Thrombin–thrombomodulin activation of protein C facilitates the activation of progelatinase A

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Received 16 December 2000; revised 7 March 2001; accepted 8 March 2001

First published online 19 March 2001

Edited by Pierre Jolles

Abstract The activation of the matrix metalloproteinase progelatinase A (MMP-2) has been of keen interest because an increase in MMP-2 activity has been implicated in disease states such as cancer and atherosclerosis. Activation of MMP-2 occurs on the surface of specific cell types in two steps. In the first step, primary cleavage of MMP-2 by a membrane-type matrix metalloproteinase generates an intermediate. A secondary cleavage and activation of the intermediate is thought to occur autocatalytically. Previous studies have shown that thrombin can also activate progelatinase A in the presence of endothelial cells. We show that this cell-dependent mechanism of MMP-2 activation also occurs with THP-1 cells and involves binding of thrombin to thrombomodulin present on the cell surface and generation of the anti-coagulant protein, activated protein C. We demonstrate that activated protein C is directly responsible for activation and cleavage of the gelatinase A intermediate. This work contributes new mechanistic insights into the activation of MMP-2 and provides a novel link between matrix metalloproteinase activation and anti-coagulation. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Matrix metalloproteinase; MMP-2; Thrombin; Thrombomodulin; Anti-coagulation; Progelatinase A

1. Introduction

Matrix metalloproteinases (MMPs) are a class of zinc-utilizing proteolytic enzymes that function to degrade extracellular matrix components. These proteins are present in healthy individuals and play central roles in wound healing and during pregnancy [1,2]; however, overexpression and activation of MMPs or underexpression of the specific inhibitors of metalloproteinases (TIMPs) can contribute to diseases such as metastasis [3,4] and atherosclerosis [5,6]. Understanding the mechanisms by which these enzymes are generated and activated is therefore of significant interest.

Activation of MMPs requires cleavage of a propeptide that blocks the active site cleft in an event that comprises an important feature of MMP regulation. This cleavage results in a conformational change that disrupts a cysteine–zinc interaction (the 'cysteine switch'), allowing the enzyme to become active [7]. The activation of progelatinase A (MMP-2) is particularly unique among the MMP family. Progelatinase A is not cleaved by elastase, trypsin, stromelysin, and several other endopeptidases that have been shown to activate related MMPs such as progelatinase B, procollagenase, and prostromelysin [8]. In addition, progelatinase A is produced constitutively in relatively high concentrations by many cell types and is a normal component of human blood plasma [9]. The activation of progelatinase A is therefore particularly critical in the control of this enzyme's activity.

There is now strong evidence that the activation of progelatinase A can be accomplished through a membrane-associated mechanism. The interaction of progelatinase A with an intrinsic membrane MMP, MT1-MMP, has been shown to facilitate the conversion of this enzyme to an active form [10,11]. In an interaction dependent upon the presence of TIMP-2, MT1-MMP cleaves progelatinase A, generating a 64 kDa inactive intermediate. A process believed to occur autocatalytically that removes an additional 43 amino acids and generates the fully active enzyme [10] follows this cleavage.

Recently, Zucker et al. reported that thrombin can activate progelatinase A in a process dependent upon the presence of endothelial cells, but not involving the thrombin receptor [14]. Thrombin is a serine protease that plays a central role in blood coagulation [13]. Thrombin activity contributes to the activation of platelets, the formation of fibrin from fibrinogen, and the activation of various profactors and proenzymes in the coagulation cascade. The binding of thrombin to the endothelial cell membrane protein thrombomodulin, however, dramatically alters the substrate specificity of thrombin. The ability of thrombin to cleave fibrinogen and to activate platelets and other procoagulant factors is inhibited, while its ability to activate protein C is enhanced greatly. Activated protein C, in turn, inactivates coagulation factors Va and VIIIa and stimulates fibrinolysis [14]. Based on the reported requirement of endothelial cells for the activation of progelatinase A by thrombin and because this mechanism occurs independently of the thrombin receptor [12], we determined whether thrombomodulin conferred on thrombin the ability to activate progelatinase A. We demonstrate here that thrombomodulin and thrombin, in the presence of serum, activate progelatinase A and that activated protein C alone can convert the 64 kDa gelatinase A intermediate to the fully active form. These results are the first such evidence for the involvement of activated protein C in the activation of gelatinase A and provide a novel link between anti-coagulation and MMP activation.

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PII: S0014-5793(01)02296-7

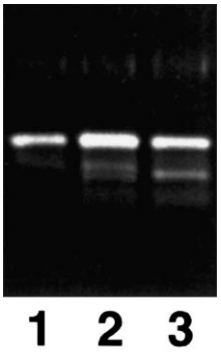

Fig. 1. Effect of thrombin on the activation of progelatinase A in the presence of endothelial cells. Progelatinase A (5 nM) was incubated with thrombin (50 nM) in the presence of 1×10^5 HUVEC and 1% FBS. After 48 h at 37°C, reaction mixes were subjected to zymography. Shown is a representative zymogram of reactions conducted with progelatinase A alone (lane 1), HUVEC and progelatinase A (lane 2) and HUVEC, progelatinase A and thrombin (lane 3). The top band represents the 72 kDa progelatinase A, and the bottom two bands the 64 kDa intermediate and the 62 kDa fully active enzyme.

2. Materials and methods

2.1. Zymography

Zymography was performed according to previously published methods [15]. Briefly, samples were electrophoresed on a 10% Tris–glycine polyacrylamide electrophoresis gel with 0.1% gelatin as a substrate. Precast zymogram gels were run at 125 V for 90 min and were then incubated under the following conditions (room temperature unless specified): 30 min in renaturing buffer (2.5% w/v Triton X-100), 30 min in developing buffer (0.1 M Tris base, 0.4 M Tris–HCl, 67 mM $CaCl_2$, 0.2% w/v Brij 35), and 18–20 h in developing buffer at 37°C. Gels were stained according to the method of Leber and Balkwill [16]. Briefly, gels were rinsed with water and then stained in a solution containing 30% methanol, 10% glacial acetic acid, and 0.02% stain derived from PhastBlue tablets (Pharmacia, Uppsala, Sweden) for 2–4 h. Gels were then rinsed with distilled water for 15 min, soaked in drying solution (30% ethanol and 5% glycerol) for 15 min, and dried on cellophane sheets overnight.

2.2. Cell culture

THP-1 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) [17]. The cells were maintained between 1×10^5 and 4×10^5 cells/ml in RPMI 1640 medium (Sigma, St. Louis, MO, USA), with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (Gibco Laboratories, Gaithersburg, MD, USA), and 0.02 mM 2-mercaptoethanol (Sigma).

Human endothelial cells were isolated from the veins of discarded umbilical cords. The cells were isolated by collagenase digestion as previously described [18] and maintained in a medium composed of M-199 medium, 15% FBS, 0.1% endothelial cell growth supplement prepared from bovine hypothalami [19], and antibiotics. Cultures were not used beyond the sixth passage.

2.3. Enzymatic reactions

All enzymatic reactions were performed in either 100 μl reaction buffer (20 mM Tris–Cl, 100 mM NaCl, 10 mM $CaCl_2$, and 10 μM $ZnCl_2$, pH 7.5) or 250 μl RPMI 1640 with 1% FBS in the presence of THP-1 cells or HUVEC at a concentration of 1×10^5 cells/ml. Reactions were carried out for 48 h at 37°C.

The activation of progelatinase B as a positive control was accomplished by incubation of the enzyme in 0.05 M borate (pH 9.0) containing 0.01 mM $ZnCl_2$, 5 mM $CaCl_2$, and 0.5 mM 4-aminophenyl mercuric acetate for 2 h at 37°C. Human recombinant progelatinase A and progelatinase B were from Calbiochem (San Diego, CA, USA), rabbit lung thrombomodulin and human thrombin were from Haematologic Technologies Inc. (Essex Junction, VT, USA), and the unactivated and activated protein C were from Sigma and Haematologic Technologies Inc.

3. Results

3.1. Thrombin–thrombomodulin activation of progelatinase A

We determined the effect of thrombin on the activation of progelatinase A in the presence and absence of HUVEC and THP-1 cells, cell types also known to express thrombomodulin and cells actively involved in the acute inflammatory and atherogenesis processes. Initial studies examined the activation of progelatinase A in the presence of thrombin and endothelial cells. Fig. 1 presents results confirming that thrombin and endothelial cells convert the progelatinase A into the active gelatinase A (compare lane 2 with no thrombin to lane 3 containing thrombin). Almost all of the activity that was associated with the 64 kDa intermediate has now been converted to the fully active state. Similarly, as shown in Fig. 2, thrombin in the presence of THP-1 cells and 1% FBS was able to activate progelatinase A (compare lanes 2 and 3). An increase in the fully active, 62 kDa gelatinase A was balanced by a corresponding decrease in the 72 kDa progelatinase A and disappearance of the 64 kDa intermediate. However, in the presence of 1% FBS alone, thrombin was unable to activate progelatinase A (lane 5), indicating that the THP-1 cells were contributing an essential component for thrombin-medi-

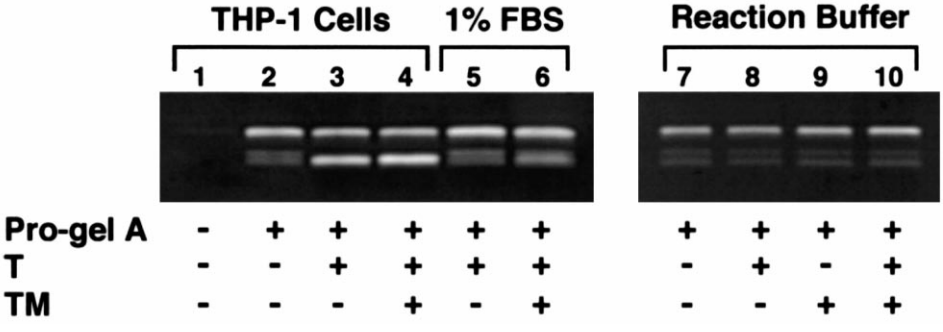

Fig. 2. Effect of thrombin and thrombomodulin on the activation of progelatinase A in THP-1 cells. Progelatinase A (pro-gel A, 5 nM) was incubated in the presence of thrombin (T, 50 nM) ± thrombomodulin (TM, 50 nM) in the presence of 1×10^5 THP-1 cells and 1% FBS, in 1% FBS alone, or in reaction buffer at 37°C. After 48 h, reaction mixes were subjected to zymography. Shown are representative zymogram gels of reactions conducted in the presence of THP-1 cells and 1% FBS (lanes 1–4), in 1% FBS alone (lanes 5 and 6), or in reaction buffer (lanes 7–10). The top band represents the 72 kDa progelatinase A, and the bottom two bands the 64 kDa intermediate and the 62 kDa fully active enzyme.

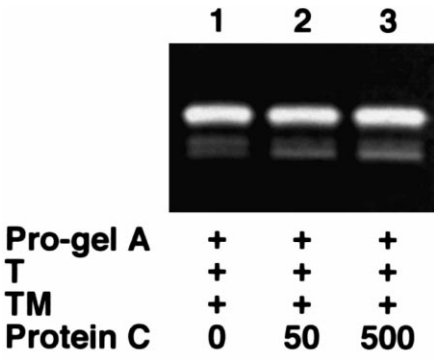

Fig. 3. Effect of thrombin, thrombomodulin, and protein C on the activation of progelatinase A. Progelatinase A (pro-gel A, 5 nM) was incubated in the presence of thrombin (T, 50 nM), thrombomodulin (TM, 50 nM), and increasing concentrations (nM) of protein C in reaction buffer for 48 h at 37°C. Shown is a representative zymogram gel of reaction mixtures after incubation. The top band represents the 72 kDa progelatinase A, and the bottom two bands represent the 64 kDa intermediate and the 62 kDa fully active enzyme.

ated progelatinase A activation. The component was not a secreted factor as THP-1 cell conditioned media failed to mediate the progelatinase A activation by thrombin (data not shown). To determine whether or not thrombomodulin was responsible for thrombin-stimulated progelatinase A activation, mixtures of rabbit lung thrombomodulin were incubated with thrombin and progelatinase A. Although it is unclear whether the addition of thrombomodulin changed the progelatinase A activation in the presence of THP-1 cells (Fig. 2, lane 4), the co-factor protein did facilitate progelatinase A activation by thrombin in the absence of THP-1 cells (Fig. 2, lane 6). The presence of 1% FBS was required for this activation, as shown by the finding that the combination of thrombin and thrombomodulin had no effect on progelatinase A when the reaction was performed in reaction buffer alone (Fig. 2, lane 10).

3.2. Protein C activation of progelatinase A

The ability of thrombin and thrombomodulin to activate progelatinase A in the presence, but not the absence, of FBS indicated that there was an additional molecule(s)

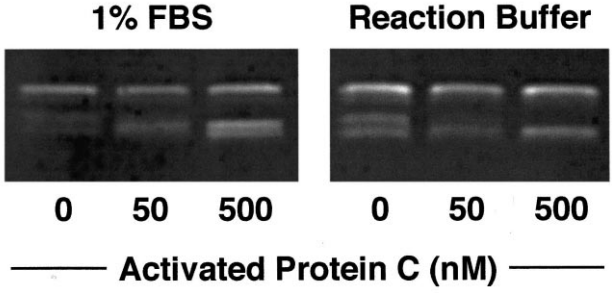

Fig. 4. Effect of activated protein C on the activation of progelatinase A. Increasing concentrations of activated protein C were added to 5 nM progelatinase A in the presence of THP-1 cells and 1% FBS or in reaction buffer alone for 48 h at 37°C. Shown are representative zymogram gels of reactions conducted in the presence of THP-1 cells and 1% FBS, or in reaction buffer. The top band represents the 72 kDa progelatinase A, and the bottom two bands represent the 64 kDa intermediate and the 62 kDa fully active enzyme.

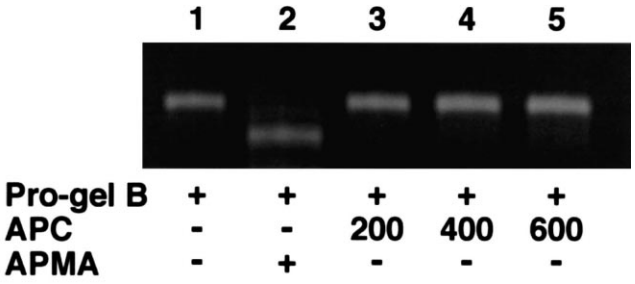

Fig. 5. Effect of activated protein C on the activation of gelatinase B. Shown is a representative zymogram depicting increasing concentrations (nM) of activated protein C (APC) added to progelatinase B (pro-gel B, 3 nM) in reaction buffer for 48 h at 37°C (lanes 3–5). Lane 1 represents unactivated gelatinase B, and lane 2, a positive control lane depicting fully active gelatinase B produced by treating progelatinase B with 0.5 mM 4-aminophenyl mercuric acetate (APMA) for 2 h at 37°C.

present in serum that was required for thrombin-mediated activation of progelatinase A. We therefore tested the ability of protein C, a known substrate for the thrombin–thrombomodulin complex, to substitute for the 1% serum in the activation of progelatinase A. As shown in Fig. 3, the addition of increasing concentrations of protein C in the presence of thrombin and thrombomodulin led to an increase in active gelatinase A in reaction buffer alone, indicating that activation of protein C by thrombin and thrombomodulin is involved in the activation of progelatinase A.

To confirm that activated protein C can act directly upon progelatinase A, the effect of activated protein C on progelatinase A was studied. As shown in Fig. 4, activated protein C enhanced formation of active gelatinase A in the presence of THP-1 cells and 1% FBS, and in reaction buffer alone. Activated protein C was therefore concluded to act upon gelatinase A directly. Here, the increase in active gelatinase A was due to the conversion of the 64 kDa intermediate to the final product while the amount of the 72 kDa progelatinase remained unchanged.

3.3. Activated protein C effects on progelatinase B

Plasmin is a serine protease that has previously also been shown to mediate the conversion of the 64 kDa gelatinase A

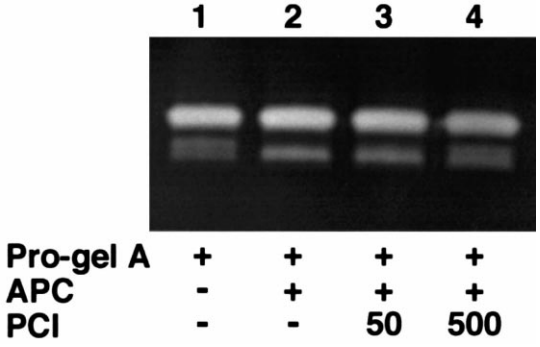

Fig. 6. Effect of protein C inhibitor on the activation of gelatinase A by activated protein C. Shown is a representative zymogram depicting increasing concentrations (nM) of protein C inhibitor (PCI) added to activated protein C (APC, 500 nM) and progelatinase A (pro-gel A, 5 nM) in reaction buffer for 48 h at 37°C. The top band represents the 72 kDa progelatinase A, and the bottom two bands represent the 64 kDa intermediate and the 62 kDa fully active enzyme.

intermediate and gelatinase B to the fully active forms [20]. As activated protein C and plasmin have similar effects on progelatinase A, we tested whether activated protein C was also able to activate progelatinase B. Increasing amounts of activated protein C were added to 3 nM progelatinase B in reaction buffer. As shown in Fig. 5, up to 600 nM of activated protein C had no effect on progelatinase B.

To rule out the presence of contaminating plasmin or other potential protease activity in the activated protein C preparations, the effect of protein C inhibitor on activation of gelatinase A was examined. As shown in Fig. 6, increasing the concentration of protein C inhibitor to 500 nM effectively eliminated the activation of gelatinase A, strongly indicating that activated protein C is the enzyme responsible for this activation.

4. Discussion

We demonstrate here that protein C, activated by thrombin and thrombomodulin, can also function to facilitate the activation of progelatinase A by converting the 64 kDa intermediate to the fully active 62 kDa form. These results demonstrate an important link between the anti-coagulant properties of thrombin and matrix turnover through the activation of progelatinase A. Baramova et al. have shown that the fibrinolytic protease, plasmin, can also cleave the gelatinase A intermediate to the active product in the presence of HT1080 cells [20]. In these studies, like those here, plasmin cannot initiate progelatinase processing, but can convert the 64 kDa intermediate to the mature, active form. It is interesting to note that both of these mechanisms are involved in anti-coagulation, suggesting that the activation of progelatinase A may play an important role in this process as well; however, the activation of a similar metalloproteinase, gelatinase B, appears specific to plasmin, as activated protein C was unable to activate this enzyme.

The differences we observed in the activation of progelatinase A between the thrombin/THP-1 and endothelial cell activation and activated protein C suggest that there is more to the ability of thrombin to activate gelatinase A than just the activation of protein C. Whereas the thrombin/THP-1 cell activation results in a conversion of the 72 kDa progelatinase enzyme to the active 62 kDa form, activated protein C only facilitates the conversion of the 64 kDa intermediate to the 62 kDa protein. The MT-MMP proteins are known to be able to process the 72 kDa proenzyme to the 64 kDa intermediate and it may be that on the cell surface, thrombomodulin coordinates with one of these proteins during progelatinase A activation. Although we have no direct evidence to support this conclusion, a similar mechanism of coordination has been suggested for plasmin [20].

Our results showing that thrombin alone is insufficient to activate the 72 kDa progelatinase A or the 64 kDa intermediate are in contrast with those of Galis et al. who reported that thrombin alone can activate progelatinase A [21]. The reaction conditions of Galis et al., however, included the presence of smooth muscle cell conditioned medium and serum. The differences between the results are thought to be due to the presence of serum containing protein C. It is also interesting to note that Galis et al. reported that thrombin can activate progelatinase A in the presence of cultured smooth muscle cells, as cultured smooth muscle cells have been shown to express thrombomodulin [22]. As the present experiments show, thrombin activated progelatinase A in the presence of THP-1 and endothelial cells, both of which express thrombomodulin on their surfaces.

Together, the results help to clarify the role of thrombin in the activation of progelatinase A and provide the first evidence for the involvement of activated protein C in metalloproteinase processing. Furthermore, these studies provide additional evidence that the intermediate to active conversion of gelatinase A may not necessarily occur autocatalytically as has been suggested, but may be accomplished by plasmin and activated protein C. Further clarification of these processes may in turn aid in the development of therapies and protocols for the treatment of diseases in which these cellular events run awry.

Acknowledgements: This work was supported by National Institutes of Health Grants HL-07751 (S.R.P.), HL-38779 (P.E.B.), and HL-36526 (R.L.H.).

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