MMP and CD13 internalization was influenced by RECK. To this end, we again used HT1080 cells that had been transfected with FLAG-tagged MT1-MMP, together with or without Myc-tagged RECK. These cells were treated with fluorogenic anti-FLAG antibody, dextran, and transferrin as was done above. In the absence of RECK, FLAG-MT1-MMP signal was preferentially colocalized with transferrin during internalization. However, notably, in the presence of RECK, FLAG-MT1-MMP signal was preferentially colocalized with dextran (Fig. 6, A and C). An identical RECK-induced variation in the endocytic cargo was observed in the case of endogenous CD13 (Fig. 6, B and C). These findings indicate that RECK alters the route of MT1-MMP and CD13 internalization to one that is preferred by RECK.

Endogenous RECK Influences the Selection of the Internalization Route for MT1-MMP and CD13—Thus far, we have discussed the internalization routes of MT1-MMP and CD13 that were analyzed by using fibrosarcoma cells (HT1080 cells) expressing exogenously introduced RECK. We addressed whether endogenous RECK possesses the same function proposed by studies using exogenously transduced RECK. To this end, FLAG-tagged MT1-MMP was introduced into HUVECs in which endogenous RECK was depleted by using two kinds of siRNAs independently (Fig. 7A). These cells were treated with fluorogenic anti-FLAG or anti-CD13 antibody, dextran, and transferrin as done above. The frequency of both dextran-MT1-MMP and dextran-CD13 colocalization was significantly reduced in RECK-depleted cells as compared with that in the control cells (Fig. 7, B and C). This result suggests that endogenous RECK contributes to the selection of the internalization route for MT1-MMP and CD13.

RECK Accelerates the Internalization and Decay of MT1-MMP and CD13—Finally, we addressed whether RECK-mediated variation of the MT1-MMP and CD13 endocytic pathways is correlated with change in their fate. We first quantified the
internalization of RECK, MT1-MMP, and CD13 in HT1080 cells that were pulse-biotinylated exclusively at the cell surface by treating the cells with membrane-impermeable sulfosuccinimidy l 2-(biotinamido) ethyl-1,3-dithiopropionate biotin at 4 °C. This type of biotin can be separated from labeled proteins by using the membrane-impermeable reducing agent glutathione in the event that the labeled proteins remain at the cell surface; however, it is resistant to washing (S-S cleavage (−)) once the labeled proteins have been internalized into the cytoplasm (13). In short, we detected only internalized proteins in the S-S cleavage (−) lanes (Fig. 8A). At 4 °C, very few molecules of RECK, MT1-MMP, or CD13 were internalized (Fig. 8A, lanes 3 and 4), while at 37 °C, a significant amount of these molecules were internalized (Fig. 8A, lanes 7 and 8). Notably, the internalization of MT1-MMP and CD13 was significantly accelerated in the presence of RECK (Fig. 8A, compare lanes 7 and 8). The densitometry analysis of multiple experiments revealed that RECK significantly accelerated the internalization of MT1-MMP and CD13 (Fig. 8B).
At last, again by using pulse-biotinylated cells but without washing out cell surface-bound biotins, we measured the life span of MT1-MMP and CD13 proteins and assessed the effect of RECK on it. The result indicated that RECK significantly accelerated the decay of MT1-MMP and CD13 (Fig. 8, C and D). These findings indicate that RECK induced variation in the endocytic pathway of its target molecules is significantly correlated with the alternations in their biochemical fate.

**DISCUSSION**

Many of GPI-anchored proteins efficiently associate with DRMs because during their sorting from the Golgi apparatus to the cell membrane they are carried by secretory vesicles that are rich in cholesterol and sphingolipids (39). In this study, we focused on this trait of GPI-anchored proteins and explored the novel function of RECK in addition to its enzymatic interaction with its previously known and newly identified target molecules. Indeed, we found that the presence of RECK significantly influences the selection of the endocytic pathways by which MT1-MMP and CD13 are internalized. The mechanism most probably involves the complex formation and altered behavior of these molecules on the cell membrane; this needs to be further investigated.

Currently, the mechanisms of the endocytosis are primarily classified as either clathrin-dependent or -independent. More recent studies allowed the clathrin-independent mechanism to be further subclassified (37, 38). Of these, one related to the endocytosis of GPI-anchored proteins is achieved in a clathrin- and caveolae-independent manner (24). As discussed above, this pathway that has been proposed by Mayor and co-workers (24) shares many characteristics with the endocytic pathway involving RECK. Although the biological significance of data obtained by cholesterol depletion is controversial (40), it is noteworthy that both the GEEC-mediated endocytic pathway (24) and the pathway involving RECK are sensitive to MBCD treatment.

Our data demonstrate that in the presence of RECK the endocytic mechanism negatively contributes to MT1-MMP function. However, we have yet to explain why RECK-mediated variation in the MT1-MMP and CD13 endocytic pathways is correlated with their accelerated internalization and decay. Given that RECK is internalized via the GEEC-mediated endocytic pathway, after early phase endocytosis the endosomal compartment containing RECK and its target molecules is thought to enter the recycling endosomal compartment in a manner identical to other compartments. However, reportedly, it exits the recycling endosomal compartment at a rate three times slower than that from other compartments (41). By this mechanism, RECK may alter the balance between the recycling and the decay of endocytosed molecules; however, the correct answer awaits more efforts.

Integrating the information obtained thus far, here we propose that RECK modulates the function of MT1-MMP in dual manners, *i.e.* one is by directly inhibiting its proteolytic activity and the other is by affecting its half-life by modulating the endocytic pathway. Then, which of these contributes more to the attenuation of MT1-MMP function in cells? Our previous study has indicated that the soluble RECK possesses a significant ability to suppress the invasive behavior of tumor cells without attenuating MT1-MMP activity to promote proMMP-2 maturation (1), suggesting that the GPI anchor-independent function of RECK is biologically and clinically important. On the other hand, the $K_i$ value at which RECK inhibits MT1-MMP (<19.3 nM) was significantly higher than the $K_i$ value at which TIMP-2 inhibits MT1-MMP (<150 pM) (42). This signifies that as an inhibitor of MT1-MMP RECK is less potent than TIMP-2; this may account for the low efficiency of soluble RECK to attenuate MT1-MMP activity in living cells. Therefore, we speculate that particularly in the case of MT1-MMP, RECK-mediated variation of the endocytic pathway is more rate-limiting to the suppression of its activity than the effect of competitive inhibition. CD13/APN emerged as a prospective intrinsic target of RECK. We further demonstrated that in the presence of RECK this molecule is internalized in a manner similar to the internalization of MT1-MMP. The biological significance of the complex formation between CD13 and MT1-MMP was partially tested. Depletion of CD13 did not generate any detectable impact on MT1-MMP function in promoting proMMP-2 maturation in HT1080 cells. Because CD13 is implicated in various human diseases, it is of great interest to further seek the biological significance of the interaction between RECK and CD13.

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