Low Density Lipoprotein Receptor-related Protein Mediates Endocytic Clearance of Pro-MMP-2/TIMP-2 Complex through a Thrombospondin-independent Mechanism*

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The low density lipoprotein receptor-related protein (LRP) mediates the endocytic clearance of various proteins and proteinase-inhibitor complexes, including thrombospondin (TSP)-dependent endocytosis of matrix metalloproteinase (MMP)-2 (or gelatinase A), a key effector of extracellular matrix remodeling and cancer progression. However, thezymogen of MMP-2 (pro-MMP-2) mostly occurs in tissues as a complex with the tissue inhibitor of MMPs (TIMPs)-2. Here we show that clearance of the pro-MMP-2/TIMP-2 complex is also mediated by LRP, because addition of receptor-associated protein (RAP), a natural LRP ligand antagonist, inhibited endocytosis and lysosomal degradation of [125I]-pro-MMP-2/TIMP-2. Both TIMP-2 and the pro-MMP-2 collagen-binding domain independently competed for endocytosis of [125I]-pro-MMP-2/TIMP-2 complex. Surface plasmon resonance studies indicated that pro-MMP-2, TIMP-2, and pro-MMP-2/TIMP-2 directly interact with LRP in the absence of TSP. LRP-mediated endocytic clearance of [125I]-pro-MMP-2 was inhibited by anti-TSP antibodies and accelerated upon complexing with TSP-1, but these treatments had no effect on [125I]-pro-MMP-2/TIMP-2 uptake. This implies that mechanisms of clearance by LRP of pro-MMP-2 and pro-MMP-2/TIMP-2 complex are different. Interestingly, RAP did not inhibit binding of [125I]-pro-MMP-2/TIMP-2 to the cell surface. We conclude that clearance of pro-MMP-2/TIMP-2 complex is a TSP-independent two-step process, involving (i) initial binding to the cell membrane in a RAP-insensitive manner and (ii) subsequent LRP-dependent (RAP-sensitive) internalization and degradation.

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that mediate tissue remodeling both in physiological and pathological situations. In particular, MMP-2 (or gelatinase A) has been originally associated with the metastatic potential of tumor cells. After intradermal implantation of cancer cells, mice devoid of MMP-2 show markedly reduced angiogenesis, tumor growth, and metastasis. Most MMPs are secreted as inactive proenzymes and are subsequently activated by other MMPs or by serine proteinases, either in the extracellular matrix or nearby the plasma membrane. Membrane-type (MT)-MMPs are anchored to the cell membrane by a C-terminal transmembrane domain or by a glycosylphosphatidylinositol anchor. The catalytic activity of MMPs is specifically inhibited by tissue inhibitors of MMPs (TIMPs) and gelatinases A (MMP-2) and B (MMP-9) mostly occur in biological tissues as latent enzymes complexed to their specific inhibitor, TIMP-2 and TIMP-1, respectively. Both gelatinase and pro-enzyme repeats homologous to fibronectin type II modules inserted in their catalytic domain, whereby they interact equally well with thrombospondin (TSP-1) or TSP-2. MMPs are multidomain macromolecules that function as regulators of cell behavior by interacting with specific cell surface receptors, cytokines, growth factors, and proteases.

Beside their ability to degrade extracellular matrix molecules, MMPs process or degrade numerous bioactive peripheral substrates, including other proteinases, proteinase inhibitors, chemotactic molecules, latent growth factors, growth factor-binding proteins, cell surface receptors, and cell-cell adhesion molecules. The control of pericellular MMP activities therefore requires tight regulation, via local activation or silencing. Activation of pro-MMP-2 involves ternary interactions. The C-terminal domain of pro-MMP-2 specifically interacts with the C-terminal domain of TIMP-2 to form a heterodimeric pro-MMP-2/TIMP-2 complex. Next, binding of the TIMP-2 moiety of this complex via its N-terminal part to the catalytic site of one MT1-MMP molecule localizes the pro-MMP-2 moiety to the cell surface, allowing its pericellular activation by associated or neighboring MT1-MMP unoccupied.

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1 The abbreviations used are: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinases; TSP, thrombospondin; LRP, lipoprotein receptor-related protein; CBD, collagen-binding domain; SPR, surface plasmon resonance; EGF, epidermal growth factor; DME, Dulbecco’s modified Eagle medium; TBS, Tris-buffered saline; MT, membrane-type.
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by TIMP-2 (14). Conversely, the membrane-associated glycoprotein, RECK, inhibits MMP-2, as well as MMP-9 and MT1-MMP catalytic activities (15). In addition, endocytosis, a general clearance mechanism for extracellular material, also contributes to the elimination of MMP-2, -9, and -13 by a receptor-mediated process, which is not yet fully understood (16–18).

The low density lipoprotein receptor-related protein (LRP), a member of the low density lipoprotein receptor superfamily, is a heterodimeric endocytic receptor comprising a large ligand-binding, integral membrane subunit (the 515-kDa heavy chain), and a non-covalently associated transmembrane partner (the 85-kDa light chain) (19). LRP mediates the endocytic clearance of several classes of ligands, including urokinase-type plasminogen activator (uPA) complexed to plasminogen activator inhibitor-1 (20, 21), TSP-1 (22), TSP-2 (23), fibronectin (24) as well as several matrix metalloproteinases: MMP-2 (16), MMP-9 (17), and MMP-13 (18). LRP-ligand complexes are first transported into endosomes where they dissociate in the acidic environment. Ligands are then delivered to lysosomes for degradation, whereas LRP is recycled to the cell surface for a new round of receptor-mediated endocytosis. A 39-kDa protein, originally identified by its co-purification with LRP (25) and thereby termed receptor-associated protein (RAP), interacts with its C-terminal heparin-binding sites with this receptor at a high affinity (26, 27). RAP efficiently blocks the binding and uptake of all known ligands of LRP (28) but also of other members of the low density lipoprotein-receptor superfamily, such as megalin. There is some evidence that TSP may promote LRP-mediated endocytosis of MMP-2 and its subsequent lysosomal degradation (16). Interaction between MMP-2 and TSP was suggested by a yeast two-hybrid screening of TSP-1 and -2 partners (10), but no direct evidence was provided that TSP could bind latent or active MMP-2 complexed with TIMP-2.

In the present report, we investigated the possible interaction with LRP and fate of pro-MMP-2-TIMP-2 complex in HT1080 human fibrosarcoma cells as a model system, where this or related processes have been previously studied by other groups (29–31). First, LRP involvement was tested by the effect of RAP on the accumulation of endogenous pro-MMP-2 and TIMP-2 in the culture medium, as well as on radioligand surface binding and receptor-mediated endocytosis. Second, the role of TSP in the endocytosis of pro-MMP-2-TIMP-2 was explored. Because MMP-2 equally binds to TSP-1 and -2, which are internalized and degraded through similar pathways (22, 23), we compared the effect of precomplexing with TSP-1, or of antibodies blocking its interaction with either MMP-2 or LRP, on the uptake and degradation of pro-MMP-2 and pro-MMP-2-TIMP-2 complex. Third, to analyze the role of each partner of the complex, we tested the ability of CBD 123, a recombinant protein comprising the entire MMP-2 collagen-binding domain (32), and of TIMP-2 to compete for LRP-mediated endocytosis and degradation of the 125I-pro-MMP-2-TIMP-2 complex and their potential for cross-competition. Finally, all relevant interactions with LRP were measured by surface plasmon resonance (SPR).

EXPERIMENTAL PROCEDURES

Materials—Cell culture medium, fetal calf serum, and other cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Albumin from bovine serum albumin or human serum albumin, chloroquine, and cycloheximide were obtained from Sigma. Na125I and Iodogen® pre-coated tubes were from Amersham Biosciences and Pierce, respectively. The ECL immunoblotting detection kit was from PerkinElmer Life Sciences.

Proteins and Antibodies—Recombinant human pro-MMP-2 and TIMP-2 were expressed in HEK-293 cells using the mammalian expression vector pCEP4 (Invitrogen) (33). Pro-MMP-2 was purified using gelatin-Sepharose affinity chromatography and gel filtration with S-200 (Amersham Biosciences) as described previously (34). TIMP-2 was purified using Green A Dynatex affinity chromatography (Millipore, Billerica, MA) and gel filtration with S-200 (33). Pro-MMP-2-TIMP-2 complex was purified from conditioned medium of human uterine cervical fibroblasts, as described (34). CBD 123 protein, the recombinant collagen-binding domain (CBD) comprising the three fibronectin type II-like modules of human MMP-2, was expressed in Escherichia coli and purified by gelatin-Sepharose 4B chromatography as reported previously (35). Purified full-length human LRP (36) was kindly provided by Dr. S. K. Møestrup (University of Aarhus, Aarhus, Denmark). Recombinant RAP was prepared as described (37). Epidermal growth factor (EGF) was purchased from R&D Systems Europe (Lille, France). TSP-1 from human platelets and anti-TIMP-2 mouse monoclonal antibody (clone T2–101) were from Calbiochem. Anti-LRP mouse monoclonal antibody 3F11 was a generous gift from Prof. D. K. Strickland (American Red Cross, Rockville, MD). Four anti-TSP mouse monoclonal antibodies (Ab-1, -3, -9, and -11) were purchased from NeoMarkers (Lab Vision Co., Fremont, CA). Ab-1 recognizes an epitope localized in the properdin-like type 1 repeats of TSP-1, a domain that interacts with both MMP-2 and MMP-9 (10). In contrast, Ab-9 is directed against an epitope present in the N-terminal, heparin-binding domain, which mediates interaction with LRP. Ab-11 recognizes an epitope localized in the C-terminal part of TSP-1 and does not prevent its interaction with LRP (38). Ab-11 is a mixture of monoclonal antibodies designed for sensitive detection by Western blotting.

Cell Culture and RAP Treatment—Human fibrosarcoma HT1080 cells were purchased from American Type Culture Collection and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% (v/v) fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM t-glutamine, and 25 mM HEPES buffer at 37 °C in a humid atmosphere (5% CO2 and 95% air). In the experiments involving RAP treatment, subconfluent cells were cultured in the absence or presence of 500 nM recombinant RAP for 24 h in serum-free medium. These 24-h-conditioned media were then analyzed by gelatin zymography to detect MMP-2 and by Western blotting to detect TIMP-2 and TSP-1.

Gelatin Zymography—Sample volumes, normalized to cell protein content, were dialyzed in non-reducing 2% (w/v) SDS sample buffer and electrophoresed on 10% polyacrylamide SDS gels containing 1% (w/v) gelatin. After electrophoresis, gels were washed at room temperature for 1 h in 2.5% (v/v) Triton X-100 or 2% SDS buffer in water, incubated at 37 °C for 16 h in 50 mM Tris-HCl buffer, pH 7.5, containing 200 mM NaCl and 5 mM CaCl2. After incubation, gels were stained for 30 min with 0.1% (w/v) C-250 Coomassie Blue in 45% (v/v) methanol, 10% (v/v) acetic acid glacial, and destained in the same solution without dye.

Western Blotting—Equal amounts of proteins of conditioned medium (20 μl) were resolved by SDS-PAGE using 15% (v/v) or 10% (v/v) gels containing 15% (v/v) SDS and electrophoresed on 10% SDS-polyacrylamide gels. After electrophoresis, gels were washed at room temperature for 2 h in 2.5% (v/v) trichloroacetic acid (TCA) buffer. Proteins were stained with a 0.5% (w/v) Coomassie Blue solution in 45% (v/v) methanol, 10% (v/v) acetic acid glacial, and destained in the same solution without dye.

Radiiodination of Proteins—Proteins were labeled with Na125I and Iodogen® according to manufacturer’s recommendations. Specific activities ranged from 5 to 20 μCi/mg. Trichloroacetic acid-perprecipitable radioactivity of radioligands used was always >97%.

Radioligand Binding onto Cultured Cells—HT1080 cells (3 × 105) were plated onto 35-mm dishes and cultured overnight, then washed twice with assay medium (DMEM containing 0.1% bovine serum albumin) adjusted to pH 7.5. Cells were then incubated with 10 nM 125I-pro-MMP-2-TIMP-2 complex in assay medium at 4 °C for 2 h, in the absence or presence of 500 nM RAP. After careful rinsing (nine times with cold phosphate-buffered saline, on ice), cells were surface-digested with 0.1% (v/v) Pronase in DMEM at 4 °C to degrade surface-bound ligands and cause cell detachment. After cell collection by centrifugation, radioactivity released in supernatant (Pronase-sensitive), measured by γ counting, was defined as surface-bound.
ligand. Binding of 125I-pro-MMP-2-TIMP-2 complex to plastic dishes without cells did not exceed 8% of total bound radioactivity.

**Endocytosis and Degradation Assays**—Alternatively, cells adapted to 4 °C were incubated with 10 nM 125I-pro-MMP-2-TIMP-2 complex in assay medium at 4 °C for 2 h, in the absence or presence of 1 μM unlaeled CBD 123, 1 μM unlabeled TIMP-2, or 500 nM RAP. In other experiments, cells were incubated with assay medium containing 10 nM 125I-CBD 123, in the absence or presence of 1 μM unlabeled TIMP-2 or 500 nM RAP, or containing 10 nM 125I-TIMP-2, in the absence or presence of 1 μM unlabeled CBD 123 or 500 nM RAP. After binding, cells were carefully rinsed nine times with cold phosphate-buffered saline and further cultured in assay medium pre-warmed at 37 °C for the indicated times. To distinguish surface-binding from intracellular accumulation, cells were washed twice with cold phosphate-buffered saline, and surface-digested with Pronase as above. Radioactivity associated with pelleted cells (Pronase-resistant) was defined as internalized ligand. After precipitation of the medium by 10% (w/v) trichloroacetic acid and centrifugation, radioactivity in the supernatant was taken to indicate the amount of degraded 125I-ligand. To inhibit lysosomal activity, 100 μM chloroquine was added to some cultures 2 h before radioligand binding. Internalization and degradation were then studied as above after 2 h at 37 °C, in the continued presence of chloroquine.

**Effect of TSP-1 on the Binding and Degradation of 125I-Pro-MMP-2-TIMP-2 Complex**—After two washes in serum-free DMEM, cells were cultured at 37 °C for 18 h in fresh serum-free medium supplemented with 10 μg/ml of Ab-3, Ab-9, or preimmune mouse IgG1 as control. After cooling dishes on melting ice for 1 h, cells were incubated with 10 nM 125I-pro-MMP-2-TIMP-2 complex or 125I-Pro-MMP-2 at 4 °C for 2 h, washed extensively, and incubated again at 37 °C to follow internalization and degradation as above. Alternatively, serum-free DMEM was supplemented with 20 μg/ml cycloheximide for 30 min to inhibit protein synthesis. TSP-1-Ab-1 complex was prepared extemporaneously by mixing TSP-1 with anti-TSP Ab-1 monoclonal antibody (10 nM each) at 37 °C for 30 min. 10 nM TSP-1 or TSP-1-Ab-1 complex was then mixed with 10 nM 125I-pro-MMP-2-TIMP-2 at 37 °C for 30 min (10). Cycloheximide-treated cells were rinsed twice, then incubated with 10 nM 125I-pro-MMP-2, 125I-Pro-MMP-2-TIMP-2, 125I-Pro-MMP-2-TIMP-2 complex, or 125I-Pro-MMP-2-TIMP-2/TSP-1-Ab-1 mixtures, in the absence or presence of 500 nM RAP and in the continued presence of cycloheximide. Internalization and degradation assays were performed as described above.

**Surface Plasmon Resonance Assay**—Interaction of various ligands with LRP was tested using a BIAcore X system (BIAcore AB, Uppsala, Sweden). LRP was immobilized on a CM5 sensor chip by amine coupling at 25 °C. LRP was immobilized on a CM5 sensor chip by amine coupling at 25 °C. LRP was immobilized on a CM5 sensor chip by amine coupling at 25 °C.

**Table I**

**Kinetics of ligand binding to LRP measured by surface plasmon resonance**

| Ligands       | $k_a \times 10^6$ | $k_d \times 10^{-7}$ | $K_D \times 10^{-7}$ |
|---------------|-------------------|-----------------------|-----------------------|
| Pro-MMP-2     | 1.0 ± 0.4         | 3.2 ± 0.1             | 3.5 ± 1.4             |
| CBD 123      | 0.4 ± 0.1         | 1.3 ± 0.7             | 2.7 ± 1.1             |
| TIMP-2       | 0.8 ± 0.1         | 5.2 ± 0.3             | 6.6 ± 1.5             |
| Pro-MMP-2/TIMP-2 complex | 3.2 ± 1.0 | 1.3 ± 0.3 | 6.5 ± 0.2 |
| TSP-1        | 21 ± 10           | 2.5 ± 1.1             | 0.01 ± 0.01           |

**RESULTS**

The **LRP Competitor, RAP, Causes Accumulation of Pro-MMP-2, TIMP-2, and TSP-1 in Medium Conditioned by HT1080 Cells**—Using HT1080 human fibrosarcoma cells that express a functionally active LRP at their surface (21), we found that treatment of cultures with RAP, a classic competitor of LRP-ligand interaction (41), caused a 3- to 4-fold increase of the accumulation of pro-MMP-2, TIMP-2, and TSP-1 in conditioned media (Fig. 1). These results confirm that LRP mediates the cellular uptake of pro-MMP-2 (16) and TSP-1 (22) and suggest for the first time its role in TIMP-2 receptor-mediated endocytosis as well.

**Direct Interaction of LRP with Pro-MMP-2, TIMP-2, and Pro-MMP-2-TIMP-2 Complex**—Because pro-MMP-2 is naturally complexed with TIMP-2 (7), we next investigated whether not only pro-MMP-2 or TIMP-2, but also pro-MMP-2-TIMP-2 complex can physically interact with LRP, using purified LRP immobilized on a BIAcore sensor chip. As shown in Table I, a direct interaction was demonstrated for each candidate ligand. Affinity was in the submicromolar range of affinity, and was 5- to 10-fold higher for the complex as compared with its individual partners. TSP-1 also showed a direct interaction with LRP, with a nanomolar range of affinity, in good agreement with a previous report based on radioligand competition assay in LRP-coated multiwells (22). Because the affinity for the pro-MMP-2-TIMP-2 complex was almost two orders of magnitude lower than for TSP-1, we used a protein that is not expected to interact at all with LRP to obtain a limit of significance. For this purpose, we used EGF, which has been previously shown by similar SPR studies not to bind to immobilized LRP (40). Although a significant peak was obtained for pro-MMP-2-TIMP-2 complex at a concentration as low as 0.3 μM, no signal was detected after injection of EGF at 6 μM. Similarly, human serum albumin at 15 μM was found not to bind to LRP (data not shown). The lack of interaction of these two proteins with LRP contrasts with the well known behavior of another member of...
The low density lipoprotein-receptor superfamily, megalin.

**RAP Sensitivity Allows the Dissection of Two Steps in Receptor-mediated Internalization and Degradation of 125I-Pro-MMP-2/TIMP-2 Complex in HT1080 Cells**—Because pro-MMP-2/TIMP-2 complex can physically interact with LRP, we next investigated whether RAP prevents cellular binding, which leads to accelerated endocytosis and degradation. When added at 4°C in saturating concentrations, high amounts of 125I-pro-MMP-2/TIMP-2 complex were found to bind to the surface of HT1080 cells (about 240,000 complexes/cell); surprisingly, binding was not sensitive to RAP (Fig. 2). In contrast, after transfer to 37°C to allow endocytosis to proceed, bound 125I-pro-MMP-2/TIMP-2 complex was internalized and degraded by a RAP-sensitive mechanism (Fig. 2). The endocytic rate (−0.6%/min⁻¹; Fig. 3, top panel) is rather slow for receptor-mediated endocytosis (typically reaching −10%/min⁻¹ for transferrin and up to −30%/min⁻¹ for occupied growth factor receptors) (42), but this value is comparable to those previously found for other LRP ligands, such as TSP (22), uPA (43), and MMP-13 (18). Intracellular 125I-pro-MMP-2/TIMP-2 complex leveled off after 1 h (Fig. 3, top panel). Internalized counts were progressively released into the extracellular medium as trichloroacetic acid-soluble material (Fig. 3, bottom panel). There was no detectable release of intact ligand: after 2 h at 37°C, the sum of surface-bound, intracellular, and released trichloroacetic acid-soluble counts was equal to initial surface-bound values at 4°C (Fig. 2). Because the release of trichloroacetic acid-soluble radioactivity was suppressed by chloroquine treatment, known to inhibit access to lysosomes and to block lysosomal proteolysis (44), the plateau of intracellular ligand reflected a balance between entry and lysosomal degradation. The absence of release of intact ligand points to an irreversible sequestration and eventual full degradation by the cells (i.e., efficient clearance). Altogether, these data indicate that 125I-pro-MMP-2 complexed with TIMP-2 first interacts with a RAP-insensitive receptor that secondarily leads to a RAP-sensitive internalization leading to lysosomal degradation.

**TSP Is Not Required for LRP-mediated Clearance of 125I-Pro-MMP-2/TIMP-2 Complex in HT1080 Cells**—MMP-2 can interact with TSP (10), and this association promotes internalization by LRP (16). To determine if TSP also promotes LRP-mediated endocytosis of pro-MMP-2/TIMP-2 complex by HT1080 cells, we first resorted to immunological interference of TSP-1 interaction with LRP. To this aim, HT1080 cells were maintained overnight in serum-free medium (thereby allowing secretion of endogenous TSP-1), in the presence of monoclonal antibody Ab-9 directed against the N-terminal heparin-binding domain of TSP-1, by which it interacts with LRP, or, for a rigorous negative control, with monoclonal antibody Ab-3 directed against its C-terminal end, which is not involved in the TSP-LRP interaction (38). Then, 125I-pro-MMP-2 was added at 4°C without washing, either alone or as complex with TIMP-2, and internalization and degradation were studied as in Fig. 2. Blocking Ab-9 antibody significantly inhibited (by −40%) internalization (Fig. 4, top panel) and subsequent degradation of 125I-pro-MMP-2 added alone (Fig. 4, bottom panel) but not those of 125I-pro-MMP-2/TIMP-2 complex. The non-blocking Ab-3 antibody affected neither pro-MMP-2 nor pro-MMP-2/TIMP-2 complex processing (Fig. 4). We conclude that endogenous TSP-1 promotes LRP-mediated pro-MMP-2 clearance but is not required for that of pro-MMP-2/TIMP-2 complex.

To verify this conclusion more directly, we examined whether pre-complexing with exogenous TSP-1 would promote endocytic clearance and degradation of 125I-pro-MMP-2, but not of 125I-pro-MMP-2/TIMP-2 complex. To this aim, medium was depleted of endogenous TSP-1 by pre-treating HT1080 cells with cycloheximide for 30 min, so as to inhibit short-term protein synthesis without affecting the capacity of receptor-mediated endocytosis via LRP (45). Under these conditions, pro-MMP-2 (zymography) and TIMP-2 and TSP-1 (Western blotting) were no longer detected in the conditioned media (not shown). 125I-pro-MMP-2 alone or complexed with TIMP-2 was
first mixed with free TSP-1, or with TSP-1 complexed with Ab-1 antibody that blocks its interaction with MMP-2 (10), then presented to HT1080 cells. As previously reported by Yang et al. (16), complexing with TSP-1 promoted both internalization and degradation of $^{125}$I-pro-MMP-2 (Fig. 5, left). This increase was suppressed when TSP-1 had been preincubated with Ab-1 antibody (prior to mixing with $^{125}$I-pro-MMP-2). In contrast, TSP-1 was unable to promote receptor-mediated endocytosis and degradation of $^{125}$I-pro-MMP-2-TIMP-2 complex (Fig. 5, right). We conclude that LRP-mediated endocytic clearance of pro-MMP-2-TIMP-2 complex occurs independently of TSP.

Independent Involvement of Collagen-binding Domain of MMP-2 and TIMP-2 in LRP-mediated Internalization and Degradation of $^{125}$I-Pro-MMP-2-TIMP-2 Complex by HT1080 Cells—To further dissect the interaction of pro-MMP-2-TIMP-2 complex with LRP at the cell surface, we tested its two components separately. Table I already showed that pro-MMP-2 and TIMP-2 directly interact with immobilized LRP on a BIAcore chip. To simplify the analysis, we restricted ourselves to the recombinant collagen-binding domain of MMP-2 (32), which includes the heparin-binding site known to be recognized by LRP (27, 46). BIAcore studies confirmed that CBD 123 is sufficient for recognition by immobilized LRP and measured its $K_D$ value at $2.7 \times 10^{-7}$ M, comparable to the affinities of pro-MMP-2 and pro-MMP-2-TIMP-2 complex (Table I). Comparison of the various kinetics revealed that association rate constants of pro-MMP-2, CBD 123, and TIMP-2 were almost identical, with $k_a$ values ranging from $0.4$ to $1.0 \times 10^4$ M$^{-1}$s$^{-1}$, and ~4-fold faster than for pro-MMP-2-TIMP-2 complex. In addition, the dissociation constant measured for the complex, which was comparable to that measured for CBD 123, was ~2-fold slower than that of pro-MMP-2, with $k_d$ values at $1.3$ and $3.2 \times 10^{-3}$ s$^{-1}$, respectively.

In cultured HT1080 cells, a 100-fold molar excess of either CBD 123 or TIMP-2 efficiently competed for LRP-mediated endocytic clearance and degradation of $^{125}$I-pro-MMP-2-TIMP-2 complex (Fig. 6). These results suggest that both MMP-2, via its collagen-binding domain, and TIMP-2 participate in the internalization and subsequent degradation of pro-MMP-2-TIMP-2 complex mediated by LRP. Conversely, we directly studied HT1080 cell association and LRP-mediated endocytosis of $^{125}$I-CBD 123 and $^{125}$I-TIMP-2 (Fig. 7). As found for pro-MMP-2-TIMP-2 complex, RAP had no effect on the binding at 4°C of $^{125}$I-CBD 123 and $^{125}$I-TIMP-2 (see time zero of reincubation), but strongly inhibited their endocytosis and degradation in a chloroquine-sensitive compartment. There was no competition between CBD 123 and TIMP-2 for receptor-mediated endocytosis. We conclude that the two components of the pro-MMP-2-TIMP-2 complex first interact with a RAP-insensitive receptor on the cell surface and are next recognized by distinct binding sites of LRP, which mediates internalization and causes transfer to lysosomes for degradation.
LRP-mediated Clearance of Pro-MMP-2-TIMP-2 Complex

This report analyzes the molecular mechanisms of LRP-mediated clearance of gelatinase A, a key effector of extracellular matrix remodeling in cancer progression, when it is presented as pro-MMP-2 complexed with TIMP-2, which is the major form occurring in biological tissues (5). These experiments were initiated by the observation that addition of RAP, a LRP antagonist, caused a strong accumulation not only of pro-MMP-2, but also of its specific inhibitor, TIMP-2, in culture medium of the human fibrosarcoma HT1080 cell line. This result pointed to LRP as an essential mediator of the catabolism of the complex. Direct interaction of pro-MMP-2, TIMP-2, and pro-MMP-2-TIMP-2 complex with immobilized LRP was demonstrated by SPR analysis. Cell uptake experiments established that LRP mediates the endocytosis and degradation of pro-MMP-2-TIMP-2 complex or of its two components added separately and discriminated a two-step cell surface process, based on RAP sensitivity. The use of monoclonal antibodies blocking TSP-1 interaction with either pro-MMP-2 or LRP demonstrated that LRP mediates endocytosis of pro-MMP-2-TIMP-2 complex independently of TSP, contrary to non-complexed pro-MMP-2 (16).

The detailed kinetic SPR analyses of LRP interaction with pro-MMP-2-TIMP-2 complex and its two components confirm the relatively low affinity indicated by comparative radioligand competition studies using LRP-coated microtiter wells (17). In this type of assay, the reference was pro-MMP-9-TIMP-1 complex, the affinity of which to immobilized LRP was estimated at ~20 nM, whereas the affinity of LRP to immobilized pro-MMP-9-TIMP-1 reached 0.6 nM, leading to the suggestion that LRP may contain several binding sites for this complex. Using the same assay, these authors reported that pro-MMP-2-TIMP-2 complex was a much poorer ligand to LRP; the affinity was predicted to be at least 10- to 100-fold weaker than for pro-MMP-9-TIMP-1, as confirmed by the exact value determined with the BIACore system. This weak affinity might explain why Yang et al. (16) could not detect direct interaction between MMP-2 and immobilized LRP. Compared with more conventional techniques, such as radioligand competition assays, SPR allows the detection of macromolecular interactions in real-time, label-free mode, i.e. the kinetics of association and dissociation. Both BIACore and radioligand competition studies on HT1080 cells demonstrate that each component of the complex can interact independently with LRP. Therefore, one would have expected that simultaneous interaction of the two components would greatly increase the affinity of the complex for LRP by slowing down the dissociation rate, because it is known for the difference between the bivalent IgG and its monovalent Fab fragment (47). Because the affinity was similar for both partners and the pro-MMP-2-TIMP-2 complex, we conclude that either LRP immobilized on the sensor chip does not interact simultaneously with pro-MMP-2 and TIMP-2 in the complex (in other words, interactions of each component of the complex are mutually exclusive due to steric hindrance), or that changes in three-dimensional structure due to complexing offset the benefit of the bivalent interaction.

The strong interaction of proMMP-9-TIMP-1 complex with LRP readily accounts for its RAP-sensitive internalization and degradation, by a process independent of other partners such as TSP (17). LRP mediates the endocytic clearance of fibronectin (24), which interacts with TSP (48), which shows itself to have a high affinity for LRP (~1 nM, see Table I). Similarly, because pro-MMP-2 exhibits a weak interaction with LRP but can also interact with TSP, TSP-MMP-2 complexing was predicted to promote LRP-mediated MMP-2 clearance, as reported by Yang et al. (16) and confirmed by this study. However, whereas pro-MMP-2 shows a low affinity for TSP-2 (in the micromolar range) (49), it displays a high affinity for TIMP-2 (in the subnanomolar range) (50). These values explain why pro-MMP-2 predominantly occurs in tissues as pro-MMP-2-TIMP-2 complex (7), and predict that pro-MMP-2-TSP-2 complex would only exist when TSP-2 is in large molar excess over TIMP-2. We therefore addressed whether TSP could also promote LRP-mediated clearance of pro-MMP-2-TIMP-2 complex. Because TSP-1 and TSP-2 are equivalent in terms of pro-MMP-2 binding and cellular uptake (10, 22, 23), we verified that TSP-1, like TSP-2 (16), promotes endocytosis of pro-MMP-2. Using two complementary approaches (immunological interference with the formation of complex between pro-MMP-2-TIMP-2 and endogenous TSP-1; or addition of this pre-formed complex as radioligand), we found that TSP had no detectable role in LRP-mediated clearance of pro-MMP-2-TIMP-2 complex.

Whereas RAP is a potent inhibitor of the endocytic clearance of pro-MMP-2-TIMP-2 complex by HT1080 cells at 37 °C, its inability to compete for surface binding at 4 °C suggests that LRP is not the primary cell surface binding site for pro-MMP-2-TIMP-2 complex, as previously reported for some of its other ligands. Several of the primary co-receptors of LRP have been identified. LRP-mediated catabolism of TSP (22), lipoprotein lipase (51), and tissue factor pathway inhibitor (52) depends on cell surface proteoglycans. Similarly, MMP-13 first binds to an unidentified 170-kDa receptor, then is transferred to LRP for internalization (18). Primary binding to a non-endocytic receptor might function to concentrate ligands in specialized microdomains of the plasma membrane, which would facilitate transfer to LRP for internalization and degradation. Lipid rafts, generated by glycosphingolipid, sphingomyelin, and cholesterol packaging in the plasma membrane (53), embed glycosylphosphati-

![Fig. 6. Independent competition for LRP-mediated endocytosis and degradation of 125I-pro-MMP-2-TIMP-2 complex by CBD 123 and TIMP-2. HT1080 cells were incubated with 10 nM 125I-pro-MMP-2-TIMP-2 complex at 4 °C for 2 h to allow surface binding, in the absence or presence of 1 μM CBD 123 or TIMP-2. After washing, cells were reincubated in fresh medium pre-warmed at 37 °C and supplemented as above, for 2 h. Intracellular (top panel) and degraded tracer (bottom panel) were determined as in Fig. 2. Values are means ± S.D. of three dishes. This experiment was performed twice with similar results.](http://www.jbc.org/doi/10.1074/jbc.M105100200)
dylinositol-anchored proteins (54) and are reported to contain LRP (55). It has been suggested that association of LRP with rafts is essential for internalization of uPA/plasminogen activator inhibitor-1 complexes after their primary binding to the glycosylphosphatidylinositol-anchored uPA receptor (21).

Together with the SPR studies, inhibition of LRP-mediated endocytosis of $^{125}$I-pro-MMP-2/TIMP-2 complex by CBD 123 or TIMP-2 and the lack of competition between CBD 123 and TIMP-2 demonstrate that the two components bind at the different sites of LRP on HT1080 cells and presumably to the RAP-insensitive primary receptor as well. Several cell surface binding sites reported for pro-MMP-2 and TIMP-2 could represent co-receptors of LRP (56, 57). MT1-MMP acts as a cell surface receptor for TIMP-2 bound to pro-MMP-2 (13), and both MT1-MMP and pro-MMP-2 have been localized in rafts (58–60). In addition, MT4-MMP, a glycosylphosphatidylinositol-anchored proteinase that binds TIMP-2 (61), similarly colocalizes with uPA receptor in rafts (62). The mechanism of entry and fate of internalized TIMP-2 is less clear. Three groups addressed this issue in HT1080 cells. Maquoi et al. (29) showed that, after binding to MT1-MMP, TIMP-2 is rapidly internalized and subsequently degraded in lysosomes. Remacle et al. (30) reported that MT1-MMP mediates TIMP-2 internalization by concomitant clathrin-dependent and -independent pathways. In contrast, Zucker et al. (31) reported that, following binding to MT1-MMP on the cell surface, internalized TIMP-2 is released primarily as an intact functional molecule.

The endocytic receptor LRP is strongly regulated in physiopathological conditions. For example, down-regulation of proteolytic activities in the endometrium during the secretory phase prevents inappropriate degradation of extracellular matrix and is regarded to offer optimal conditions for successful implantation of the embryo. Expression of LRP mRNA significantly increases from the proliferative to the secretory phase, when progesterone concentration is the highest (63). This observation could explain why, in human endometrial stromal cells, uPA activity decreases upon stimulation by progesterone (64). Another example is tumor invasion and metastasis, a hallmark of which is extracellular matrix breakdown. Invasive cancer cells derived from human prostate or breast tumors express lower levels of LRP compared with their non-invasive counterparts (65). Similarly, LRP expression decreases in late stages of melanocytic tumor progression (66) and invasive endometrial carcinoma (63). LRP-defective embryonic fibroblasts...
exhibit accelerated migration on vitronectin, a property associated with cell invasive potential (67), and invalidation of MMP gene expression stimulates HT1080 cells to migrate on vitronectin and to invade reconstituted basement membrane matrices (68). Further, the ability of MT1-MMP to degrade LRP (69) may represent a powerful mechanism for malignant cells to down-regulate the clearance of matrix-degrading pro- teases by LRP and thus contribute to their aggressive phenotype. These various observations point to an inverse correlation between extracellular matrix breakdown and MMP expression and functionality and underline the role of LRP-mediated endocytosis in the regulation of pericellular MMP activities.

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