Gelatinase A and membrane-type metalloproteinase (MT1-MMP) were able to process human procollagenase-3 (M, 60,000) to the fully active enzyme (Tyr36 N terminus; M, 48,000). MT1-MMP activated procollagenase-3 via a M, 56,000 intermediate (Ile36 N terminus) to 48,000 which was the result of the cleavage of the Glu84–Tyr85 peptide bond. We have established that the activation rate of procollagenase-3 by MT1-MMP was enhanced in the presence of progelatinase A, thereby demonstrating a unique new activation cascade consisting of three members of the matrix metalloproteinase family.

In addition, procollagenase-3 can be activated by plasmin, which cleaved the Lys38–Glu39 and Arg76–Cys77 peptide bonds in the propeptide domain. Autoproteolysis then resulted in the release of the rest of the propeptide domain generating Tyr36 N-terminal active collagenase-3. However, plasmin cleaved the C-terminal domain of collagenase-3 which results in the loss of its collagenolytic activity.

Concanavalin A-stimulated fibroblasts expressing MT1-MMP and fibroblast-derived plasma membranes were able to process human procollagenase-3 via a M, 56,000 intermediate form to the final M, 48,000 active enzyme which, by analogy with progelatinase A activation, may represent a model system for in vivo activation. Inhibition experiments using tissue inhibitor of metalloproteinases, plasminogen activator inhibitor-2, or aprotinin demonstrated that activation in the cellular system was due to MT1-MMP/gelatinase A and excluded the participation of serine proteases such as plasmin during procollagenase-3 activation. We have established that progelatinase A can considerably potentiate the activation rate of procollagenase-3 by crude plasma membrane preparations from concanavalin A-stimulated fibroblasts, thus confirming our results using purified progelatinase A and MT1-MMP. This new activation cascade may be significant in human breast cancer pathology, where all three enzymes have been implicated as playing important roles.

Degradation of the extracellular matrix during tumor invasion is thought to result from a combined action of several proteolytic enzyme systems, including the collagenases and other matrix metalloproteinases (MMPs)1, 2 and serine proteases, such as plasmin generated by the urokinase pathway of plasminogen activation (3).

Human collagenase-3 (MMP-13), a new member of the MMP family, is expressed by breast tumors and is likely to play a crucial role in the modulation of extracellular matrix degradation and cell-matrix interactions involved in metastasis (4). Procollagenase-3 comprises three distinct domains which include an 85-amino acid residue propeptide that is lost during activation (5), and in which the conserved sequence PRGVPD is responsible for the latency of the MMPs (6). This sequence is followed by the catalytic domain containing the active site of the enzyme linked via a short hinge sequence motif to the third, C-terminal domain, that shows homology to vitronectin and which is essential for the collagenolytic activity of collagenase-3.2 Collagenase-3 is a powerful collagenolytic and gelatinolytic enzyme that preferentially cleaves type I collagen, and it can therefore be implied that this enzyme may play a considerable role in connective tissue turnover (5). One of the key events in the regulation of extracellular collagenolytic activity is the activation of procollagenase-3, but there are currently only limited data available on how this may occur in vivo. We have recently shown that procollagenase-3 can be directly activated by stromelysin (5); however, other mechanisms may be of physiological and pathophysiological significance.

Increasing evidence is accumulating that the newly discovered membrane associated MMPs (MT-MMPs) act as cell surface activator(s) of progelatinase A (proMMP-2) under physiological or pathophysiological conditions (7-12). This mechanism was thought to be specific for progelatinase A, since other MMPs such as progelatinase B, fibroblast procollagenase, neutrophil procollagenase, and prostromelysin are not activated by MT1-MMP (13).3 These cell surface MT-MMPs th-

1 The abbreviations used are: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; MT1-MMP, membrane type metalloproteinase (MMP-14); CT1399, N-hydroxy-N1-(S-morpholinosulfonyl)aminomethylalanylcarbonyl)-2-cyclohexyl-ethyl-2-(R)-(4-chlorophenylpropyl)succinamide): PAI-2, plasminogen activator inhibitor; ConA, concanavalin A; McaPLGLDpaAR, (7-methoxycoumarin-4-ylacetroyl-Pro-Leu-Gly-Leu-[3-(2,4-dinitrophenyl)-2,3-diaminopropionyl]Ala-ArgNH2; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

2 V. Knauper and G. Murphy, unpublished results.

3 G. Murphy, H. Will, and V. Knauper, unpublished results.
selves may be activated intracellularly within the constitutive secretory pathway since they contain a RRXXRK/R furin recognition site, and it has been demonstrated that stromelysin-3, which also contains a similar motif, can be activated by furin (14).

Here we describe the activation of procollagenase-3 by recombinant gelatinase A and MT1-MMP which leads to the generation of extracellular collagenolytic activity. MT1-MMP and gelatinase A act by forming a proteolytic activation cascade which results in rapid activation of procollagenase-3. ConA-stimulated fibroblast monolayers expressing MT1-MMP and plasma membranes derived from them both activate procollagenase-3, which results in rapid activation of procollagenase-3. ConA-stimulated fibroblast monolayers expressing MT1-MMP and plasma membranes derived from them both activate procollagenase-3, thus providing evidence for a cell-surface activation cascade mechanism for procollagenase-3 similar to that described for progelatinase A.

MATERIALS AND METHODS

Expression of Recombinant Procollagenase-3 (ProMMP-13).—Wild Type, and Glu179→Ala Progelatinase (ProMMP-2).—Purification of the Pro-Human and Human Recombinant Procollagenase-3 and recombinant wild type and Glu179→Ala progelatinase A were expressed using stably transfected NSO mouse myeloma cells and purified from serum-free conditioned cell culture medium as recently described (5, 15, 16). Plasmin was generated by activation of plasminogen (1 mg/ml; Sigma) using streptokinase (10 µg/ml; Sigma) at 37°C for 15 min.

Expression and Purification of Recombinant TIMP-1, TIMP-2, and TIMP-3.—Recombinant tissue inhibitors of metzaloproteinase (TIMPs) were prepared from the culture medium of transfected NSO myeloma cells essentially as described elsewhere (17–19).

Expression of Recombinant Catalytic Domain of Membrane Type Matrix Metalloproteinase (ProMT1-MMP; MMP-14) and Purification—A protein fragment consisting of the propeptide and catalytic domain of MT1-MMP (Δψ99, numbering includes the signal sequence) was synthesized in Escherichia coli. In order to generate an expression plasmid the sequence of nucleotides 183–991 of the MT1-MMP cDNA was amplified by polymerase chain reaction using the primers 5'-CCGCCATGGCCTCCCTCGGCTGC-3' and 5'-ACCTGGTGACACCACTGACTCAC-3', thereby introducing NcoI and BstEII restriction sites (underlined). The amplified cDNA was inserted into the NcoI and BstEII restriction sites of an E. coli expression plasmid derived from pUC119 which contains a lacZ promoter and synthetic oligonucleotides encoding the pel B leader sequence (pel B: pectate lyase B of Escherichia coli was synthesized in in vitro transcription—translation in the presence of 720 ng of active gelatinase A for the time intervals (Fig. 1, lane 1) or ConA-stimulated fibroblast membrane preparations (Fig. 8B, lane 1) identified the M, 60,000 band of the proenzyme and the M, 48,000 band of the active form.

Processing of Procollagenase-3 (ProMMP-13) by Fibroblast Monolayers—Human foreskin fibroblasts were plated in 24-well Liser dishes and grown to confluence in DMEM in the presence of 10% fetal calf serum, washed with serum-free DMEM, and stimulated for 24 h with 50 µg/ml ConA in serum-free DMEM. The cell monolayers were incubated with either 100 ng of procollagenase-3 alone or in the presence of 100 ng or 10 µg of plasminogen in 300 µl of serum-free DMEM for 24 h at 37°C. In some experiments either TIMP-1, TIMP-2, TIMP-3, the peptide hydroxamate CT1399 (N4-hydroxy-N1-(L-(5-morpholinolinsulfonylaminoethylamino)carbonyl)-2-cyclohexyl-ethyl)-2-(R)-(4-chlorophenyl)-propanamide), aprotinin, or PAI-2 were included in order to demonstrate inhibition of processing. The conditioned medium from three wells was concentrated using Amicon microcon concentrators (M, 10,000 cutoff) and analyzed by SDS-PAGE followed by Western blotting. In all instances the conditioned media were incubated with human recombinant procollagenase-3 raised in sheep, and antigen-antibody complexes were detected using peroxidase-conjugated donkey anti-sheep IgG (Dako) using the Pierce enhanced chemiluminescent detection kit and the recommended protocol.

Preparation of Procollagenase-3 (ProMMP-13) by Con A-stimulated Human Foreskin Fibroblast Membranes—Fibroblast membrane preparations were prepared from fibroblast cultures grown to confluence and suspended with 50 µg/ml ConA essentially as described previously (13). Resuspended membranes (15 µl; 1 mg/ml protein) were incubated with 50 ng of procollagenase-3 alone or in the presence of 50 ng of progelatinase A for 4–16.5 h at 37°C. Procollagenase-3 was incubated either alone or in the presence of gelatinase A in buffer as the appropriate control for processing. The collagenolytic activity of 16.6 ng of activated collagenase-3 was monitored by incubation with 2.5 µg of type II collagen for 16 h at 25°C and analyzed by SDS-PAGE. Molecular mass changes of 33 ng of procollagenase-3 during the membrane-mediated activation were demonstrated by Western blotting as described above.

RESULTS

Activation of Procollagenase-3 (ProMMP-13) by Gelatinase A (MMP-2)—Human procollagenase-3 was processed by active gelatinase A (23:1 molar ratio) which resulted in the rapid generation of enzymatic activity within 60 min (Fig. 1) with a concomitant loss of the propeptide domain, thereby reducing the molecular mass of the proenzyme (M, 60,000) to 48,000 (Fig. 1, lanes 2–4). N-terminal sequence determination re-
revealed that the Glu\textsuperscript{84}–Tyr\textsuperscript{85} peptide bond was hydrolyzed during the activation reaction (Fig. 2). Due to the lack of specific MMP inhibitors which would block the activity of each enzyme we have been unable to exclude the possibility that transient intermediate forms may be generated.

Activation of Procollagenase-3 (ProMMP-13) by the Recombinant Catalytic Domain of MT1-MMP (MMP-14)—Human procollagenase-3 was susceptible to cleavage by the active form of the catalytic domain of MT1-MMP which resulted in activation of the proenzyme as demonstrated in Fig. 3. The relative molecular mass of the proenzyme (M, 60,000) was reduced to 48,000 which coincided with the increase in enzymatic activity (Fig. 3, lanes 2–5). At a 9:1 molar ratio nearly all the proenzyme was converted to the M, 48,000 active species after 240 min of incubation. The activation of procollagenase-3 by MT1-MMP proceeded without the generation of visible intermediates in the absence of inhibitors. However, activation of procollagenase-3 in the presence of TIMP-1 by MT1-MMP revealed that an intermediate species was generated with an apparent M, of 56,000 (Fig. 4, lane 2), as also observed in cell activation experiments (see “Processing of Procollagenase-3 (proMMP-13) by ConA-stimulated Fibroblast Monolayers and Influence of Plasmin in the Processing Rate”). N-terminal amino acid sequence analysis revealed that the Gly\textsuperscript{35}–Ile\textsuperscript{36} peptide bond within the propeptide domain was initially hydrolyzed by MT1-MMP which was followed by the cleavage of the Glu\textsuperscript{84}–Tyr\textsuperscript{85} peptide bond (Fig. 2). This suggests that either final processing occurs by an autoproteolytic step which is prevented by the presence of TIMP-1 through complex formation with the intermediate collagenase-3 species, or the collagenase-3-TIMP-1 complex is not susceptible to further cleavage by MT1-MMP. It is currently not possible to distinguish between these two processing routes due to a lack of MMP inhibitors which block either collagenase-3 or MT1-MMP specifically. Processing of procollagenase-3 was completely inhibited by recombinant TIMP-2 (Fig. 4, lane 3) or TIMP-3 (not shown) indicating that at least the initial cleavage was due to MT1-MMP. TIMP-2 and TIMP-3 are more effective inhibitors of MT1-MMP with k\textsubscript{on} values comparable to those for gelatinase A while TIMP-1 reacts extremely slowly with an insignificant k\textsubscript{on} value.\textsuperscript{4}

Potentiation of the Procollagenase-3 (ProMMP-13) Activation Rate by MT1-MMP (MMP-14) in the Presence of Progelatinase A (ProMMP-2)—Activation of procollagenase-3 by MT1-MMP was performed in the presence of either wild type progelatinase A or an inactive progelatinase A mutant where the active site glutamic acid residue Glu\textsuperscript{375} had been altered to alanine (Glu\textsuperscript{375} → Ala progelatinase A) (14) or buffer. Analysis of the reaction mixtures revealed that the activation rate of procollagenase-3 was enhanced in the presence of wild type progelatinase A while activation in the presence of the mutant corresponded to the rate observed with MT1-MMP alone (Fig. 5). The concentration of MT1-MMP activated gelatinase A used to potentiate procollagenase-3 activation was not high enough to lead to detectable substrate hydrolysis in the assay and furthermore the active MT1-MMP showed likewise no hydrolysis of McaPLGLDPaAR under these conditions. Analysis of the reaction products by SDS-PAGE revealed that the molecular mass of procollagenase-3 was reduced to M, 48,000 during activation which corresponds to the active species (Fig. 6, A and B). Activation in the presence of progelatinase A was more rapid
and the reaction was completed within about 200 min (Fig. 6A, lane 6). In contrast, MT1-MMP alone (not shown) or in the presence of Glu
Glu

Ala progelatinase A was much slower and the conversion to the M, 48,000 species was only partially completed after 380 min (Fig. 6B, lane 8; about 45% conversion). This indicated that MT1-MMP generated active gelatinase A during procollagenase-3 activation reaction, which then significantly contributed to the conversion of procollagenase-3 to the active enzyme (Fig. 6A and see also Figs. 1 and 5). The low concentration of active gelatinase A generated in the incubation mixture was not sufficient to initiate substrate hydrolysis, since the appropriate control experiment, i.e. the activation of progelatinase A by MT1-MMP in the presence of buffer, showed no activity (Fig. 5, ●). The decline in collagenase-3 activity following activation by gelatinase A and MT1-MMP is due to instability of the active enzyme and has also been observed during APMA or trypsin activation (5).

Activation of Procollagenase-3 (ProMMP-13) by Plasmin—Human procollagenase-3 was activated by plasmin in a time-dependent fashion (Fig. 7), and fully active enzyme was generated after about 120 min as assessed by the synthetic substrate assay. Analysis of the activation process by SDS-PAGE performed under reducing conditions indicated that, concomitant with the increase in activity, there was a drop in M, of the proenzyme from 60,000 to 48,000 (Fig. 7, lane 2). The procollagenase-3 used in the activation experiments displayed about 5% activity prior to activation, but, as can be seen from Fig. 7, no further activation was observed for the buffer control. Thus the reduction in M, was due to plasmin activity and could be blocked by plasmin inhibitors such as aprotinin (Fig. 7, lane 3). No intermediate enzyme forms were initially detectable by SDS-PAGE during activation. However, activation in the presence of the synthetic MMP inhibitor CT1399 showed that plasmin generated a transient M, 56,000 species (Fig. 7, lane 4), and N-terminal sequence determination revealed that plasmin generated two intermediate forms through cleavage of the Lys
Lys

Glu
Glu

Ala peptide bond followed by cleavage of the Arg
Arg

Cys
Cys

Tyr
Tyr

peptide bond within the propeptide domain (Fig. 2). It was observed that plasmin activation of procollagenase-3 led to the accelerated release of the C-terminal domain of the enzyme, even in the presence of the MMP inhibitor CT1399 (not shown) at concentrations sufficient to completely inhibit collagenase-3 activity. The final conversion to Tyr
Tyr

N-terminal collagenase-3 was inhibited in the presence of CT1399, indicating that this step is due to autoproteolysis, which leads to the release of the rest of the propeptide domain, and this was observed only in the absence of CT1399. Amino acid sequence analysis of the final reaction product revealed that the Glu
Glu

Tyr
Tyr

peptide bond was hydrolyzed by collagenase-3. The loss of the C-terminal domain of collagenase-3 was also observed during MT1-MMP, gelatinase A, and p-aminophenylmercuric acetate activation, but at a slower rate. This was due to the autoproteolytic hydrolysis of the Ser
Ser

Leu
Leu

peptide bond summarily could be inhibited by CT1399. Thus plasmin could transiently generate active collagenase-3, which was followed by cleavage within the hinge sequence motif leading to loss of the collagenolytic activity of the enzyme (not shown).

Processing of Procollagenase-3 (ProMMP-13) by ConA-Stimulated Fibroblast Monolayers and Influence of Plasmin on the Processing Rate—Human procollagenase-3 was partially processed via a M, 56,000 intermediate form to the final M, 48,000 active enzyme by ConA-stimulated fibroblast monolayers incubated for 24 h at 37°C (Fig. 8A, lane 2), while nonstimulated fibroblast monolayers had no effect (Fig. 8A, lane 1). The addition of 100 ng of plasminogen had no effect on the processing rate of procollagenase-3 by either nonstimulated or ConA-stimulated fibroblast monolayers (Fig. 8A, lanes 3-6), although the plasminogen was converted to plasmin (not shown). However, high concentrations of plasminogen (6 μg/ml) led to fragmentation of collagenase-3, and the enzyme was destroyed completely (not shown). Processing of procollagenase-3 by ConA-stimulated fibroblast monolayers was inhibited by TIMP-2, TIMP-3, and the synthetic MMP inhibitor CT1399 (not shown). PAI-2 or aprotinin inhibited fragmentation in the presence of plasminogen, but activation via the M, 56,000 intermediate form to the M, 48,000 active collagenase-3 in the cellular activation experiments was not affected. From these results it may
be concluded that activation of procollagenase-3 in the cellular system is predominantly regulated by MT1-MMP, and a physiological role for activation by plasmin can be excluded. TIMP-1 did not inhibit processing by ConA-stimulated fibroblast monolayers at concentrations sufficient for inhibition by TIMP-2 or TIMP-3 (not shown). This is probably due to the extremely slow association rates for complex formation between TIMP-1 and human procollagenase-3 (proMMP-13) by MT1-MMP as discussed above.4

Processing of Procollagenase-3 (ProMMP-13) by Plasma Membranes Derived from ConA-stimulated Fibroblasts—Crude membranes derived from ConA-stimulated fibroblasts were also able to process procollagenase-3 to the M₆₅, 56,000 intermediate form. In contrast to the cell activation experiments, the M₅₆, 56,000 intermediate form was not visible during membrane processing of procollagenase-3. However, processing of procollagenase-3 was partially inhibited by TIMP-1 and the intermediate collagenase-3 species (M₅₆, 56,000) accumulated in the presence of TIMP-1 (Fig. 8B, lane 4), preventing conversion to the final active enzyme. In addition, the processing rate of procollagenase-3 by membranes was enhanced when gelatinase A was included in the experiment (Fig. 8B, lane 14), which was due to activation of gelatinase A by membranes and the activated gelatinase A then contributed significantly to the activation of procollagenase-3.

**DISCUSSION**

Human procollagenase-3 is expressed by breast tumors (4) and interleukin-1β-stimulated chondrocytes (25), which implies that the enzyme may play a substantial role in breast cancer pathology as well as in the arthritides. Activation of human procollagenase-3 is one of the critical control mechanisms regulating extracellular collagenolytic activity and ultimately connective tissue integrity. It is therefore an important goal to elucidate mechanisms leading to proenzyme activation in order to understand extracellular matrix turnover. We have demonstrated that human procollagenase-3 is directly activated by MT1-MMP or gelatinase A, thereby establishing a new possible in vivo activation cascade.

The latency of procollagenase-3 and other MMPs is due to the propeptide domain which is comprised of a separate folding unit containing three α-helices and an extended peptide that contains the conserved Pro-Arg-Cys-Gly-Val/Asn-Pro-Asp sequence motif as revealed from the x-ray crystallographic analysis of a C-terminal deletion mutant of prostromelysin (6). This peptide lies in the active site cleft of the enzyme and the free Cys residue provides the fourth ligand for the catalytic zinc ion. The propeptide makes several β-structure-like hydrogen bonds with the active site of the enzyme which stabilizes the structure through formation of a three stranded β-sheet. The amino-to-carboxyl direction of the propeptide chain within the groove of the active site is opposite to that adopted by synthetic peptide MMP inhibitors and the S₃'-pocket remains empty in the proenzyme. Activation of procollagenase-3 involves the proteo-
lytic or autoprotolytic removal of the propeptide domain (5) by the cysteine switch activation mechanism (6, 26, 27). We have shown that procollagenase-3 is efficiently activated by MT1-MMP, which is the result of stepwise proteolytic removal of the propeptide domain. It can be assumed that the propeptide domain of procollagenase-3 may show a similar overall structure to the corresponding domain of stromelysin (6). The initial cleavage by MT1-MMP within procollagenase-3 occurs at the Gly\textsuperscript{35}–Ile\textsuperscript{36} peptide bond which would be just downstream from cleavage by MT1-MMP within procollagenase-3 occurs at the first \(\alpha\)-helix, in a region lacking electron density. This part of the molecule is therefore most likely solvent exposed and accessible to proteolytic attack by MT1-MMP. This is followed by a secondary cleavage, which generates Tyr\textsuperscript{85} N-terminal active collagenase-3, and probably only proceeds after a substantial conformational rearrangement of the remaining propeptide domain. It is currently not clear whether the secondary cleavage is due to MT1-MMP or due to autoproteolysis by collagenase-3. The two other human collagenases, MMP-1 and MMP-8, are resistant to proteolysis by MT1-MMP\textsuperscript{2} and remain inactive. Thus activation by MT1-MMP is specific for collagenase-3 within the collagenase subfamily. Furthermore, we have established that gelatinase A also activates procollagenase-3 and activates much faster than MT1-MMP. We also showed that MT1-MMP activation is potentiated in the presence of progelatinase A, which is itself activated by MT1-MMP. Activation of procollagenase-3 by gelatinase A or MT1-MMP in the presence of progelatinase A was rapid and accompanied with the removal of the propeptide domain leading to the generation of Tyr\textsuperscript{85} N-terminal active collagenase-3 which represents the fully active form of collagenase-3. Other work has shown that following the removal of the propeptide domain, a substantial rearrangement of the first 6 amino acid residues occurs in the active MMPs (6). The first amino acid in the active form of stromelysin is more than 17 Å away from the corresponding position in the proenzyme which is due to the establishment of a salt link between the NH\textsubscript{2}-terminus of Phe\textsuperscript{83} and the side chain of Asp\textsuperscript{237} in helix C of the catalytic domain (6). This interaction is...
also observed in the structures of the Phe\textsuperscript{79} neutrophil collagenase catalytic domain and active porcine collagenase and has been associated with the superactive collagenases (28–30). Thus the conservation of this salt link in the structures of stromelysin, neutrophil collagenase and fibroblast collagenase suggests that this might be a general feature in the structures of fully active MMPs, and it can be assumed that Tyr\textsuperscript{85} N-terminal collagenase-3 shares identical features.

In the light of data associating increased extracellular MMP activity with the metastatic phenotype of certain tumors, cell surface activation of progelatinase A by MT1-MMP is an important in vivo activation mechanism for the generation of extracellular gelatinolytic and type IV collagenolytic activity (7–11, 31). It was thought that this mechanism is specific for progelatinase A, since the homologous progelatinase B cannot be activated by crude membrane preparations containing MT1-MMP or by Con A stimulated fibroblasts (13). Here we have shown using our ConA-stimulated fibroblast monolayer system and crude plasma membranes from ConA-stimulated fibroblasts, that procollagenase-3 is activated by a similar mechanism, establishing that MT1-MMP may also play a role in initiating interstitial collagenolytic activity thereby potentiating extracellular matrix degradation. However, this mechanism is specific for procollagenase-3 activation since the two other human procollagenases (MMP-1 and MMP-8) are not activated by MT1-MMP (Knäuper & Murphy, unpublished results).

Progelatinase A activation by MT1-MMP is dependent on cell surface localization of the enzyme as assessed using a transmembrane deletion mutant and a chimeric MT1-MMP fused to the interleukin-2 receptor \( \alpha \)-chain (32). In addition, cell surface activation of progelatinase A can be enhanced by small amounts of TIMP-2. Strongin et al. (11) isolated a trimolecular complex consisting of MT1-MMP-TIMP-2-gelatinase A and concluded that the MT1-MMP-TIMP-2 complex acts as a cell surface receptor for progelatinase A, which binds through its C-terminal domain. These results agree with our recent studies showing that the C-terminal domain of progelatinase A is essential for processing (31). Following cell surface binding, MT1-MMP then cleaves progelatinase A at the Asp\textsuperscript{72}–Leu\textsuperscript{73} peptide bond which is followed by autoproteolytic release of the remaining propeptide domain thereby generating fully active Tyr\textsuperscript{85} N-terminal gelatinase A (10).

Procollagenase-3 activation by either the catalytic domain of MT1-MMP, by ConA-stimulated fibroblast plasma membranes, or by ConA-stimulated fibroblast monolayers shows remarkable similarities to the progelatinase A processing described above. Activation proceeds via a \( M_r \), 56,000 intermediate form to the final active collagenase-3 (\( M_r \), 48,000) in an apparent two-step mechanism which was demonstrated in the three different systems, e.g. using purified MT1-MMP, ConA-stimulated fibroblast monolayers, and membranes from ConA-stimulated fibroblasts. The precise mechanism of cell-associated processing of procollagenase-3 by MT1-MMP needs to be addressed. Initial studies have shown that membranes from ConA-stimulated fibroblasts process only full-length procollagenase-3, while a C-terminal deletion mutant is not affected, which indicates that, like progelatinase A, C-terminal domain interactions may be crucial for procollagenase-3 processing in the cellular system. It is currently not clear whether cellular processing of procollagenase-3 can be enhanced by low levels of TIMP-2, as has been described for progelatinase A activation by cells expressing MT1-MMP (11). By analogy, it is not known whether a MT1-MMP-TIMP-2 complex acts as a cell surface receptor for procollagenase-3 as it does for progelatinase A, which binds through the C-terminal domain. This is in agreement with our results on procollagenase-3 activation by ConA-stimulated fibroblast monolayers and membranes, and indicates that activation was due to an MMP expressed by these cells. This is most likely to be MT1-MMP. Activation of procollagenase-3 by fibroblasts or membranes may be due to combined action of MT1-MMP and gelatinase A, because both purified enzymes where able to activate procollagenase-3 on their own. However, cellular MT1-MMP (full-length) is able to activate procollagenase-3 in the absence of gelatinase A, since membranes from myeloma cells cotransfected with MT1-MMP and TIMP-2 were able to process procollagenase-3 (not shown). These membranes were shown to be free of gelatinolytic activity as assessed by zymography, and it can therefore be concluded that MT1-MMP alone is able to activate procollagenase-3, albeit at a slower rate than gelatinase A. We postulate that MT1-MMP, as well as gelatinase A, may be able to activate procollagenase-3 alone or in concert, thereby establishing a new activation cascade consisting of three members of the MMP family.

It is interesting to note that rodent collagenase-3 has been described in association with the surface of rat osteoblasts, and a specific receptor has been proposed which acts as a clearance mechanism for the enzyme (33); it is, however, not clear whether this is due a membrane-associated MMP.

A second possible activation pathway proceeds via the plasma serine proteinase plasmin which activates procollagenase-3 at relatively high concentrations. Procollagenase-3 activation by plasmin involves the proteolytic removal of parts of the propeptide domain by plasmin specific cleavage, which is completed by autoproteolysis, thereby generating the Tyr\textsuperscript{85} C-terminal collagenase-3. The homologous fibroblast procollagenase (MMP-1) is activated by a similar mechanism, but plasmin activation on its own does not generate active MMP-1 with high specific collagenolytic activity (34, 35). Activation of MMP-1 by combined treatment with plasmin and stromelysin generates enzyme with high specific collagenolytic activity (36, 37) through cleavage of the Gin\textsuperscript{80}–Phe\textsuperscript{81} peptide bond, which corresponds to Tyr\textsuperscript{85} N-terminal collagenase-3. However, activation of procollagenase-3 by plasmin is accompanied by loss of the C-terminal domain of the enzyme, which leads to a decrease in collagenolytic activity. This process is pronounced in the case of collagenase-3, since this enzyme contains a Lys\textsuperscript{260}–Thr\textsuperscript{261} peptide bond within the hinge sequence, which is relatively exposed in the molecule, as deduced from the x-ray crystallographic analysis of the homologous pig collagenase (29), and therefore susceptible to serine proteinases and is indeed hydrolyzed by trypsin. Therefore, activation by plasmin does not lead to the generation of enzyme displaying high specific collagenolytic activity. Furthermore, plasminogen activator inhibitors or plasmin inhibitors did not affect processing of procollagenase-3 in our cellular model system thereby excluding a role for these serine proteinases in proenzyme activation. We conclude that plasmin may only play a minor role in cellular processing of procollagenase-3, if at all.

The generation of extracellular collagenolytic activity through a membrane type MMP alone or in combination with gelatinase A represents a new activation cascade consisting of three members of the MMP family and is unique. This may well be of great importance in vivo since MT1-MMP, gelatinase A and collagenase-3 have all been implicated independently in human breast cancer pathology (38–40) and may well act in concert. Thus these three enzymes could form a proteolytic cascade in vivo which leads to the dissolution of the extracel-

\[ ^5 \text{S. Atkinson, personal communication.} \]

\[ ^6 \text{S. Atkinson, M. Butler, and G. Murphy, unpublished results.} \]
lular matrix, thereby allowing growth and spreading of tumors expressing MT1-MMP, gelatinase A and collagenase-3.

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