Collagenase-3 Binds to a Specific Receptor and Requires the Low Density Lipoprotein Receptor-related Protein for Internalization*

Olga Y. Barmina, Hobart W. Walling, Gerald J. Fiacco, José M. P. Freije‡, Carlos López-Otín‡, John J. Jeffrey§, and Nicola C. Partridge¶

From the Department of Pharmacological and Physiological Science, St. Louis University School of Medicine, St. Louis, Missouri 63104, [Department of Bioquímica y Biología Molecular, Universidad de Oviedo, 33006 Oviedo, Spain, and] Department of Biochemistry, Albany Medical College, Albany, New York 12208.

We have previously identified a specific receptor for collagenase-3 that mediates the binding, internalization, and degradation of this ligand in UMR 106-01 rat osteoblastic osteosarcoma cells. In the present study, we show that collagenase-3 binding is calcium-dependent and occurs in a variety of cell types, including osteoblastic and fibroblastic cells. We also present evidence supporting a two-step mechanism of collagenase-3 binding and internalization involving both a specific collagenase-3 receptor and the low density lipoprotein receptor-related protein. Our data suggest that the 170-kDa protein is a specific collagenase-3 receptor. Low density lipoprotein receptor-related protein-null mouse embryo fibroblasts bind but fail to internalize collagenase-3, whereas UMR 106-01 and wild-type mouse embryo fibroblasts bind and internalize collagenase-3. Internalization, but not binding, is inhibited by the 39-kDa receptor-associated protein. We conclude that the internalization of collagenase-3 requires the participation of the low density lipoprotein receptor-related protein and propose a model in which the cell surface interaction of this ligand requires a sequential contribution from two receptors, with the collagenase-3 receptor acting as a high affinity primary binding site and the low density lipoprotein receptor-related protein mediating internalization.

Collagenase-3 (MMP-13) is a member of the matrix metalloproteinase family of enzymes, which participates in extracellular matrix remodeling (1). Members of this family have a number of structural and functional features in common. In addition to sharing a similar domain structure, all are synthesized in inactive form, function at neutral pH, and require intrinsic zinc and calcium ions for their activity. Collagenase-3 is a highly regulated enzyme that cleaves native fibrillar collagens of types I, II, and III. The 57-kDa proenzyme is converted to its active 52-kDa form by the plasmin activation cascade, as well as by cathepsin B, stromelysin, and plasma kallikrein (2, 3). Collagenase-3 activity is inhibited by a family of tissue inhibitors of metalloproteinases (4, 5). A range of hormones and agents can also regulate expression of collagenase-3 (6). Parathyroid hormone is one of the hormones participating in this process. Osteoblastic cells respond to parathyroid hormone by increasing collagenase-3 synthesis (6–8), plasminogen activator activity (9), and tissue inhibitors of metalloproteinases expression (8). In addition, experiments with UMR 106-01 rat osteosarcoma cells showed that over 80% of exogenous rat collagenase-3 was removed from the medium after 8 h of incubation (10). This rapid removal of rat collagenase-3 from the medium suggested the existence of a specific receptor that represents another level of regulation of this enzyme. Previous work (10) established the existence of this receptor and showed that it has a high affinity for collagenase-3 (Kd = 5 × 10⁻⁹ M), with approximately 12,000 receptors per UMR 106-01 cell. In this report, we show that the receptor is specific for collagenase-3 among the matrix metalloproteinases, demonstrate that binding activity is present on other cells, and describe the two-step process of binding and internalization that requires both a specific 170-kDa collagenase-3 receptor and LDL-receptor-related protein (LRP).

EXPERIMENTAL PROCEDURES

Materials—Cell culture media, fetal bovine serum (FBS), and other cell culture reagents were purchased from the Washington University Tissue Culture Support Center, St. Louis, MO. The following chemicals were purchased from Sigma: ascorbic acid, bovine serum albumin, chloramine T, proteinase E (Pronase), sodium iodide, sodium metabisulfite, Tween 20 and Tween 80, isopropylthio-β-D-galactoside, glutathione and glutathione-agerone, thrombin inhibitor, CHAPS, insulin, transferrin. Na⁺/I⁻ and ECL immunoblotting detection kit were purchased from Amersham Pharmacia Biotech. Bovine serum albumin was purchased from Roche Molecular Biochemicals. SDS-polyacrylamide gel electrophoresis materials and nonfat dry milk were purchased from Bio-Rad and Amresco.

Proteins, Antibodies, and Plasmids—Purified rat collagenase-3 was isolated from media of cultures of post-partum rat uterine smooth muscle cells as described previously (11). The pGEX-receptor-associated protein (RAP) expression construct was a kind gift from Dr. Joachim Herz (University of Texas Southwestern Medical Center, Dallas, TX). The rabbit polyclonal antibody raised against the RLP receptor was a gift from Dr. Dudley Strickland (American Red Cross, Rockville, MD). Human RAP from the pGEX-RAP expression vector was expressed in bacteria and prepared as described previously (12). Human collagenase-3 was produced in a vaccinia virus-based expression system as described (13). 92 kDa and 72 kDa gelatinases were kind gifts from Dr. Howard Welgus (Washington University, St. Louis, MO). Human stromelysin was a generous gift from Dr. Paul Cannon (Syntex, Palo Alto, CA).

This paper is available on line at http://www.jbc.org

Received for publication, March 31, 1999, and in revised form, July 26, 1999.

* This work was supported by National Institutes of Health Grants AR 40661 (to N. C. P.) and HD 05291 (to J. J. J.) and National Aeronautics and Space Administration Grant NAG 5-4538. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed; Dept. of Pharmacological and Physiological Science, St. Louis University School of Medicine, 1402 S. Grand Blvd., St. Louis, MO 63104. Tel.: 314-577-8551; Fax: 314-577-8554; E-mail: partrinc@slu.edu.

‡ The abbreviations used are: MMP, matrix metalloproteinase; FBS, fetal bovine serum; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]1-propanesulfonic acid; RAP, receptor-associated protein; LRP, low density lipoprotein receptor-related protein; LDL, low density lipoprotein; MEM, Eagle’s minimal essential medium.
Cell Culture—The following cell lines were cultured according to ATCC recommendations: the human osteosarcoma cell line SAOS-2 (ATCC HTB 85); the mouse embryo fibroblast cell line NIH 3T3 (ATCC CRL 1658). UMR 106-01 rat osteosarcoma cells were cultured as described previously (5), but 5% FBS was used instead of 10% FBS. The rat breast carcinoma BC-1 cell line was cultured in 1:1 Dulbecco’s modified Eagle’s medium:Ham’s F-12 medium with 25 mM HEPES, pH 7.1, 5 mM/ml insulin, 1 mM/mg transferrin, 5 mg/ml bovine serum albumin, 10 units penicillin/ml, and 10 μg streptomycin/ml. The rat osteosarcoma cell line ROS 17/2.8 was cultured in Ham’s F-12 medium with 5% FBS, 1% glutamine, 10 μg of streptomycin/ml, 1×10^6 M HEPS. Normal rat osteoblasts were isolated from newborn rat calvariae as described previously (14) and cultured in Eagle’s minimal essential medium (MEM) containing 10% FBS, nonessential amino acids, 10 units penicillin/ml, 10 μg streptomycin/ml. After cells reached confluence, the culture medium was changed to BGJb medium containing 10% FBS, 10 units of penicillin/ml, 10 μg of streptomycin/ml, 50 μg/ml ascorbic acid, and 2.16 mg/ml β-glycerophosphate to allow differentiation and mineralization. Wild-type (MEF-1) and LRP-null (MEF-2) mouse embryo fibroblasts were generous gifts from Dr. Joachim Herz. These cells were cultured in Dulbecco’s MEM with 10% FBS, 10 units penicillin/ml, 10 μg streptomycin/ml. Radioiodination of Proteins—Protein labeling with 125I was done using the chloramine T method (15). The proteins had specific activities ranging 19 to 27 μCi/μg.

Binding Assays—For all binding experiments, cells were seeded into 2 cm² wells. After the cells reached approximately 95% confluence, the medium was replaced with fresh medium containing 1 mg/ml bovine serum albumin, and the cells were assayed for binding 4 h later. The cells were first washed with maintenance medium, then incubated in the same medium with 0.01% Tween 80 containing 125I-labeled rat collagenase-3 or other ligated ligands at 4°C for 2 h. Nonspecific binding was assessed by adding a 50–100-fold excess of cold ligand to the medium. After washing, the cells were blocked with 5% nonfat dried milk in buffer containing 50 mM NaCl, pH 7.1, 5 mM MgCl₂, 0.25 M sucrose, 1 mM phenylmethylsulfonyl fluoride. The 100,000 g membrane pellet was resuspended in buffer containing 50 mM Tris-HCl, pH 8.5, 2 mM CaCl₂, 80 mM NaCl (16). The samples of cell membranes were subjected to 4–15% SDS-polyacrylamide gel electrophoresis under non-reducing conditions at 50 V for 3 h and then electrotransferred to polyvinylidene difluoride filters in transfer buffer containing 10% methanol, 192 mM glycine, 56 mM Tris at 15 V for 16 h at 4°C. The filters were blocked with 5% nonfat dried milk in buffer containing 50 mM Tris-HCl, pH 8.0, 80 mM NaCl, 2 mM CaCl₂, and 0.1% Triton X-100 (binding buffer) for 1 h at room temperature. The filters were then incubated for 16 h at 4°C in the same buffer supplemented with 1% nonfat dried milk in the presence of 20 pmol of 125I-labeled rat collagenase-3 or 20 pmol of 125I-GST-RAP in the presence or absence of the same unlabeled ligands (30–40-fold excess of rat collagenase-3, 170-fold excess of GST-RAP). The filters were then washed with the same buffer, dried, and subjected to autoradiography.

Western Blot Analysis—The filters used for ligand blot analysis were wetted with methanol for 2 s, rinsed with H₂O, and equilibrated with buffer containing 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20. The filters were incubated 2 h at room temperature in the same buffer containing 5% nonfat dried milk. The filters were subsequently incubated with anti-LRP antibodies (1:2,000) in the same buffer containing 1% nonfat dried milk for 16 h at 4°C. A 1,000,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG in the same buffer containing 1% nonfat dried milk was incubated with the filters for 1 h at room temperature to detect the primary antibodies. Detection was performed using an ECL kit.

RESULTS

Previous work has shown that the rat collagenase-3 receptor is present on UMR 106-01 rat osteosarcoma cells (10). To characterize this receptor, we first tested a variety of other cells for rat collagenase-3 binding to ensure that this was not just restricted to a transformed rat osteosarcoma line. Specific binding was found in normal rat osteoblasts, rat embryo fibroblasts, and rat osteosarcoma cells, ROS 17/2.8 (Fig. 1). The binding of rat collagenase-3 to normal rat osteoblasts and normal rat embryo fibroblasts was higher than binding to the UMR 106-01 cells. We observed very low levels of binding in rat epithelial breast carcinoma cells, BC-1, mouse NIH 3T3 fibroblasts, and human osteosarcoma cells, SAOS-2. It has been demonstrated that osteoblastic cells in vitro can secrete a number of matrix metalloproteinases including collagenase-2 (8, 17–19), 72-kDa and 92-kDa gelatinase (19–22), and stromelysin-1 (19). These proteinases are thought to play an active role in extracellular matrix remodeling in bone tissue. As we have shown in our previous competition experiments (10), none of the various proteins we used was able to compete with rat collagenase. However, of the matrix metalloproteinases (MMPs), only human collagenase-1 (MMP-1) had been...
TABLE I
Analysis of 125I-labeled proteinases binding to UMR cells

Confluent UMR cells were incubated with 8 nM or 10 nM 125I-labeled rat collagenase-3 (rat MMP-13), human fibroblast collagenase-1 (MMP-1), human stromelysin-1 (MMP-3), human collagenase-3 (human MMP-13), 92-kDa gelatinase (MMP-9), and 72-kDa gelatinase (MMP-2) for 2 h at 4 °C. Nonspecific binding was assessed by addition of a 100-fold molar excess of the same unlabeled proteins. After 2 h, cells were washed, lysed, and assessed for radioactivity. The displayed values represent means ± S.E. for triplicate wells.

| Ligand | Specific binding |
|--------|------------------|
|        | fmol/10^6 cells  |
| Rat MMP-13 | MMP-1 | MMP-3 | Human MMP-13 | MMP-9 | MMP-2 |
| 8      | 95.8 ± 3.8       | 0.4 ± 5.4 | 0.0 ± 0.0 | 135.8 ± 6.5 | 0.0 ± 0.0 |
| 8      | 76.2 ± 4.2       | 0.0 ± 0.0 | 0.0 ± 0.0 | 4.2 ± 0.4 | 15.0 ± 4.2 |
| 10     | 107.3 ± 4.6      | 0.0 ± 0.0 | 0.0 ± 0.0 | 135.8 ± 6.5 | 0.0 ± 0.0 |

tested. Since the publication of our initial observations on the rat collagenase receptor (10), human collagenase-3 (MMP-13) had been cloned and shown to be homologous to the rat and mouse collagenases (13). In addition, supplies of the other MMPs had become available. To show the specificity of the rat collagenase receptor in UMR 106-01 rat osteosarcoma cells, we investigated the ability of these cells to bind other MMPs. Ligand binding assays were performed using rat collagenase-3 (rat MMP-13), human fibroblast collagenase-1 (MMP-1), human stromelysin-1 (MMP-3), human collagenase-3 (human MMP-13), human 92-kDa gelatinase (MMP-9), and human 72-kDa gelatinase (MMP-2). As shown in Table I, only human collagenase-3 was comparable to rat collagenase-3 in binding to UMR cells. This was expected since human collagenase-3 has 86% homology to rat collagenase-3 (13). Human collagenase-3 also competes effectively with 125I-labeled rat collagenase-3 for binding to the collagenase receptor (Fig. 2). This result argues for the existence of a specific receptor for collagenase-3 on osteoblastic cells, in contrast to collagenase-1, which has never been observed to be produced by these cells nor to bind or compete for binding. We next conducted a binding assay on UMR 106-01 cells using 125I-labeled rat collagenase in the presence and absence of Ca²⁺ to investigate the requirements of ligand-receptor interaction for this ion (Table II). The results showed that Ca²⁺ is necessary for rat collagenase-3 binding to its receptor.

To determine the molecular weight of the rat collagenase-3 receptor, we next performed a ligand blot assay using partially purified UMR 106-01 cell membranes. It was found that 125I-labeled rat collagenase-3 bound to two proteins with molecular masses of about 600 kDa (●) and 170 kDa (Fig. 3, panel 1,*). 125I-Collagenase binding was highly specific, since a 40-fold excess of unlabeled rat collagenase abolished binding to both proteins (Fig. 3, panel 2).

As described previously (23), rat collagenase-3 undergoes a process of binding, internalization, and degradation following secretion from UMR 106-01 cells. We hypothesized that the mechanism might be similar to the internalization of the members of the low density lipoprotein (LDL) receptor superfamily (24, 25). Therefore, we proposed that one of the proteins that showed collagenase-3 binding on ligand blot analysis might be a member of the LDL receptor superfamily. Among members of this superfamily, only two have molecular masses around 600 kDa: LRP and gp330/megalin. None of the LDL superfamily, except for LRP, we next performed a ligand blot assay using partially purified UMR 106-01 cell membranes. It was found that 125I-labeled rat collagenase-3 binding to these cells (Fig. 6), whereas MEF-2 cells do not (Fig. 4). Ligand blot and Western blot analyses showed that 125I-labeled rat collagenase-3 specifically binds to the large subunit of the LRP in UMR 106-01 and MEF-1 but not MEF-2 cell membranes (Fig. 5, panels 1, 2, and 5, ●). Also, 125I-RAP binds only the large subunit of the LRP in UMR 106-01 and MEF-1 cell membranes (Fig. 5, panels 1–5, ●). Furthermore, all three of these cell lines show binding of 125I-collagenase-3 to the 170-kDa protein (Fig. 5, panel 1,*). We have also noticed that both MEF-1 and MEF-2 cells have an additional protein with molecular mass of approximately 200 kDa, which specifically binds 125I-labeled rat collagenase-3 (Fig. 5, panel 1, ●).

125I-Collagenase-3 binding assays were performed with MEF-1, MEF-2, and UMR 106-01 cells. The results showed no significant difference in binding between wild-type and LRP-deficient cells, suggesting that the LRP is not required for collagenase-3 binding to these cells (Fig. 6). We have also...
shown that RAP does not inhibit 125I-labeled rat collagenase binding to the UMR cells, although it is known to inhibit binding of most ligands for the LRP (Fig. 7). These data suggest that the 170-kDa protein is a specific receptor for collagenase-3 in UMR 106-01 cells.

Although the LRP is not required for rat collagenase-3 binding to the cell, it might be required for ligand internalization. Therefore, we performed internalization assays with 125I-labeled rat collagenase-3 using MEF-1 and MEF-2 cells. The results showed that despite equal binding, MEF-2 cells cannot internalize rat collagenase-3. This suggests that the LRP is required for collagenase-3 internalization (Fig. 8). It is known that RAP inhibits internalization of ligands by the LRP (12, 33–35). Therefore, we next performed internalization assays using 125I-labeled rat collagenase-3 as a ligand and RAP as a competitor. Our results showed that internalization of 125I-labeled rat collagenase-3 was inhibited by RAP by approximately 70% in UMR 106-01 cells (Fig. 9). Kinetic studies also suggested that RAP acts as a competitive inhibitor for rat collagenase-3 binding to LRP for internalization (Fig. 9, inset).

We next compared the ability of RAP to inhibit internalization of 125I-labeled rat collagenase-3 in UMR 106-01 osteoblastic cells and normal rat osteoblasts. The presence of 100 mM RAP in binding medium reduced the intracellular accumulation of 125I-collagenase by 79% in UMR 106-01 cells and by 43% in normal mineralizing rat osteoblasts (Table III). The difference in inhibition might be explained by the presence of mineralized extracellular matrix in normal rat osteoblast cultures as well as the possibility that an additional mechanism may also operate for internalization of rat collagenase-3 in these cells. However, inhibition of collagenase-3 internalization by...
RAP in both transformed osteoblastic cells and normal osteoblasts suggests that the same type of receptor operates in both cell types.

To investigate the mechanism by which RAP regulates internalization of collagenase-3, we performed an experiment where excess unlabeled RAP or rat collagenase-3 was prebound to UMR 106-01 cells. Binding and internalization of 125I-labeled rat collagenase-3 and RAP were then allowed to proceed. The data showed that although prebound RAP inhibited rat collagenase-3 internalization, prebound rat collagenase-3 had almost no effect on RAP internalization (Fig. 10, A and B).

**Discussion**

In this paper we describe collagenase-3 interaction with the cell and show that it involves two receptors; the specific collagenase-3 receptor acts as the primary binding site, whereas the LRP is required for internalization. The LRP belongs to the LDL receptor superfamily (36). This superfamily consists of endocytic receptors that mostly participate in the recognition and endocytosis of lipoproteins (25). The receptors have high affinity for their ligands and broad specificity. They recognize not only lipoproteins but also a variety of nonlipoprotein ligands, including urokinase and tissue plasminogen activator with their inhibitors and participate in different physiological processes (26, 37–45). Ten members of this family are known to date: the LDL receptor itself, α2-macroglobulin receptor/low density lipoprotein receptor-related protein (α2MR/LRP), very low density lipoprotein receptor, Heymann nephritis antigen/megalin/gp330, chicken vitellogenin receptor, Drosophila yolks, chicken LR5B, placental calcium sensor protein (29), the newly discovered apolipoprotein E receptor 2 (apoER2) (30), and LR11 (31). These receptors share a similar structure, with a single transmembrane domain and numerous ligand binding domains organized as cysteine-rich repeats arranged in clusters, followed by two epidermal growth factor-like repeats separated from a third one by a spacer region containing a YWTD consensus sequence and an NPXY internalization signal in the cytoplasmic domain.

Binding assays showed that the collagenase-3 receptor is present mostly in osteoblasts and fibroblasts. Interestingly, cell surface binding of collagenase-3 does not necessarily correlate with expression of collagenase-3 by these cells. For example, ROS 17/2.8 cells do not express collagenase-3, but the binding of the enzyme to ROS 17/2.8 cells was comparable to that of
and structural features, our data demonstrate high specificity of the collagenase receptor for rat collagenase-3 and human collagenase-3 but almost no binding of other MMPs. Similarly, we have shown that mouse collagenase-3 binds equally well as the rat enzyme (data not shown). Nevertheless, we cannot rule out the possibility that the receptor may have ligands other than collagenase-3.

Ligand and Western blot analyses showed that rat collagenase-3 can specifically bind to the large subunit of the LRP receptor and a protein with a molecular mass of approximately 170 kDa, which is present in membranes of UMR 106-01, MEF-1, and MEF-2 cells. Equal levels of rat collagenase-3 binding to UMR 106-01, wild-type (MEF-1), and LRP-null (MEF-2) cells suggested that the collagenase-3 receptor is present in all of these cell lines and that the LRP receptor does not participate in primary binding of collagenase-3 to the cell surface. Although MEF-1 and MEF-2 cells bind rat collagenase-3 equivalently, our experiments showed that MEF-2 cells cannot internalize the bound ligand. We have also observed that rat collagenase-3 internalization by UMR 106-01 cells was abolished in the presence of RAP. Therefore, we conclude that collagenase-3 interaction with the cell is a two-step process.

First, a specific collagenase receptor of 170 kDa acts as a primary binding site for collagenase-3 on the cell surface. Interaction between the LRP and the enzyme-receptor complex then occurs, resulting in internalization of collagenase-3. A similar process has been reported for urokinase-type plasminogen activator-plasminogen activator inhibitor type 1, tissue plasminogen activator-plasminogen activator inhibitor type 1, and urokinase-type plasminogen activator-recombinant protease nexin-1 complexes (46–48). In each case, the serine protease binds to a specific receptor as a primary event. The inhibitor then binds to the receptor-ligand complex, which leads to its rapid internalization and degradation by the LRP. In our studies, this latter process is similarly inhibited by RAP, which implicates the LRP. The exact mode by which transfer and internalization of the collagenase-3 ligand-receptor complex takes place is not completely understood. It is possible that the ligand dissociates from the specific receptor to bind to the LRP. Alternatively, the LRP might bind and internalize the entire receptor-ligand complex. Similarly, it is not known if collagenase-3 is internalized alone or in complex with its inhibitor and/or specific receptor. Recent data suggest that LRP-mediated internalization of the plasminogen activators involves the endocytosis of the primary binding receptor, which may then be recycled to the cell surface (49, 50).

Ligand blot studies showed that mouse embryo fibroblasts have an additional protein with a molecular mass of approximately 200 kDa, which also specifically bound 125I-labeled rat collagenase-3. We thus concluded that in these cells, three membrane proteins might be involved in collagenase-3 clearance, indicating that our proposed mechanism might vary somewhat in different cell types.

The results of inhibition studies showed that RAP abolished rat collagenase-3 internalization in UMR 106-01 cells, whereas collagenase-3 does not change the level of RAP internalization. Thus, collagenase-3 does not compete for binding to RAP sites on the LRP. In addition RAP may be a physiological modulator of collagenase-3 internalization by the LRP. It has been shown that RAP is coexpressed with either LRP or gp330 (51). However, it is still unknown whether RAP is expressed in osteoblastic cells. Further experiments may show the presence of RAP in bone tissue.

In conclusion, the two-step mechanism of collagenase-3 interaction with the cell may serve as one more link in the chain of fine regulation of collagenase-3 activity contributing to homeostasis of the extracellular matrix.

Acknowledgments—We thank Sandra Winchester and Joseph Lemker for assistance with tissue culture.

Note Added in Proof—The 170-kDa collagenase-3-binding protein was purified by affinity chromatography with recombinant collagenase-3. Sequencing identified this band as homologous to a previously cloned gene named a novel type C lectin (52).

REFERENCES
1. Nagase, H., Barrett, A. J., and Woessner, J. F. (1992) Matrix 1, (suppl.) 421–424
2. Eeckhout, Y., and Vaes, G. (1977) Biochem. J. 166, 21–31
3. Murphy, G., Cockett, M. I., Stephens, P. E., Smith, B. J., and Docherty, A. J. P. (1987) Biochem. J. 248, 265–268

a N. Selvamurugan, R. J. Brown, and N. C. Partridge, manuscript in preparation.
