Extracellular Collagenases and the Endocytic Receptor, Urokinase Plasminogen Activator Receptor-associated Protein/Endo180, Cooperate in Fibroblast-mediated Collagen Degradation*

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The collagens of the extracellular matrix are the most abundant structural proteins in the mammalian body. In tissue remodeling and in the invasive growth of malignant tumors, collagens constitute an important barrier, and consequently, the turnover of collagen is a rate-limiting process in these events. A recently discovered turnover route with importance for tumor growth involves intracellular collagen degradation and is governed by the collagen receptor, urokinase plasminogen activator receptor-associated protein (uPARAP or Endo180). The interplay between this mechanism and extracellular collagenolysis is not known. In this report, we demonstrate the existence of a new, composite collagen breakdown pathway. Thus, fibroblast-mediated collagen degradation proceeds preferentially as a sequential mechanism in which extracellular collagenolysis is followed by uPARAP/Endo180-mediated endocytosis of large collagen fragments. First, we show that collagen that has been pre-cleaved by a mammalian collagenase is taken up much more efficiently than intact, native collagen by uPARAP/Endo180-positive cells. Second, we demonstrate that this preference is governed by the acquisition of a gelatin-like structure by the collagen, occurring upon collagenase-mediated cleavage under native conditions. Third, we demonstrate that the growth of uPARAP/Endo180-deficient fibroblasts on a native collagen matrix leads to substantial extracellular accumulation of well defined collagen fragments, whereas, wild-type fibroblasts possess the ability to direct an organized and complete degradation sequence comprising both the initial cleavage, the endocytic uptake, and the intracellular breakdown of collagen.

The physiological mechanisms responsible for collagen degradation have long been subject to investigation. Due to their unique structural features, collagens can only be degraded by a minority of mammalian extracellular proteases, but certain matrix metalloproteases (MMPs), such as MMP-1, MMP-2, MMP-8, MMP-13, and the membrane-bound MMP-14 and -15, are indeed active against native collagens (2–10). The initial attack of these proteases leads to the generation of well defined collagen fragments, which, while still in the extracellular environment, may be subject to further degradation by gelatinases, MMP-2 or MMP-9, or other types of proteases (11–13).

Importantly, however, collagen may also be degraded through an intracellular turnover pathway (11, 14). Recent studies have shown that an endocytic route of collagen breakdown, mediated by the collagen internalization receptor, uPARAP/Endo180 (15–17) (hereafter designated uPARAP), is a rate-limiting factor in collagenolysis and invasive growth of breast tumors in mice (18). This new observation underscores the importance of understanding the function of uPARAP and the interplay between cellular and extracellular events in collagen and matrix turnover.

uPARAP is a type-1 transmembrane protein and a member of the mannose receptor protein family of constitutive endocytosis receptors (reviewed in Refs. 19 and 20). The extracellular portion includes a cysteine-rich domain, a fibronectin type-II domain, and eight consecutive C-type lectin-like domains (20). The receptor binds directly to various types of collagen through interactions governed, wholly or in part, by the fibronectin type-II domain (15, 21–23). uPARAP also has carbohydrate binding activity, mediated through the second C-type lectin-like domain (24), and on certain cell types it interacts with the urokinase plasminogen activator receptor (15, 25) in a process.
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implicated in signal transduction in connection with cell migration (26, 27).

The molecular interaction between uPARAP and collagen is reflected in part in a decreased initial adhesion of uPARAP-deficient cells on a collagen matrix, but a much more striking effect is noted on the ability of fibroblasts to internalize solubilized collagen, which is lost completely upon gene targeting of uPARAP (17, 23, 28). uPARAP has also been shown to be responsible for the uptake of collagen in hepatic stellate cells (29). Following endocytosis from clathrin-coated pits (30, 31), uPARAP is routed to early endosomes and later recycled to the cell surface, whereas, the initially bound collagen is routed to lysosomes for degradation (23, 28). The latter process is primarily undertaken by cysteine proteases (11, 32).

Consistent with a role in matrix turnover, the expression of uPARAP is clearly associated with sites of tissue remodeling. Thus, the receptor is expressed in osteoblasts of developing bone (33) and in myofibroblasts and certain other cell types in wound healing tissue (34). In connection with cancer, expression is observed in the myoepithelium of mammary carcinoma in situ, in the myofibroblasts of invasive mammary carcinoma, and in fibroblast-like cells in oral cancer (35, 36).

Little is known, however, about the interplay between the intracellular and extracellular processes that govern the breakdown of extracellular matrix components. In this work, we show that fibroblasts, a dominant cell type in collagen turnover, direct an organized degradation pathway that combines extracellular cleavage and uPARAP-mediated endocytosis. These observations assign a new function to uPARAP as an efficient endocytic receptor for early collagen cleavage products.

EXPERIMENTAL PROCEDURES

Proteins and Cultured Cells—Skin fibroblasts from newborn homozygous uPARAP-deficient mice and littermate control wild-type mice were isolated and cultured as published previously (17). Skin fibroblasts from mice homozygously deficient for MMP-2 (37), MMP-13 (38), or MMP-14 (7) were isolated and cultured in the same manner, in each case accompanied by wild-type control cultures obtained from littermate control mice. Recombinant, soluble uPARAP fusion protein (suPARAP), comprising the extracellular portion (Met1–Ser1402) of the human uPARAP sequence fused to a purification tag (the third domain of the urokinase receptor), was expressed in Drosophila Schneider S2 cells and purified as described (39). The monoclonal mouse anti-uPARAP antibody 2h.9:F12 was raised against purified suPARAP and produced as described previously (36). The following proteins were purchased from the commercial sources indicated: native trypsin-resistant collagen type I from rat tail (BD Biosciences), FITC-collagen type I from MD Biosciences (Zürich, Switzerland), cysteine protease inhibitor E-64d and recombinant, human pro-MMP-13 (Calbiochem, San Diego, CA), 4-aminophenylmercuric acetate (APMA) (Sigma-Aldrich), interleukin 1β, and tumor necrosis factor-α (Peprotech, Rocky Hill, NJ), and polyclonal rabbit antibody against collagen type I (Rockland, Gilbertsville, PA).

Cleavage of Collagen under Non-denaturing Conditions—For activation of pro-MMP-13, the pro-enzyme was diluted to 50 μg/ml in reaction buffer (100 mM Tris-Cl, 10 mM CaCl2, 1 μM ZnCl2, 100 mM NaCl, pH 7.0). APMA was added from a freshly prepared 20 mM stock solution in 0.1 mM NaOH to a final concentration of 1 mM, after which the sample was incubated for 30 min at 37 °C. For the removal of APMA after the activation step, the sample was subjected to four rounds of buffer exchange, each cycle including centrifugation through an Amicon Ultrafree-MC 10,000 NMWL Filter Unit, to 10% of the starting volume and replenishment of reaction buffer to 100% volume. For cleavage of collagen, collagen was diluted to 250 μg/ml in reaction buffer, after which the freshly activated MMP-13 was added to a final concentration of 8.75 μg/ml (~175 nM). The samples were incubated at 25 °C for 24 h.

Internalization Studies with Collagen and Collagen Fragments—Labeling of proteins with 125I and radioligand internalization assays with cultured newborn mouse fibroblasts were performed as described before (17) and included incubation of cells with radioligand in 24-well tissue culture plates for 3 h at 37 °C, followed by isolation of the intracellular fraction and quantification of the internalized radioactivity. Before the assay, the protein concentration and the specific radioactivity of each of the radiolabeled protein preparations was estimated by SDS-PAGE and silver staining, by careful comparison with a narrow dilution series of a known standard of the same protein preparation in the non-labeled form, run on the same gel. 30 ng of labeled intact or cleaved collagen was added to each well with near-confluent cells in a volume of 300 μl. In some experiments, the labeled collagen ligands were preincubated at elevated temperature before the assay, as indicated. Incubation of cultured fibroblasts with FITC-labeled intact and cleaved collagen-I (15 μg/ml) followed by confocal fluorescence microscopy with a Zeiss LSM 510 microscope was performed as described previously for fluorescence-labeled, intact collagen-IV (28), using an incubation period of 22 h in the presence of 20 μM E-64d.

Surface Plasmon Resonance Analysis—Interaction analyses with intact and cleaved collagen and immobilized suPARAP were done using a BIAcore2000 instrument (BIAcore, Uppsala, Sweden). Before interaction analysis, the monoclonal antibody 2h.9:F12, against uPARAP (36), was coupled to the surface of a CM5-type BIAcore chip by amine-directed coupling, following the instructions provided by the manufacturer. This chip was then used as a catching support for suPARAP, injected subsequently, to obtain a level of immobilized suPARAP of ~5500 resonance units. Due to the binding characteristics of the 2h.9:F12 antibody, this non-covalent immobilization step was irreversible under the conditions used, because the elution conditions for collagen (below) led to no release of bound suPARAP from the chip. Cleaved or intact collagen type-I was preincubated in assay buffer (0.01 M Hepes, 0.15 M NaCl, 1 mM CaCl2, 0.005% surfactant P20, pH 7.4) at 37 °C, or at elevated temperature as indicated, after which various concentrations of the protein were injected. After each round of binding and dissociation, the chip was regenerated by injection of 10 mM glycine/HCl, pH 2.0, to obtain complete dissociation of all bound collagen material. A parallel flow channel, containing coupled antibody but no suPARAP, served as a control for unspecific binding and buffer bulk subtraction. No binding of intact or cleaved collagen was obtained with antibody alone. The
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BIACore interaction analyses were otherwise performed as described (40), monitoring binding and dissociation at 20 °C in assay buffer at a flow rate of 10 μl/min. Curve fitting to kinetic models and calculation of kinetic parameters were done using the BIACeval software, version 3.2 RC1, supplied with the instrument.

Analysis of Collagen Turnover in Growing Fibroblasts—A stock solution of collagen type-I in 0.02 M acetic acid was brought to neutral pH by addition of the appropriate volume of phosphate buffer to obtain a final concentration of 300 μg/ml collagen, after which a trace amount of 125I-labeled collagen type-I was added to obtain a radioactivity of ~1,500,000 cpml/mg. Immediately thereafter, each well of a 24-well tissue culture plate was coated with 350 μl of the tracer-containing collagen solution. The collagen was gelled by incubation at 37 °C for 3 h and allowed to dry in a flow hood. For rehydration, the dried gel was washed with three changes of water, after which the wells were incubated with phosphate-buffered saline at room temperature for 24 h. Phosphate-buffered saline was removed, and 1 × 10⁶ cells were added to each well in a volume of 300 μl of Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, covering the bottom of the well. The cells were allowed to attach for 4 h, after which the medium was removed and replaced with 400 μl of Dulbecco’s modified Eagle’s medium, including 1 nM interleukin 1β and 10 nM tumor necrosis factor-α. After 5 days of cell culture, the conditioned medium from each well was collected. The media were analyzed by SDS-PAGE followed by Western blotting or automated phosphorimaging analysis as described previously (17). After harvest of the media, the cells and residual matrix were washed with phosphate-buffered saline. Cells were then detached by trypsinization, after which the matrix was stained with Coomassie Blue for visualization of zones of collagen clearance, as described before (7).

RESULTS

uPARAP-dependent Internalization and Degradation of Collagen Fragments—Because the extracellular routes of collagen turnover are initiated by a well defined cleavage in the triple helical structure (41), we wanted to learn whether the same initial event might serve as a starting point for the endocytic turnover are initiated by a well defined cleavage in the triple helical structure (41), we wanted to learn whether the same initial event might serve as a starting point for the endocytic degradation route in fibroblasts. Consequently, we undertook, studying the endocytic activity of fibroblasts from wild-type and littermate uPARAP-deficient mice (uPARAP−/−) enabled us to also test this uPARAP dependence for unknown samples, such as the collagen fragments.

This study revealed that both the intact and the cleaved collagen are taken up by wild-type (uPARAP+/+) cells (Fig. 2A, black columns). Strikingly, however, the uptake of the cleaved protein (column 5) was much more efficient than the internalization of the untreated collagen (column 1). This increased efficiency was indeed the result of proteolytic cleavage, because preincubation of intact collagen at 25 °C without collagenase-3 did not affect the internalization (column 3). The uptake of both cleaved and uncleaved collagen was completely uPARAP-dependent, because fibroblasts from littermate uPARAP−/− mice displayed no internalization (gray columns). To ensure that the uptake of the collagen fragments was indeed part of a degradation pathway, we also studied the effect of blocking lysosomal cysteine proteases in the fibroblasts. To this end, a similar experiment was set up with the inclusion of E-64d (Fig. 2B), an inhibitor that has been shown previously to block lysosomal degradation of internalized collagen-IV (28). Comparison of parallel samples obtained in the absence and presence of E-64d revealed that the blocking of intracellular degradation led to a marked increase in intracellular radioactivity in the uPARAP+/+ cells (Fig. 2B, black columns). Whereas this was the case both for intact collagen (columns 1 and 3) and cleaved...
The amount of collagen fragments accumulated in the cells was much larger than the amount of intact collagen, also in the presence of the inhibitor. The presence of E-64d did not affect collagen uptake in uPARAP/H11002/H11002 cells (gray columns).

To enable the visualization of the collagen fragments after endocytosis, FITC-labeled collagen type-I was treated with collagenase-3, leading to complete cleavage into fluorescent products with an electrophoretic migration pattern indistinguishable from that obtained with unlabeled collagen (Fig. 1A, lanes 3 and 4). The fluorescent fragments, as well as FITC-labeled intact collagen, were added to live fibroblasts from wild-type mice or littermate uPARAP-deficient mice, after which endocytosis was allowed to proceed in the presence of E-64d (Fig. 3).

Examining the wild-type cells by fluorescence microscopy after 22 h of endocytosis, a strong fluorescence was observed in intracellular vesicles in the cells incubated with the cleaved FITC-collagen (Fig. 3B). The same cellular distribution was observed with FITC-labeled intact collagen-I (Fig. 3D), but strikingly, the amount of accumulated, fluorescence-labeled intact collagen was much lower than that of the collagen fragments, although the amounts of added fluorescent protein were identical. Again, this endocytosis was completely uPARAP-dependent, because no uptake or intracellular accumulation occurred with uPARAP-deficient cells (Fig. 3, A and C). The perinuclear, vesicular distribution of fluorescent protein, obtained with both cleaved and intact collagen-I, was indistinguishable from that obtained with fluorescence-labeled collagen-IV (result not shown), confirming results reported previously where the latter ligand was shown to accumulate in lysosomes in response to the arrest of lysosomal degradation (28).

Altogether, these results showed that the defined proteolytic fragments of collagen-I are taken up much more efficiently than intact collagen by a uPARAP-dependent mechanism, through which they are directed to further lysosomal degradation.
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obtained with the lowest concentration (2 μg/ml) of collagen tested, with only marginal (<2 resonance units) deviations from the theoretical curve, as shown in the residual plot (Fig. 4A, lower panel). For this binding curve, an apparent $K_d$ in the sub-nanomolar range was estimated (see figure legend for kinetic parameters and curve fitting), in accordance with competition studies performed previously with uPARAP on intact cells (15).

Binding analyses were then performed on the cleaved collagen in the same system (Fig. 4B). Again, a strong interaction with the immobilized uPARAP was observed, but, as expected for a fragment mixture, the binding curves could in this case not be fitted satisfactorily to a 1:1 Langmuir binding model. Nevertheless, the sensorgrams still revealed some important new features. Apparently, the complexes formed were somewhat less stable than those obtained with intact collagen (compare the dissociation phases, $\sim 440 – 800$ s of Fig. 4, A and B), but an even much more pronounced difference was noted in the association phase. Comparing the early injection phase ($\sim 70 – 120$ s of Fig. 4 (A and B), it was evident that one or several components in the cleaved collagen had a much higher association rate with uPARAP than the intact collagen.

The conformational properties of collagens are highly sensitive to limited proteolysis. This is connected to the fact that collagen undergoes quite well defined denaturation transitions as a function of temperature (44). Thus, whereas intact triple helical collagen is conformationally stable at body temperature, the $\frac{1}{3}$ and $\frac{2}{3}$ fragments are both known to be conformationally unstable, gradually entering a gelatin-like state at $37^\circ C$ (45, 46). Intact collagen undergoes a very similar conformational transition but only after heating to higher temperatures (45). In the standard conditions employed above (see “Experimental Procedures”), all samples were kept at $37^\circ C$ before injection to present the collagen in the same state as adopted in the internalization assays. Consequently, a possible explanation for the increased uPARAP association rate in the samples with cleaved collagen could be the acquisition of a gelatin-like state in the fragments.

This hypothesis was supported by studies on heat-denatured collagen and fragments. Thus, preincubation of intact collagen at $65^\circ C$ before injection led to a binding curve with uPARAP characterized by a fast initial binding phase (Fig. 4C, upper curve), clearly distinct from the curve obtained with native, intact collagen (Fig. 4A) but very similar to the situation obtained with collagen fragments, irrespective of whether the latter had been incubated at $37^\circ C$ (Fig. 4B) or at $65^\circ C$ (Fig. 4C, lower curve).

Altogether, these experiments showed that collagen fragments, preincubated at body temperature, displayed an increased uPARAP association rate relative to intact collagen. This increase, however, could be mimicked by heat-denatured, intact collagen. Thus, the acquisition of a gelatin-like state in the fragments was responsible for the effect observed.

**Increased Uptake of Cleaved Collagen Is Mimicked by Heat-denatured, Uncleaved Collagen**—An increased association rate with uPARAP might explain the initial finding that cleaved collagen was internalized more efficiently than intact collagen by uPARAP-mediated endocytosis. If so, a high internalization
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FIGURE 5. Endocytosis of cleaved and heat-denatured collagen. Samples of 125I-labeled cleaved and intact collagen, respectively, were either preincubated at 65 °C for 20 min before the assay or used for internalization assay directly, as indicated. Internalization assays with uPARAP+/+ and uPARAP−/− cells were then carried out as described in the legend to Fig. 2. Error bars indicate standard deviations of triplicate samples.

This experiment revealed that the gelatin-like product, obtained by heating uncleaved collagen-I to 65 °C, was internalized much more efficiently than the unheated protein (compare columns 1 and 3). Actually, the uptake observed was very close to that obtained with the same amount of unheated cleaved collagen (column 5). Furthermore, in the case of the cleaved collagen, heating to 65 °C before the assay led to only a marginal increase in the internalization efficiency (column 7). Thus, in the latter case, the major part of the molecular transition responsible for the high efficiency had occurred already at 37 °C, closely reflecting the molecular behavior observed above during the BIACore binding studies. Importantly, none of these binding reactions were the result of a loss of specificity in the denatured state, because the cellular internalization was completely uPARAP-dependent for all the protein samples. This was shown using uPARAP-deficient cells as the negative control (Fig. 5, columns 2, 4, 6, and 8).

FIGURE 6. Fibroblasts direct the sequential cleavage of collagen and uptake of collagen fragments. A and B, fibroblasts from newborn uPARAP+/+ and littermate uPARAP−/− mice, respectively, were grown on a reconstituted collagen type-I matrix with a tracer of 125I-labeled collagen type-I included. After 5 days of cell culture, the conditioned culture supernatants were harvested and analyzed by non-reducing SDS-PAGE, followed by Western blotting with a polyclonal antibody against collagen-I (A) or by phosphorimaging analysis of the gel for localization of radiolabeled collagen and its degradation products (B). In each panel, the culture supernatants from the uPARAP+/+ and uPARAP−/− cells are shown in lanes 3 and 4, respectively. The following purified reference proteins are shown: A: lane 1, unlabeled, intact collagen type-I; lane 2, unlabeled collagen type-I, cleaved with collagenase-3 as described in the legend to Fig. 1A. B: lane 1, 125I-labeled, intact collagen type-I; lane 2, 125I-labeled, cleaved collagen type-I. The 1/4 and 3/4 collagen fragments are indicated. C, after harvest of the cell culture media analyzed in A and B, cells were detached, and the remaining collagen matrix was stained with Coomassie Blue. Upper sample: uPARAP+/+ cells. Lower sample: uPARAP−/− cells. D, upper row, fibroblasts homozygously deficient for MMP-13 (left), MMP-14 (center), and MMP-2 (right) were cultured as above, after which cells were detached followed by Coomassie staining of the matrix, performed as in C. Lower row, for each genotype, a parallel wild-type control sample was included, representing a fibroblast culture obtained from a littermate control wild-type mouse. Note the complete lack of collagen clearance in the MMP-14-deficient cell sample and the partial effect in the MMP-2-deficient sample.

This model might also be expected for heat-denatured collagen, because this ligand displayed the same steep association phase as the collagen fragments. Consequently, we compared the fibroblast-mediated internalization of radiolabeled collagen and collagen fragments after preincubation at different temperatures (Fig. 5).

Fibroblasts Direct the Sequential Cleavage of Collagen and the uPARAP-mediated Endocytosis and Degradation of Collagen Cleavage Products—Finally, we wanted to learn whether a sequential collagen degradation mechanism, involving both initial extracellular cleavage and endocytosis-dependent complete breakdown, is actually operative in living cells. Therefore, we seeded uPARAP-expressing and uPARAP-deficient fibroblasts on a reconstituted, trypsin-resistant collagen-I matrix and analyzed the content of the culture supernatants after 5 days of cell culture using Western blotting with an anti-collagen antibody for the detection of released collagen fragments (Fig. 6A). A striking difference was observed. In the culture supernatant of the uPARAP-deficient cells, well defined collagen fragments had accumulated (lane 4). A strong signal was observed at the electrophoretic position of the 1/4 collagen fragment (compare with the sample of cleaved purified collagen; lane 2) and the 3/4 fragment could also be detected, although with a lower band intensity. Strikingly, both of these fragments were absent in the culture supernatant of the wild-type cells (lane 3). In the latter sample, the only high molecular weight
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component recognized by the antibody was intact collagen-I, which was present in very low amounts. This material was likely to reflect diffusion into the medium of minor contents of non-cross-linked collagen from the matrix, because the same component was found in wells incubated with growth medium in the absence of cells (data not shown). In the low molecular mass region (<15 kDa), unidentified collagen fragments resulting from extensive degradation were evident. These degradation products, however, were present in the culture supernatants of both of the cell types.

In addition to being the primary cell type involved in collagen clearance, fibroblasts also synthesize collagen (47). Therefore, it was important to verify that the collagen fragments observed above were indeed derived through cleavage of the extracellular collagen compartment and were not just degradation products of aberrant neosynthetic collagen. This was ascertained through the incorporation of 125I-labeled collagen in the reconstituted collagen matrix. An autoradiogram of an SDS gel with the culture supernatant samples (Fig. 6B) revealed the same well defined collagen fragments in the uPARAP+/− supernatants as observed in Western blotting. Again, these fragments were absent in the uPARAP+/+ supernatant.

These experiments demonstrated that fibroblasts grown on a collagen matrix direct an initial collagen cleavage step, which leads to well defined fragments that accumulate in the extracellular space if uPARAP is not present on the cells. On the other hand, when the uPARAP pathway is operative, these fragments are efficiently endocytosed and degraded.

According to this notion, whereas uPARAP has a primary role in the clearance of the cleaved collagen, the endocytic event would not be expected to be the major determinant in the initial attack on the collagen matrix under these conditions. This was confirmed by comparing the zones of collagen clearance in the solid matrix after cell culture. Coomassie staining of the collagen matrix after detachment of the cells (7) revealed extensive zones of clearance, which were indistinguishable when comparing uPARAP-expressing and uPARAP-deficient cells (Fig. 6C). We therefore studied the effect of deletion of various candidate collagenase genes on this initial event, using primary fibroblast cultures from the respective gene-deficient mice and matched wild-type cell cultures from littermate control mice in each case (Fig. 6D). In accordance with results published previously (7), deficiency for the membrane-bound collagenase, MMP-14 (MT1-MMP), completely abolished collagen clearance (center wells). In contrast, deficiency for the soluble collagenase, MMP-13 (collagenase-3), led to zones of clearance indistinguishable from those in the wild-type control (wells to the left). Because MMP-14 acts both to degrade collagen directly (9, 48–50) and to activate MMP-2 (gelatinase-A (51)), which is a third protease with collagenolytic activity (3), we also studied the effect of MMP-2 deficiency (wells to the right). In this case, the gene deficiency led to a clearly discernible reduction in the degree of clearance but not a complete abolition. Thus, part of the function of MMP-14 in this system might be exerted through MMP-2 activation, but because the effect of MMP-14 deficiency was stronger than that of MMP-2 deficiency, a direct MMP-14-mediated collagen cleavage seemed to be central to the process, as also suggested previously (7).

Altogether, these results document the existence of a sequential collagen degradation pathway, initiated by pericellular collagenolytic activity and completed by uPARAP-mediated fragment uptake and intracellular degradation.

DISCUSSION

In this work, we show that uPARAP-mediated endocytosis of defined collagen fragments serves as a distinct step in a consecutive mechanism of clearance that involves extracellular collagen cleavage, uPARAP-mediated internalization, and lysosomal degradation. This observation adds an important new role of uPARAP to the previously established reactivity toward intact collagen (15, 17, 18, 22).

In the cell culture system studied, the uPARAP-dependent step may be indispensable, because the fragments from the initial cleavage accumulated in its absence. On the other hand, fragments of a much lower molecular weight did also appear with uPARAP-deficient cells, thus suggesting that parallel degradation systems are also operative (Fig. 6, A and B). Most likely, this alternative route is exclusively extracellular and involves the attack of a gelatinase such as MMP-2 on the fragments initially formed by collagenase cleavage (12). However, irrespective of any alternative mechanism, the lack of uPARAP makes the degradation of the initial fragments a rate-limiting step in the total process. Furthermore, comparing the defined, major fragments found in the cell culture system with uPARAP-deficient cells, the accumulation of the 1/4 fragment was much more pronounced than that of the 3/4 fragment. Therefore, any alternative route of degradation may be directed primarily against the larger fragment in this system.

An important question is which enzymes in the extracellular compartment serve to perform the initial cleavage of the triple helical collagen in the consecutive mechanism of collagen degradation described here. Because different proteases with collagenolytic activity exist, and because these enzymes have different expression patterns, it is likely that this cleavage can be performed by more than one enzyme in vivo and that the details of this event have at least some tissue specificity. Fibroblasts are generally recognized as a primary cell type in collagen turnover (47, 52). In our assay system with fibroblasts in vitro, MMP-14 was indispensable, whereas MMP-2 deficiency had only a partial effect. MMP-13, a third collagenase with expression in fibroblasts (5), could be deleted without any detectable effect in this assay system.

In addition to the sequential process described here, it is likely that alternative, parallel routes of collagen breakdown are also operative in vivo, depending on the physiological process in question. Thus, whereas MT1-MMP is centrally engaged in the initial collagen cleavage in the combined mechanism in our cell culture system, both uPARAP and MT1-MMP also take part in collagen turnover processes independently. This follows from the fact that mice with double deficiency for both of these proteins have an even more compromised collagenolytic function than mice deficient for either component alone.4 Furthermore, it is well established that uPARAP is also active against intact, 4 R. Wagenaar-Miller, T. H. Bugge, and K. Holmbeck, unpublished observations.
The complete lack of internalization of cleaved as well as intact collagen in fibroblasts from uPARAP-deficient mice suggests that uPARAP is the exclusive collagen clearance receptor in fibroblasts. It is noteworthy, however, that collagen interactions have also been reported for other members of the same protein family, i.e. the mannose receptor (57, 58) and the phospholipase A₂ receptor (59), which, thus, may serve to fulfill a similar function in other cell types. This has indeed been suggested in the case of the mannose receptor (58), although an actual endocytic function of this receptor with collagen or its fragments remains to be demonstrated.

Altogether, our work suggests that the interplay between extracellular proteolysis and specific endocytic mechanisms is a crucial factor in the directed breakdown of extracellular matrix material. This organized process is an important subject in the attempts to elucidate and manipulate matrix turnover in conjunction with several pathological processes, including those of cancer invasion.

Acknowledgments—We thank Dr. Z. Werb for generously providing the MMP-13-deficient mice for establishment of cell culture. The excellent technical assistance of Suzanne K. Møller and Katharina H. Stegmann is gratefully acknowledged, as is the expert photographic work of John Post.

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