Intermolecular Autolytic Cleavage Can Contribute to the Activation of Progelatinase A by Cell Membranes*

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Membrane-type matrix metalloproteinase (MT-MMP) messenger RNA and protein expression were shown to be elevated in human fibroblasts following treatment with concanavalin A, coincident with the induction of the ability to process progelatinase A. CHO cells transfected with the cDNA for MT-MMP were able to process both wild type progelatinase A and a catalytically inactive mutant, E375A progelatinase A. Both proenzymes were converted to a 68-kDa intermediate (reducing gels) form, but only the wild type enzyme was processed further to a 66-kDa end product. In contrast, both forms of progelatinase were processed via the 68-kDa intermediate to 66 kDa by concanavalin A-stimulated fibroblasts.

Further study of the processing of E375A progelatinase A by plasma membrane preparations from concanavalin A-stimulated fibroblasts showed that addition of active gelatinase A enhanced processing to the mature form. It was concluded that cell membrane-mediated activation of progelatinase A could be via a cascade involving both MT-MMP and intermolecular autolytic cleavage.

The matrix metalloproteinase (MMP) family of enzymes has been strongly implicated in extracellular matrix turnover, in both physiological and pathological situations. All MMPs are secreted in proenzyme forms, and much research has been aimed at elucidating the proteolytic mechanisms required to elicit their full activities. A number of possible physiological activators have been described, including plasmin, plasma kallikrein, neutrophil elastase, and cathepsin G (Nagase et al., 1991). Gelatinase A (MMP-2, 72-kDa gelatinase, type IV collagenase) is of great current interest, not only because of its possible involvement in tumor invasion and metastasis (Stetler-Stevenson et al., 1993; Tsuchiya et al., 1994) but also because the proenzyme cannot be activated by any of the suggested physiological activators of other MMPs including serine proteinases (Okada et al., 1990; Nagase et al., 1991), although activation by other MMPs, especially matrixinsin (Crabbe et al., 1994a) and collagenase (Crabbe et al., 1994b), has recently been described. Furthermore, there is evidence that activation by self-cleavage can occur at high progelatinase A concentrations (Crabbe et al., 1993).

Cell-mediated activation of progelatinase A has been the focus of much recent work by the present authors (Ward et al., 1991, 1994) by and others (Strongin et al., 1993, 1995; Brown et al., 1993). We reported that human skin fibroblasts stimulated by concanavalin A (ConA) can bind, proteolytically process, and activate progelatinase A. The importance of the C-terminal domain of the enzyme for the binding to cell membranes and subsequent activation was demonstrated (Murphy et al., 1991; Strongin et al., 1993; Ward et al., 1994). Cell membrane-mediated cleavage of progelatinase A to a 62-kDa active form (Tyr-81) occurs via a 64-kDa intermediate (Leu-38) (Strongin et al., 1993), and exogenous TIMP-2 specifically inhibits the activation process.

Sato et al. (1994) recently reported the cloning of a novel transmembrane member of the matrix metalloproteinase family (MT-MMP) and showed that cells transfected with MT-MMP cDNA can effect the activation of progelatinase A. Questions therefore arise about the precise mechanism of activation of progelatinase A by stimulated fibroblasts, especially whether MT-MMP is an obligatory component, the relative roles of other metalloproteinases, and the involvement of self-cleavage. In this study we have made use of a previously described mutant form of gelatinase A (Crabbe et al., 1994c), in which the active site glutamate was replaced by alanine (E375A). The mutant can be proteolytically processed to remove the propeptide (Crabbe et al., 1994c) but yields a catalytically inactive enzyme that cannot self-process. We have investigated the hypothesis that cell membrane-mediated activation of progelatinase A is, by analogy with other matrix metalloproteinases (Murphy et al., 1987; Nagase et al., 1990), a complex activation cascade. We show that it is likely to involve MT-MMP, which can be induced in fibroblasts by ConA, and bimolecular autolysis.

MATERIALS AND METHODS

E375A mutant progelatinase A was constructed, expressed, and purified as described by Crabbe et al. (1994c). Recombinant wild type human progelatinase A, TIMP-1, and TIMP-2 were purified from media conditioned by the relevant transfected mouse myeloma cells as described (Crabbe et al., 1993; Murphy et al., 1991, 1992; Willenbrock et al., 1993). Digoxigenin-11-UTP, nylon membrane, and a Nucleic Acid Detection Kit were obtained from Boehringer Mannheim, East Sussex, UK. Other transcription reagents and pBluescript KS(+) were obtained from Stratagene Ltd., Cambridge, UK. Hybond ECL nitrocellulose membrane, ECL Kit for Western blotting autoradiography, and luminescence detection film were all from Amersham Life Sciences, Amersham, Bucks, UK. Peroxidase-conjugated sheep anti-mouse IgG was from Sigma, and the membrane-type matrix metalloproteinase (MT-
**RESULTS**

Apparent molecular masses for the three species of gelatinase A identified in these experiments differed according to the method used to assess them. In this report, molecular masses for the proenzyme, intermediate, and fully active forms, as assessed by nonreducing SDS-PAGE/gelatin zymography, were 72, 68, and 66 kDa, respectively. Where SDS-PAGE with reducing conditions was used, i.e., Coomassie-stained gel scanning (Fig. 6), the respective molecular masses were 72, 68, and 66 kDa. Molecular masses...
MT-MMP is induced by ConA stimulation of fibroblasts. A, Northern blot of RNA from fibroblast monolayers. Cells were either untreated (-) or stimulated with 50 μg/ml ConA (+) for 24 h prior to extraction of total RNA. Samples (5 μg/lane) were fractionated by electrophoresis and transferred to a nylon membrane. MT-MMP mRNA was detected by hybridization with a digoxigenin-labeled riboprobe. The positions of the 28S and 18S ribosomal bands are shown to the left. Ethidium bromide staining of the ribosomal RNA is also shown, as a measure of loading. B, 10-μl aliquots of membrane preparations from approximately equal numbers of fibroblasts (2 × 9 T175 cm² confluent flasks), which had been incubated for 48 h in either DMEM alone (-) or DMEM + 50 μg/ml ConA (+), were applied to SDS-PAGE. Samples are from two separate experiments. The electrophoresed proteins were analyzed by Western blot as detailed under "Materials and Methods." Relative mobilities of molecular mass markers are indicated on the left.

reported by other workers (Strongin et al., 1993) referred to in the introduction and under "Discussion" are those assessed by gelatin zymography performed under different conditions and are described as 66, 64, and 62 kDa, respectively.

The Membrane-type Matrix Metalloproteinase, MT-MMP, Is Induced by Concanavalin A Stimulation of Fibroblasts—Northern blot analysis of human skin fibroblast RNA (Fig. 1A) showed a low level of MT-MMP mRNA in unstimulated cells. The amount of mRNA was shown to increase in cells that had been exposed to ConA for 24 h. In both cases, a single band of MT-MMP mRNA was detected, consistent with the 4.5-kilobase transcript reported by Sato et al. (1994). MT-MMP protein was detected by Western blot analysis of isolated membrane preparations from both unstimulated and ConA-stimulated cells (Fig. 1B). A strongly reactive band at 63 kDa seen in membranes from stimulated fibroblasts (+), which corresponded to that reported by Sato et al. (1994), was also identified more weakly in the membrane preparations from unstimulated cells (-). These data clearly demonstrate that, like MT-MMP mRNA, expression of the protein is increased in fibroblasts exposed to ConA. Other weaker bands indicate reactions between the antibody and other components of the isolated membranes, which did not conform to the predicted molecular mass of MT-MMP and are not further characterized at this stage.

Wild Type Progelatinase A and E375A Progelatinase A Are Processed to Different Forms by Cells Expressing Recombinant MT-MMP—MT-MMP induces activation of progelatinase A (72 kDa) by two steps (via a 68-kDa intermediate to a 66-kDa activated form, Sato et al. (1994)). To analyze the contribution of MT-MMP and gelatinase A activities in this process, MT-MMP was expressed in CHO cells by transient transfection of the expression plasmid. When 125I-labeled progelatinase A or 125I-labeled E375A mutant were incubated with the CHO cells transiently expressing MT-MMP, wild type progelatinase A (66 kDa, nonreducing SDS-PAGE) was partially processed via a 62-kDa intermediate form to the fully active 59-kDa species (Fig. 2, lane 1). E375A mutant, however, was processed only to the 62-kDa intermediate form (lane 3). Neither enzyme was processed by CHO cells transfected with vector alone (lanes 1 and 2). Partial processing of 125I-labeled E375A mutant to the 59-kDa form as well as the 62-kDa intermediate was observed when equimolar unlabeled wild type progelatinase A was included in the MT-MMP-transfected CHO cultures together with the inactive mutant gelatinase (lane 5), but not if both enzymes were incubated with vector control CHO cells (lane 6).

E375A Progelatinase A Is Processed by ConA-stimulated Fibroblast Monolayers and by Isolated Cell Membranes—Both 125I-labeled wild type progelatinase A and 125I-labeled E375A mutant (66 kDa, nonreducing SDS-PAGE) were partially processed via a 62-kDa intermediate form to 59 kDa by incubation at 37 °C for 24 h with fibroblasts prestimulated with ConA (Fig. 3A). Unstimulated cells were unable to process either progelatinase A or mutant during 24 h. Fig. 3B demonstrates that isolated membrane preparations from ConA-stimulated cells were also able to process the E375A mutant progelatinase in the same way as the cell monolayers and the wild type enzyme, although with less efficiency. Processing of the E375A mutant to the 59-kDa form by either cell monolayers or isolated cell membranes occurred more slowly than that of the wild type enzyme and, unlike the wild type progelatinase A, no gelatinolytic activity was observed by gelatin zymography corresponding to the processed form of the E375A mutant revealed by autoradiography.

Inhibition of Cellular Processing of E375A Progelatinase by TIMP-1 and TIMP-2—Processing of the wild type enzyme and E375A mutant by stimulated fibroblast monolayers was fully inhibited in a dose-responsive manner by the inclusion of human recombinant TIMP-2 in the cell cultures (Fig. 4, A and B). Processing of wild type 125I-labeled progelatinase A was more complete than that of 125I-labeled E375A in the absence of TIMP-2 (Fig. 4A, lane 6; Fig. 4B, lane 6), but the inhibitor was equally effective in blocking activation (Fig. 4A, lanes 9 and 10; Fig. 4B, lanes 9 and 10). TIMP-1 was a much less effective

Gelatinase A at High Concentrations—E375A progelatinase A and wild type progelatinase A (2 μM) were incubated with active gelatinase A at 37 °C for up to 24 h at molar ratios of 1:0.01, 1:0.1, or 1:1, and the extent of proenzyme processing was assessed by SDS-PAGE (Fig. 5). At equimolar concentrations, the major product of processing of both the wild type enzyme and the E375A gelatinase was a 66-kDa form (reducing SDS-PAGE) identical with APMA-activated gelatinase A (results not shown). Lower molecular mass products also became visible with time, suggesting that the 66-kDa form is itself susceptible to further degradation (data not shown). At these high concentrations of progelatinase A, no propeptide intermediates (between 72 kDa and 66 kDa) were detectable, suggesting that the intermediate cleavage products of the propeptide seen with APMA and cell membrane processing are extremely transient. At higher concentrations of proenzyme compared to active enzyme, mutant processing became far less efficient and was negligible at a molar ratio of 1:0.01. The wild type enzyme, in comparison, was still slowly but efficiently processed, due to the production of active enzyme that was able to participate in the reaction.

Enhancement of Membrane Processing of 125I-Labeled E375A by Exogenous Active Gelatinase A—Active gelatinase A enhanced processing of 125I-labeled E375A (14 nm) by ConA-stimulated membrane preparations in a dose-responsive manner (Fig. 6). Enhancement was first observed at a molar ratio of 0.1 active enzyme (lane 3). At equimolar concentrations, approximately 50% of the proform of 125I-labeled E375A was completely converted to the 59-kDa form (lane 4). In the absence of any membrane preparation, equimolar active gelatinase A also brought about a low level of processing of 125I-labeled E375A (lane 2), although no processing was observed at lower concentrations (lane 1). Progelatinase A was also able to enhance processing in the presence of the membrane preparation (lane 5) but with gelatinase A alone, at equimolar concentrations, no processing of 125I-labeled E375A took place (lane 6). When the experiment was performed using trypsin-activated collagenase (MMP-1) instead of gelatinase A, no enhancement of processing was observed (data not shown).

**DISCUSSION**

The activation of gelatinase A by a membrane-mediated process was studied using ConA-stimulated skin fibroblasts, a model system that we described previously in our attempts to elucidate the mechanism of activation of this pro-MMP (Ward et al., 1991, 1994; Murphy et al., 1992). The recent cloning of a putative membrane-bound metalloproteinase, MT-MMP, from a tumor library, and the demonstration of the ability of the recombinant protein to process gelatinase A (Takino et al., 1995; Sato et al., 1994), was of extreme relevance to the question of the number of potential gelatinase A activation mechanisms in our model system and in vivo. In this study we have shown that MT-MMP mRNA and protein are expressed at low levels in normal skin fibroblasts but that the levels are considerably up-regulated upon treatment with ConA. We chose gelatin zymography followed by autoradiography to study the fate of a 125I-labeled inactive mutant (E375A gelatinase A (Crabbe et al., 1994c)) or 125I-labeled wild type progelatinase A when incubated in cell cultures or with isolated membranes.

Gelatin zymography was performed so that gelatin degrading activities could be identified, and autoradiography of the zymograms was chosen in preference to silver- or Coomassie-stained gels since it allowed us to detect the relatively low levels of enzyme remaining in the cell supernatants. For these reasons, the separation of the three species of gelatinase is less clear than it might have been using different techniques. Where there is rapid processing to the final (59-kDa) species, the
incubated with 125I-labeled progelatinase A and varying doses of TIMP-1. Lanes 1–5 or ConA-stimulated fibroblasts (lanes 6–10) incubated with 125I-labeled progelatinase A and varying doses of TIMP-2 (A). Lanes 1 and 6, no TIMP-2; lanes 2 and 7, 7.5 nM TIMP-2; lanes 3 and 8, 15 nM TIMP-2; lanes 4 and 9, 75 nM TIMP-2; lanes 5 and 10, 150 nM TIMP-2. B, as A, except that 125I-labeled E375A mutant was used instead of the wild type enzyme. C, unstimulated fibroblasts (lanes 1–5) or ConA-stimulated fibroblasts (lanes 7–12) incubated with 125I-labeled progelatinase A and varying doses of TIMP-1. Lanes 1 and 7, no TIMP-1; lanes 2 and 8, 6 nM TIMP-1; lanes 3 and 9, 12 nM TIMP-1; lanes 4 and 10, 24 nM TIMP-1; lanes 5 and 11, 60 nM TIMP-1; lanes 6 and 12, 120 nM TIMP-1. D, as C, except that 125I-labeled E375A was used instead of the wild type enzyme.

FIG. 5. The processing of E375A progelatinase A and wild type progelatinase A by active gelatinase A. 2 μM samples of either wild type progelatinase A (closed symbols) or E375A mutant progelatinase A (open symbols) were incubated for varying times with either 0.02 μM (○, ●), 0.2 μM (□, ■), or 2 μM (▲, ▼) APMA-activated gelatinase A. Progelatinase A is presented as the percentage of proenzyme remaining after varying times of incubation at 37 °C, as determined by densitometric scanning.

FIG. 6. Enhancement of processing of 125I-labeled E375A by membrane preparations with active gelatinase A. 125I-labeled E375A progelatinase (14 nM) was incubated at 37 °C for 20 h with increasing doses of unlabeled APMA-activated gelatinase A from which the APMA had been removed (see “Materials and Methods”) and with or without 10-μg aliquots of membrane preparations from ConA-stimulated fibroblasts. The autoradiograph shows 125I-labeled E375A mutant gelatinase A incubated with: lane 1, 0.1 molar ratio of APMA-activated gelatinase A; lane 2, 1.0 molar ratio of active gelatinase A; lane 3, membrane preparation supplemented with 0.1 molar ratio of active gelatinase A; lane 4, membrane preparation supplemented with 1.0 molar ratio of active gelatinase A; lane 5, membrane preparation supplemented with 1.0 molar ratio of progelatinase A; lane 6, 1.0 molar ratio of progelatinase A; lane 7, membrane preparation; lane 8, 125I-labeled E375A mutant alone.

Intermolecular Autolytic Cleavage of Progelatinase A

Intermolecular autolytic cleavage of progelatinase A is a naturally self-process like the wild type proenzyme in the presence of an organomercurial, can be processed efficiently at high concentrations in the presence of a high molar ratio of active gelatinase A. This is in agreement with our previous data that a truncated mutant of gelatinase A could effect the same propeptide processing of the E375A mutant as can be seen for the wild type gelatinase (Crabbe et al., 1994c). Our studies of proenzyme processing by gelatinase A (Fig. 5) measured the amount of proenzyme remaining by scanning Coomassie-stained gels. This method provided a better estimate of gelatinase “activation” in this experimental system than attempts to quantitate the amount of fully processed (66-kDa reducing SDS-PAGE) enzyme for two reasons. Firstly, the experiment adds increasing amounts of previously activated 66-kDa gelatinase A to the system. This could be circumvented by using 125I-labeled gelatinase, but, for quantitation experiments, autoradiography is less accurate than scanning of Coomassie-stained protein bands. Secondly, the 66-kDa form is itself constantly subject to further degradation at 37 °C at a rate that is
in part determined by the concentration of 66-kDa gelatinase A (Crabbe et al., 1993). The experiments show that the rate of proenzyme loss is not only governed by the amount of active enzyme added to the incubation but by the potential for the generation of further active enzyme; thus, the processing of wild type proenzyme at low initial values of added active enzyme accelerates with time, while the mutant processing rate cannot be modified. These observations suggest that the processing is by intermolecular reactions.

To investigate the role of endogenous membrane-associated gelatinase A in the processing of the E375A mutant by ConA-stimulated fibroblasts, we took a number of approaches. Initially, we confirmed that the processing was likely to be due to the action of an MMP by demonstrating that the TIMPs were effective inhibitors of processing. As previously noted (Ward et al., 1991), TIMP-2 was a more efficient inhibitor of processing than TIMP-1. This phenomenon was also observed in the inhibition of progelatinase A processing by CHO cells transfected with MT-MMP and has been reported by others (Strongin et al., 1993). It is thought to be due to tighter C-terminal domain interactions of the proenzyme with TIMP-2 (Willenbrock et al., 1993). We therefore proposed this also to be indicative of the necessity for progelatinase to bind to the cell membrane through the C-terminal domain for activation to occur. Such binding and activation can be blocked by TIMP-2, and the isolated C-terminal domain of gelatinase (Murphy et al., 1992; Strongin et al., 1993; Ward et al., 1994). We conclude that TIMP-2 is a more efficient inhibitor of E375A mutant membrane processing for the same reason.

We had noted that the preparation of membrane fractions from ConA-stimulated fibroblasts reduced both the amount of endogenous gelatinase A associated with the system and the ability of the fibroblast membranes to process progelatinases. However, we were unable to reduce further the gelatinase A content of cell membranes using solvents, acid pH etc. (data not shown). The addition of small amounts of activated gelatinase A to the system caused a dose-responsive restoration of the ability to process both the wild type and mutant progelatinases. In processing studies in the absence of membranes, high concentrations of proenzymes and high molar ratios of the active form were shown to be required for this phenomenon to occur (this paper and Crabbe et al., 1993, 1994a). In the presence of membranes, processing can occur at relatively low concentrations (150-fold less) of the reagents. Crabbe et al. (1993) and Ward et al. (1994) have proposed that the binding of gelatinases to the cell membrane may increase its localized concentration such that an intermolecular processing reaction is promoted. We therefore conclude that membrane-associated gelatinase A is involved in the activation of its proform. The differences in the rates of wild type and mutant proenzyme processing by active gelatinase A observed in the absence of cell membranes can be extrapolated to the results obtained in their presence. Thus, because the rate of wild type processing using membranes is only slightly faster than that of the mutant, the amount of active gelatinase A endogenously present on the cell surface of ConA-stimulated fibroblasts is likely to be approximately equal to the amount of bound proenzyme.

Until MT-MMP has been rigorously characterized, we are not able to assess its importance as a progelatinase processing enzyme relative to active gelatinase. By analogy with other MMP activation cascades, it is extremely likely that MT-MMP and active gelatinase A will act in concert in the cleavage of the propeptide of progelatinase A. There are numerous examples of this, such as the case of plasmin activation of stromelysin-1, where the final propeptide cleavage is autocatalytic (Nagase et al., 1990) and in the collagenase processing of gelatinase A (Crabbe et al., 1994b). Our results do not rule out the possibility that ConA induces another as yet unidentified membrane component to which progelatinase A can bind such that it becomes concentrated on the cell surface allowing intermolecular cleavage to take place. Thus, the up-regulation of MT-MMP in ConA-stimulated fibroblasts could be entirely coincidental. However, the recent availability of the isolated catalytic domain of MT-MMP has enabled us to demonstrate conclusively that the inactive mutant E375A progelatinase A is cleaved only to the intermediate species by MT-MMP in the absence of catalytically active enzyme, but can be fully processed by the inclusion of wild type progelatinase A in the incubation mixture. Preliminary immunohistochemical studies using specific antibodies to MT-MMP and gelatinase A show that CHO cells transfected with MT-MMP bind exogenous progelatinase A on the cell surface whereas vector control cells do not. Confocal microscopy and double labeling techniques have demonstrated that the bound gelatinase has the same distribution as MT-MMP. The availability of the E375A mutant which cannot self-process should prove invaluable in the further unravelling of the question of the role of different MMPs in membrane processing of progelatinase A.

While this manuscript was in preparation, Strongin et al. (1995) reported a role for TIMP-2 in the phorbol ester-induced activation of progelatinase A by HT1080 cells. These workers demonstrated that MT-MMP acts as a receptor for TIMP-2, forming a complex capable of binding progelatinase A on the cell surface that leads to its activation. Our studies using specific antisera to gelatinase A, TIMP-2, and MT-MMP and confocal microscopy indicate that in some activating cells, all three are located together on the cell surface. Levels of TIMP-2 are critical, therefore, in determining the activation status of gelatinase A in the extracellular environment, since we have clearly shown that excess TIMP-2 completely inhibits the activation of progelatinase A by ConA-stimulated fibroblasts. Further work will be required to understand the nature of the binding of the three components that leads to activation of progelatinase A without its inhibition.

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