The membrane-anchored protease regulator RECK plays important roles in mammalian development and tumor suppression. The biochemical bases of these bioactivities, however, remain poorly understood. Here we report on the properties of a recombinant RECK protein expressed in mouse fibroblasts and purified to near homogeneity. Multiple lines of evidence indicate that RECK forms dimers. Single particle reconstruction using transmission electron microscopy revealed a unique cowbell-like shaped RECK dimer. RECK is cleaved by MMP-2 and MMP-7 and competitively inhibits MMP-7-catalyzed cleavage of fibronectin. Forced expression of RECK in HT1080 cells, whose endogenous RECK expression is minimal, leads to an increase in the amount of fibronectin associated with the cell. Our data demonstrate the ability of RECK to protect fibronectin from MMP-mediated degradation.

RECK (reversion-inducing cysteine-rich protein with Kazal motifs) was initially identified as a transformation suppressor gene (1). It encodes a glycoprotein of 971 amino acid residues (~125 kDa) anchored to the membrane through C-terminal glycosylphosphatidylinositol (GPI) modification. RECK-deficient mice die around embryonic day 10.5 (E10.5) with reduced glycosylation, premature blood vessels, and the level of residual RECK expression in tumor tissues often correlates with better prognosis (7). Despite the accumulating evidence indicating the importance of RECK in mammalian development and tumor suppression, the molecular basis of its action remains poorly understood. Several characteristics of RECK, such as high cysteine content, requirement of carbohydrate modification for its bioactivity (8), and susceptibility to proteolysis, make it difficult to produce a large quantity of this protein in a laboratory. In addition, many RECK mutants and fragments have been found biologically inactive, most likely because of misfolding. It is therefore imperative to establish a system to produce full-length RECK protein satisfactory for biochemical studies.

Matrix metalloproteinases (MMPs) (~23 in humans) typically consist of a peptidase, catalytic domain, linker peptide, and hemopexin domain (9–11). Noncatalytic domains of MMPs contribute to their interaction with other molecules, including their specific substrates. MMP-7 (also known as matriylsin and pump-1) is one of the smallest members of the family, consisting only of a peptidase and the catalytic domain. Some MMP family members are up-regulated in cancer cells, and their expression is often associated with poor prognosis (12). Enhanced production of MMP-7, for instance, is found in endometrial carcinoma, and its expression is correlated with invasion and metastasis (13). MMP-2-deficient mice show reduced progression of inoculated tumors (14). Previous studies using a partially purified, soluble form of RECK expressed in CHO cells demonstrated competitive inhibition of gelatinases (MMP-2 and MMP-9) and MT1-MMP in vitro (1, 2, 15). The spectrum and modes of inhibition by RECK on other MMP family members remain to be elucidated.

Fibronectin (FN) is one of the major extracellular matrix components that can be cleaved by several MMPs (9). FN is mainly composed of three repeating units (types I–III), expressed in multiple cell types, and plays important roles in cell adhesion, migration, proliferation, and differentiation (16, 17). FN was initially identified as a glycoprotein lost in malig-
nant transformation, and later studies revealed the involvement of both transcriptional and post-transcriptional regulation in this event (18). Whether RECK down-regulation in malignant transformation plays any part in this FN loss is an interesting question yet to be addressed.

In this study, we established a system to produce a highly purified, soluble form of human RECK protein. Using this protein, we could uncover its dimeric nature and unique three-dimensional structure. We also identified MMP-7 as its novel potential target and FN as an MMP substrate protected by RECK.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—The retroviral vector, pL(RECK-His)SB, was constructed by inserting, by three parts ligation, the MluI-EagI fragment of human RECK cDNA, and the double-stranded oligonucleotides encoding the C-terminal 7 amino acids (954AHHHHHH) between the MluI and Apal site of pLXSb, a derivative of pLXSN (19) carrying the bsd marker. The virus was propagated using EcoPack 293 (Clontech).

**Establishing RECK-Producer**—MEFs were prepared as described (2). R′ M2′ MT′ MEFs were infected with L(RECK-His)SB virus and selected with blasticidin S (8 μg/ml). A transfectant clone (TOM2–6) secreting RECK-His at a high level was expanded to 20–40 150-mm dishes using selection medium. When the culture reached confluence, the medium was replaced with α-minimum essential medium containing 5% fetal calf serum and harvested every 48 h for several weeks.

**Purification of RECK-His**—The outline is presented in the supplemental material.

**Transmission Electron Microscopy (TEM)**—Purified proteins were adsorbed to thin carbon films and negatively stained with 2% uranyl acetate. Micrographs were taken using a JEOL 100CX transmission electron microscope (JEOL) at ×52,000 magnification with 100-kV acceleration voltage, and digitized using Scitex Leafscan 45 (Leaf System Inc) with a pixel size of 1.92 Å (specimen level).

**Automated Particle Selection and Image Analysis**—Three-dimensional reconstruction (20, 21) was performed using our SPINNS techniques (22–25) and the IMAGIC V algorithm (20). Dimensional reconstruction (20, 21) was performed using our automated particle selection and image analysis.

**Electrophoretic Detection of Proteins**—For immunoblot assay, proteins boiled in sample buffer without (nonreducing conditions) or with 50 mM dithiothreitol (reducing conditions; assay, proteins boiled in sample buffer without (nonreducing conditions) or with 50 mM dithiothreitol) and then incubated with N-glycosidase F (1,000 units; Roche Applied Science) in 50 mM NaPO₄ (pH 7.5), 1% Nonidet P-40.

**Mass Spectrometry** (26)—After SDS-PAGE, proteins were visualized by silver staining, excised individually from the gel, and digested in gel with trypsin (Promega). Mass analyses of the tryptic peptides were performed by matrix-assisted laser desorption ionization time-of-flight/mass spectrometry using an ultraflex TOF/TOF (Bruker Daltonics).

**Expression and Purification of Recombinant Human RECK Protein**—Our preliminary experiments indicated that a recombinant human RECK protein (RECK-His), in which the C-terminal GPI-anchoring signal (28 residues) was replaced by six

**RESULTS**

**Expression and Purification of Recombinant Human RECK Protein**—Our preliminary experiments indicated that a recombinant human RECK protein (RECK-His), in which the C-terminal GPI-anchoring signal (28 residues) was replaced by six...
histidines, could be expressed as a secreted protein in insect cells (Sf21) and that this protein could be cleaved by MMP-2 and MT1-MMP (supplemental Fig. 1A). This protein, however, was not glycosylated as extensively as RECK produced in mammalian cells (supplemental Fig. 1B). In an attempt to produce intact and fully glycosylated RECK-His in large quantity, we established a MEF line from an animal carrying null mutations in three genes, Reck, Mmp-2, and Mmp-14 (Mti-Mmp). When the RECK-His transgene was expressed in this MEF line (named R^M2^M^-MEFs) using a retroviral vector, near-full-length RECK-His could be detected in the culture supernatant (Fig. 1A). We set up a four-step purification procedure starting from this material: 1) His tag affinity fractionation; 2) cation exchange chromatography; 3) anion exchange chromatography; and 4) gel filtration chromatography. This procedure allowed us to routinely prepare RECK-His to over 90% purity (Fig. 1B) with a net yield of ~4 µg from 1-liter of culture supernatant.

***Intactness of the RECK-His Protein—***RECK is rich in cysteine (9.17%) and has Kazal motifs with conserved cysteine residues (1). When we detected the native RECK protein in the lysates of HT1080 cells overexpressing RECK (R/HT1080 cells) by immunoblot assay, a major band of ~125 kDa was found under reducing conditions (Fig. 1C, lane 1), and a band of ~95 kDa was found under nonreducing conditions (Fig. 1C, lane 2), suggesting the presence of intra-molecular disulfide bridges. A similar band shift was observed when the purified recombinant RECK-His was analyzed under reducing and nonreducing conditions (Fig. 1B, lanes 3 and 4).

In addition to the ~125-kDa band, several smaller bands (major species, ~110 and ~53 kDa) were detectable in the
lysate of R/HT1080 when the antibody recognizing the C-terminal portion of RECK was used (Fig. 1, C and D, lane 1). Many of these smaller bands were absent or less intense in the cells treated with a broad spectrum MMP inhibitor GM6001 (Fig. 1D, lane 2). Sizes of the major subfragments are similar to those of the RECK-His fragments produced by MMPs in vitro (see below). These observations suggest that the folding of the RECK-His preparation was largely intact. Furthermore, treatment with N-glycosidase F indicated that the extent of glycosylation was similar between the purified RECK-His and the RECK in R/HT1080 cell lysate (Fig. 1E).

Molecular Mass of RECK-His—We next estimated the molecular mass of nondenatured RECK-His by two methods, gel filtration chromatography (Fig. 2, A and B) and blue native-PAGE (Fig. 2C). Both experiments yielded similar results: 215.9 ± 4.2 kDa (mean ± S.D., n = 5) by gel filtration chromatography and ~210 kDa by blue native-PAGE. Because the calculated molecular mass of RECK-His polypeptide is 102 kDa, and the contribution of carbohydrate groups is typically ~10 kDa in a RECK monomer (e.g. Fig. 1E) (1, 8), the observed molecular mass is close to the value expected for a dimer: i.e. (102 + 10) x 2 = 224.

To test whether intact RECK also shows a similar molecular mass, we subjected a lysate of R/HT1080 cells to blue native-PAGE-immunoblot assay (Fig. 2D). A major band at the position corresponding to the dimer was detected. We also prepared the supernatants of the cells cultured in the presence of phosphatidylinositol-specific phospholipase C (PI-PLC); this treatment released a large fraction of RECK (probably representing the molecules displayed on the cell surface) into the medium (Fig. 2E, lanes 2 and 4). This sample again showed a

with their corresponding class averages (2nd row), surface representations of the three-dimensional reconstruction (3rd row), and projections from the reconstruction (bottom panels) along with the corresponding Euler directions (0, β, γ). Protein is displayed in bright shades. C, Euler angles of 87 adopted class averages. D, FSC function indicates the resolution of the three-dimensional map is 25.2 Å at the correlation coefficient of 0.5. E, electron microscopic images of RECK-His bound with monoclonal antibody against the C-terminal His tag. Purified RECK-His and anti-penta-His antibody (Qiagen) were mixed and incubated for 16 h at 4 °C. The RECK-His-antibody complex was separated from unbound antibody by gel filtration chromatography and negatively stained for TEM. Contours are shown below each image with RECK-His in white and the bound antibodies in gray. Scale bars, 100 Å.

FIGURE 4. Surface representation of RECK-His. A, three-dimensional reconstruction of the RECK-His dimer viewed from 25 different angles. B, size of the RECK-His dimer determined from the side and the bottom views. Scale bars, 50 Å.
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major band at the dimeric position in blue native-PAGE immunoblot assay (Fig. 2F, lane 2). Hence, a major fraction of RECK molecules, including those on the cell surface (i.e. mature form), are probably in the dimeric form.

Three-dimensional Structure of RECK-His—Given the high purity and intactness of the RECK-His preparation, we studied its gross three-dimensional structure by negative staining and TEM. Differently shaped globular objects of similar sizes, presumably representing projections of RECK-His at different angles, were observed (Fig. 3A, arrowheads). The raw images of these globular objects (n = 2,325) were picked up and analyzed to reconstruct a three-dimensional density assuming C2 symmetry (i.e. dimeric assembly) using our SPINNS method and the Imagic V program (see “Experimental Procedures”). The final reconstruction included 2,033 particles, 87.4% of all the selected images.

In Fig. 3B, representative raw images (top panels) are shown with their corresponding class averages (2nd row), the surface representations, and the projections of the reconstruction (3rd and 4th rows). Despite the large divergence in the shapes of the raw images, a higher level of consistency was observed in their size, shape, and inner structure with the corresponding averages and the projections from the final three-dimensional structure. This indicates successful three-dimensional reconstruction from the original particle images. A plot of the Euler angles of the 87 adopted class averages (Fig. 3C) shows that RECK-His was almost randomly oriented on the grid surface. The resolution (20, 21) was 25.2 Å at the correlation coefficient of 0.5 according to the FSC function (27) (Fig. 3D).

The surface representation (Fig. 4) demonstrates that the RECK-His dimer is a cowbell-shape with an inside cavity that opens widely at the bottom. From an elliptic bottom view, the estimated lengths at the widest and narrowest positions are 109 and 89 Å, respectively (Fig. 4B, lower panel). The molecular height is 120 Å (Fig. 4B, upper panel). Of note, a pair of small protrusions (~16 Å height) sticking out from the bottom cavity can be seen (Fig. 4, A, panels 11, 12 and 17, and B, upper panel). The three-dimensional map is contoured using isosurfaces and contains a volume corresponding to 235 kDa by assuming a protein density of 1.37 g/cm³. This is 115% of the dimeric RECK mass (204.2 kDa) calculated from the amino acid composition.

We also observed RECK-His decorated with anti-penta-His monoclonal antibodies (Fig. 3E). Binding of this antibody to the globular objects was clearly demonstrated, indicating that the images we analyzed indeed represent RECK-His. Moreover, globular objects bearing two antibodies were frequently found (Fig. 3E, lower Panels), further supporting the dimeric assembly of RECK-His.

Cleavage of RECK-His by MMPs—Consistent with the preliminary data pertaining to the protein

FIGURE 5. Cleavage of RECK-His by MMPs. A, cleavage of RECK-His by MMP-2. RECK-His (4.7 nM) was incubated without (lane 1) or with activated MMP-2 (60 nM (lane 2), 120 nM (lane 3), and 180 nM (lane 4)) in a 30-μl reaction mixture at 37 °C for 15 h and analyzed by immunoblot (IB) assay using anti-FN. Arrow, full-length pFN. Bracket, doublet fragments of ~175 kDa. B, cleavage of RECK-His by MMP-7. RECK-His (1.7 nM) was incubated without (lane 1) or with activated MMP-7 (62 nM (lane 2), 125 nM (lane 3), and 255 nM (lane 4)) in a 30-μl reaction mixture at 37 °C for 15 h and analyzed by immunoblot assay. C, cleavage of RECK-His by double digestion with MMP-2 and MMP-7. RECK-His (4.7 nM) was incubated with activated MMP-2 (180 nM) and/or activated MMP-7 (185 nM) in a 30-μl reaction mixture at 37 °C for 15 h and analyzed by immunoblot assay.

FIGURE 6. Inhibition of MMP-catalyzed cleavage of pFN by RECK-His. A, inhibition of MMP-2-catalyzed pFN cleavage by RECK-His. pFN (4.3 nM) was treated with activated MMP-2 (20 nM) in the absence (lane 1) or presence (lane 2) of RECK-His (183 nM) in a 30-μl reaction mixture at 37 °C for 15 h and analyzed by immunoblot (IB) assay using anti-FN. Arrow, full-length pFN. Bracket, doublet fragments of ~175 kDa. B, time course of pFN cleavage by MMP-2 in the absence or presence of RECK-His. pFN (7.1 nM) was treated with activated MMP-2 (62 nM) in the absence (−) or presence (+) of RECK-His (327 nM) in a 30-μl reaction mixture at 37 °C for the indicated period of time. The reaction products were analyzed by immunoblot assay. Arrow, full-length pFN. Bracket, doublet fragments of ~175 kDa. C, densitometric quantification of the time course data shown in B. The proportion of cleavage products (cumulative density of fragment bands) to the input substrate (cumulative density of all bands, including the full-length pFN) generated in the absence (open square) or presence (filled circle) of RECK-His was plotted against incubation time. D, substrate dose response of MMP-2-catalyzed pFN cleavage. pFN at the indicated concentrations was mixed with a fixed amount of MMP-2 (17.5 nM). −, reaction was stopped before incubation; +, reaction was stopped after incubation for 1 h at 37 °C. pFN in each reaction mixture was detected by immunoblot assay. E, substrate dose response of MMP-7-catalyzed pFN cleavage. pFN at the indicated concentrations was mixed with a fixed amount of MMP-7 (17.5 nM). −, reaction was stopped before incubation; +, reaction was stopped after incubation for 1 h at 37 °C. pFN in each reaction mixture was detected by immunoblot assay. F, effects of RECK-His on MMP-7-catalyzed pFN cleavage under substrate-excess conditions. pFN at the indicated concentrations was mixed with a fixed amount (1.75 μM) of MMP-7 without (−) or with (+) RECK-His (119 nM) and incubated for 1 h at 37 °C. pFN in each reaction mixture was visualized by immunoblot assay. G, Lineweaver-Burk plots. Initial velocities (v) of MMP-7-catalyzed pFN cleavage in the absence (filled circle) or presence (filled square) of RECK-His at different pFN concentrations (SI = 2–141 nM) were determined from immunoblot data as shown in F.
expressed in insect cells (see above and supplemental Fig. 1), RECK-His purified in this study could be cleaved by MMP-2 when the enzyme concentration was higher than the substrate concentration (Fig. 5A, lanes 2–4; [RECK-His]:[MMP-2] = 1:13, 1:26, 1:38), yielding two major fragments (≈110 and ≈53 kDa), although complete digestion was not achieved under these conditions. RECK-His could also be cleaved by MMP-7 when the enzyme was present in great excess (Fig. 5B, lanes 2–4; [RECK-His]:[MMP-7] = 1:109, 1:218, 1:326), yielding a major fragment of ≈53 kDa (Fig. 5B). Double digestion experiments (Fig. 5C; [RECK-His]:[MMP-2]:[MMP-7] = 1:38:39) indicated that MMP-2 and MMP-7 cleave RECK-His at proximate (or identical) sites to yield the ≈53-kDa fragment.

Effects of RECK-His on the Proteolytic Activity of MMPs—Next, we asked whether highly purified RECK-His can regulate MMPs. We chose to use plasma fibronectin (pFN), a natural substrate known to be cleaved by both MMP-2 and MMP-7 (9, 28). MMP-2 cleaves pFN at several sites as visualized by immunoblot technique (Fig. 6A; compare lanes 1–3). This reaction was partially inhibited by RECK-His when its concentration was higher than the concentrations of both MMP-2 and pFN (Fig. 6A, lane 2; [RECK-His]:[MMP-2]:[pFN] = 43:5:1). Under similar conditions, RECK-His could also inhibit the cleavage of pFN by MMP-7 (Fig. 6B; [RECK-His]:[MMP-7]:[pFN] = 46:9:1).

The above results indicate that RECK can inhibit MMPs, while at the same time being a substrate of MMPs. To understand why this is possible, we compared the susceptibility of pFN and RECK-His to MMP-7 under similar, substrate-excess conditions (Fig. 6, D and E). MMP-7-catalyzed cleavage of pFN was detectable over a wide range of substrate concentrations (Fig. 6D; [MMP-7]:[pFN] = 1:8–80), whereas the cleavage of RECK-His was barely detectable under similar conditions (Fig. 6E; [MMP-7]:[RECK-His] = 1.9–86). RECK-His could only be cleaved at far higher MMP-7 concentrations (see Fig. 5B), indicating that RECK-His is a poor substrate for MMP-7.

In time course studies such as the one shown in Fig. 6B, we ascertained that it was possible to determine the reaction velocity of MMP-mediated pFN cleavage by immunoblot densitometry (e.g. Fig. 6, B and C). We therefore determined initial reaction velocities at various pFN concentrations in the absence or presence of RECK-His (Fig. 6F; [RECK-His]:[MMP-7]:[pFN] = 68:1:1.1–81) and obtained Lineweaver-Burk plots (Fig. 6G). The plots yielded two lines (representing the data in the absence and presence of RECK-His) crossing near the y axis (Fig. 6G), suggesting competitive inhibition, and the following kinetic constants: 

\[ V_{\text{max}} = 44 \text{ pmol/min}, \quad K_m = 8.2 \text{ nM for pFN cleavage by MMP-7}, \quad \text{and} \quad K_i = 41 \text{ nM for inhibition of this reaction by RECK-His}. \]

Characterization of pFN Fragments—Cleavage of pFN by MMP-7 results in the appearance of a ∼175-kDa doublet, followed by the appearance of a lower molecular weight doublet (Fig. 6B). The 175-kDa doublet, particularly the upper band, remains prominent for more than 6 h in mixtures to which RECK has been added. Persistence of similar fragments was also observed when pFN was treated with MMP-2 in the presence of RECK-His (Fig. 6A, lane 2, bracket). The doublet FN fragments could be recognized by a C-terminal specific anti-FN antibody but not by an N-terminal specific anti-FN antibody (Fig. 7A; see Fig. 7C, top diagram for the positions of antigens). Partial digestion of pFN by MMP-7 also gave rise to similar doublet bands (Fig. 7B, arrowheads a and b). Mass spectrometry of these bands indicated that they share the same N-terminal end, 721LVAT—, corresponding to the beginning of the FN III2 module and that they contain a common sequence YSQRT2356 corresponding to a site near the C-terminal end of FN (Fig. 7C, lower diagrams). The size difference between the doublet bands is because of the presence or absence of the V region (residues 1991–2110 in P02751), which is consistent with the known composition of the pFN dimer (i.e. only one subunit contains the V region). Thus, the FN fragments (∼175 kDa) that persist in the presence of RECK-His are devoid of the N-terminal assembly domain, which consists mainly of type I and type II modules (Fig. 7C).

Effects of RECK Expression on FN Associated with HT1080 Cells—To assess the biological relevance of our findings in vitro, we compared the levels of FN associated with HT1080 cells transfected with the control vector (V) or RECK-expression vector (R). FN levels, both in the cell lysate and culture media, were determined by ELISA (Fig. 8A) or by immunofluorescence microscopy (Fig. 8B). The FN signal in the cell lysate was higher in cells expressing RECK compared to control cells (Fig. 8A, lanes 2 and 4), indicating that RECK expression leads to an increase in FN production. In contrast, the FN signal in the culture media was lower in cells expressing RECK compared to control cells (Fig. 8B, lanes 2 and 4), indicating that RECK expression leads to a decrease in FN release into the culture media. Thus, RECK expression can lead to an increase in FN production and a decrease in FN release into the culture media.
staining of nonpermeabilized cells (Fig. 8C, panel 3) and by immunoblot assay (Fig. 8D, lane 3), suggesting the involvement of regulated protein degradation. The major FN receptor, integrin-α5β1, was not up-regulated in RECK transfectants (Fig. 8E), making it unlikely that the observed increase in the level of cell-associated FN reflected an increase in the ability of the cells to bind FN.

Finally, we examined the localization of cell-associated FN by two approaches. In the first approach, we treated the cells with trypsin and determined the levels of FN remaining associated with the cells (Fig. 8F). Interestingly, the level of FN in the trypsin-insensitive (i.e. intracellular) compartment was also increased in RECK-expressing cells (Fig. 8F, lane 2). Second, immunofluorescent staining of permeabilized cells expressing a plasma membrane marker (YFP-mem; green signals) (Fig. 8G) indicated that the increased FN signals (red) in R/HT1080 culture (Fig. 8G, panel 2) can be found both inside and outside the plasma membrane (Fig. 8G, panel 3). Thus, RECK seems to elevate the levels of FN both inside and outside the cells.

**DISCUSSION**

Using a MEF-based expression system, we could produce a recombinant RECK protein of good quality. Despite the modest yield, the MEF-based system has its own advantages as follows: 1) their strong attachment and persistent metabolism allow us to harvest their supernatant for a long period of time (up to a few months), and 2) cells from genetically manipulated animals can be used. To facilitate purification, we used His-tagged RECK incapable of GPI anchoring. Although the events it encounters en route to the cell surface may not be identical to those encountered by intact RECK, based on several criteria no obvious difference was detected between RECK and RECK-His, e.g. mobility shift in SDS-PAGE induced by reducing agents, patterns of MMP digestion products, and the ability to form dimers. This supports our premise that RECK-His is useful for exploring the properties of native RECK protein.

Heavily glycosylated proteins are not suitable for x-ray crystallography, because large, heterogeneous, and flexible glycan moieties often hinder crystallization. Although enzymatic elimination of glycans is one way to circumvent this obstacle, this treatment often destabilizes the proteins.

A single particle reconstruction using TEM, in which various glycans on a protein are averaged, is an alternative choice (21). Moreover, this method requires far smaller amounts of sample (at least 3 orders of magnitude less) than crystallography. The molecular size of RECK (204.2 kDa as a dimer), however, is near the lower limit for single particle analysis; this was overcome by our image processing programs, including automatic pickup programs (23, 24). Three-dimensional reconstruction of objects with a lower point of symmetries is also a challenge; in the present case (i.e. 2-fold symmetry), this difficulty was surmounted by the use of the posterior Euler angle assignment program (25).

The resulting RECK-His model has a larger dimension than expected from its molecular size, most probably because of an internal large cavity. The binding patterns of the C-terminal specific antibodies are suggestive. First, when two antibodies are bound (Fig. 3E, lower panels), they tend to cluster on one...
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side of the particle, which is consistent with the polarized nature (top-bottom polarity) of the cowbell model. Second, the images, especially those with one antibody molecule (Fig. 3E, upper panels), suggest that the C-terminal (i.e. GPI anchoring) domain is located on the bell top. This raises the possibility that the RECK dimer anchors to the membrane at its bell top and holds its opening with two protuberances outward. This configuration looks advantageous for docking and interactions with MMPs and other molecules. Improvement in resolution and analyses of RECK-MMP complexes are still needed to verify this hypothesis. Nevertheless, our three-dimensional model sets a foundation for further studies on the actions of RECK at the molecular and atomic levels.

Our data suggest that MMP-2 and MMP-7 directly cleave RECK to yield two major fragments (~110 and ~53 kDa) as detected by C-terminal specific anti-RECK antibodies (Fig. 5). Similar fragments are often detected in lysates of RECK-expressing cells (e.g. Fig. 1, C and D, lanes 1), suggesting the presence of at least two regions of RECK that are susceptible to proteolysis as follows: one near the N-terminal end and one near the center. The effects of these cleavages on RECK functions need to be clarified in future studies.

Our results in vitro also indicate that RECK-His inhibits MMP-7 cleavage of pFN, probably through a competitive mechanism. This finding has several important implications. First, MMP-7 is known to be involved in many biological processes (9, 29, 30); RECK needs to be considered as a potential regulator of these events. Second, MMP-7 is composed almost exclusively of the MMP catalytic domain; RECK may therefore interact directly with this domain, and hence, the spectrum of RECKs inhibition among MMP family members may be broader than we previously expected. Third, the expression of both RECK and FN genes is known to be down-regulated by oncogenes; this may imply the presence of regulatory circuits coordinating the expression of these functionally linked molecules. Fourth, Fn-deficient mice and Reck-deficient mice share similar phenotypes (31); some of the phenotypes of RECK-deficient mice may indeed be attributable to local FN depletion.

~175-kDa pFN fragments persist when pFN is treated with MMP-2 or MMP-7 in the presence of RECK-His (Fig. 6). Intriguingly, these fragments lack the N-terminal “assembly domain” but retain the major cell-binding domain (RGD). Although the ~175-kDa fragments were undetectable in the HT1080 culture system (Fig. 8), such fragments may play a role in other cells or tissues in vivo. A 76-amino acid peptide derived from the FN III domain has been reported to show anti-angiogenic and anti-metastatic activities by enhancing FN-FN association (32, 33). Several MMPs can release FN fragments with cryptic activities to regulate adipocyte differentiation and migration (34). It would therefore be interesting to test whether the ~175-kDa FN fragments have any cryptic bioactivities. It is also important to clarify whether these fragments retain the C-terminal cysteine residues of FN (Cys2367 and Cys2371), which are essential for dimer formation and fibrillogenesis (35).

The MMP-7 cleavage site in FN yielding the ~175-kDa fragments (Ser110 and Thr111) is located in the area between FN III1 and FN III2 and is known to be susceptible to some other proteases, including thermolysin, cathepsin D, and chymotrypsin (36). Previous studies using several MMP-7 substrates, including osteopontin (37), Fas ligand (38, 39), type IIa procollagen (40), procrystypsin (41, 42), and IGF-binding protein-3 (43), indicate that cleavage occurs frequently between a polar amino acid (e.g. Ser, Gln, Arg, Lys, Asp, and Glu at subsite P1) and a hydrophobic amino acid (e.g. Leu, Ile, and Val at subsite P1’). The MMP-7 target site we identified in FN is typical in its P1’ subsite (Leu) but atypical in its P1 subsite (Pro). The data from systematic screening of MMP-7 targets using random hexapeptides (44) indicate a weak preference for Pro at P1’, moderate preference for Val at P1’2, and frequent occurrence of prolines and phenylalanines in the N-terminal side of the target sites, which is the case in our site. How these residues contribute to the interaction with MMP-7 and why RECK permits this cleavage at this particular site are interesting questions to be addressed in future studies.

RECK expression is known to be down-regulated by several oncogenes, including activated RAS (1, 45). HT1080 cells contain activated N-RAS and show minimal RECK expression (1, 45). FN fibrils are decreased in transformed cells through several mechanisms, including reduced FN expression, reduced FN receptor expression, and increased FN degradation (18). Our data indicate that forced expression of RECK alone in HT1080 cells has some effect in increasing the amount of intact FN both inside and outside the cells (Fig. 8). Of note, a substantial fraction of RECK is associated with some intracellular compartments besides the cell surface, and at least a part of it seems to be involved in endocytosis (15). The nature and functions of RECK inside the cell and their relevance to the abundant FN found inside the RECK-expressing cells are also important issues to be addressed in future studies.

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RECK Forms Cowbell-shaped Dimers and Inhibits Matrix Metalloproteinase-catalyzed Cleavage of Fibronectin

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