Cell surface-mediated activation of progelatinase A: demonstration of the involvement of the C-terminal domain of progelatinase A in cell surface binding and activation of progelatinase A by primary fibroblasts

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We report that the isolated C-terminal domain of progelatinase A is inhibitory to the activation of this proenzyme by primary skin fibroblast plasma membranes but is unable to inhibit organomercurial-induced self-cleavage and activation. Ligand binding studies demonstrate that fibroblasts stimulated with concanavalin A to activate progelatinase A have a significantly enhanced level of cell surface-associated progelatinase A. Tissue inhibitor of metalloproteinases-2 (TIMP-2), an effective inhibitor of membrane-mediated progelatinase A activation, is able to abolish the enhanced level of cell surface-associated progelatinase A that occurs following stimulation. TIMP-1, a poor inhibitor of membrane activation, is unable to inhibit the cell surface binding of progelatinase A. The enhancement in the binding of ^{125}I -

progelatinase A to fibroblasts following concanavalin A stimulation can be blocked by the inclusion of excess C-terminal gelatinase A but not by a truncated form of gelatinase A lacking the C-terminal domain. Scatchard analysis of the binding of ^{125}I -progelatinase A to concanavalin A-stimulated fibroblasts has identified 950 000 gelatinase binding sites per cell with a K_d of 1.3×10^{-8} M. Analysis of non-stimulated fibroblasts has identified 500 000 sites per cell with a K_d of 2.6×10^{-8} M. We propose that membrane-mediated activation of progelatinase A involves binding of the proenzyme through its C-terminal domain to the cell surface and that TIMP-2 can inhibit activation by interaction with progelatinase A through the C-terminal domain, thus preventing binding of the proenzyme.

INTRODUCTION

Gelatinase A (72 kDa gelatinase, type IV collagenase, MMP-2) is a metalloendopeptidase secreted by a wide range of cell types from connective tissues and tumours (Seltzer et al., 1981; Murphy et al., 1981, 1985; Collier et al., 1988; Stetler-Stevenson, 1990). It has the ability to degrade many connective tissue components including denatured fibrillar type I, II and III collagens, native type IV and V collagen and types VII and XI collagens (reviewed by Docherty and Murphy, 1990). Additionally, gelatinase A is able to degrade elastin (Murphy et al., 1991; Senior et al., 1991) and has low activity towards proteoglycan (Murphy et al., 1991), laminin and fibronectin (Okada et al., 1990). Gelatinase A is secreted as a proenzyme which can be converted by mercurial compounds to an active form via conformational change and self-cleavage (Stetler-Stevenson et al., 1989).

Progelatinase A is unique amongst the matrix metalloproteinases in that it fails to activate after treatment with exogenous proteinases such as plasmin, plasma kallikrein, neutrophil elastase or cathepsin G (Nagase et al., 1991), which have been identified as putative physiological activators of other members of this family. A number of studies have identified gelatinase activities of 58 kDa, which correspond to the activated form of gelatinase A, from sources such as human gastric and colorectal carcinoma tissue (Yamagata et al., 1991) and wounded rabbit cornea (Matsubara et al., 1991). The search for a physiological mechanism of progelatinase activation has led to a number of descriptions of cell-mediated activation processes. These have frequently involved tumour cells (Brown et al., 1990, 1993;

Azzam and Thompson, 1992; Strongin et al., 1993), but we and others have also shown that the capacity to activate progelatinase A may be elicited from primary fibroblasts when treated with concanavalin A or phorbol esters (Overall and Sodek 1990; Ward et al., 1991a; Atkinson et al., 1992). In addition we have been able to demonstrate that both normal fibroblasts and osteoblasts plated onto type I collagen can activate progelatinase A spontaneously (S. Atkinson and S. Bord, unpublished work).

We previously demonstrated the existence of a cell surface activator of progelatinase A, which can be inhibited by metalloproteinase inhibitors, and showed that tissue inhibitor of metalloproteinases-2 (TIMP-2) is a more potent inhibitor of activation than TIMP-1 (Ward et al., 1991a). Use of a C-terminally truncated progelatinase demonstrated that the C-terminal domain plays an important role in the regulation of gelatinase A by a potential physiological activator (Murphy et al., 1992). In the present study the binding of progelatinase A to concanavalin A (Con-A)-stimulated fibroblasts was investigated together with the effect of the C-terminal domain and TIMPs on the binding and activation of progelatinase A.

MATERIALS AND METHODS

Culture of human infant foreskin fibroblasts for studies on the activation of progelatinase A

For studies on the activation of progelatinase A by fibroblast monolayers, human infant foreskin fibroblasts were prepared from explants of foreskin taken at circumcision (courtesy of Dr. T. Cawston, Addenbrooke's Hospital, Cambridge, U.K.) using

Abbreviations used: TIMP, tissue inhibitor of metalloproteinases; Con-A, concanavalin A; APMA, 4-aminophenylmercuric acetate; DMSO, dimethyl sulfoxide; ^{125}I -proGLA, ^{125}I -labelled progelatinase A; DMEM, Dulbecco's modified Eagle's medium; proN-GL, C-terminally truncated $\Delta 418$ –631 progelatinase A.

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the method described for human gingival fibroblasts (Heath et al., 1982).

Preparation of human fibroblast plasma membrane-enriched fraction

Crude fibroblast plasma-membrane preparations were prepared from Con-A-stimulated human fibroblasts according to the method described by Ward et al. (1991a). Third to sixth passage fibroblasts were grown to confluence and stimulated with 50 µg/ml Con-A for 24 h. Cells were scraped from culture flasks (approx. 15 × 175 cm² flasks) and pelleted by centrifugation (1200 g, 10 min, 4 °C). Whole cell lysates were prepared by resuspending the cells at 2 × 10⁷/ml in ice-cold 5 mM Tris/HCl (pH 7.8) and incubating at 0 °C for 10 min prior to homogenization by 30 passages through a narrow gauge needle (26G). Crude plasma membrane preparations were prepared by centrifugation of the whole cell lysate at 10000 g for 10 min at 4 °C; the supernatant was retained and centrifuged at 100000 g for 1 h at 4 °C. The 100000 g pellet was washed in 20 mM Tris/HCl (pH 7.8)/10 mM CaCl₂/0.05 % Brij 35, recentrifuged (100000 g, 40 min, 4 °C) and resuspended in the pellet washing buffer at a concentration of 2 mg/ml assuming an $A_{1\text{cm}, 280\text{nm}}^{1\%}$ of 10, and stored at -70 °C.

Preparation and purification of the C-terminal domain of gelatinase A

Natural human gelatinase A [256 µg in 1.43 ml of 20 mM Tris/HCl (pH 7.8)/10 mM CaCl₂/0.02 % NaN₃/0.04 % Brij 35/1 M NaCl] purified from human gingival fibroblast-conditioned medium (Ward et al., 1991b) was autolysed by incubation with 2 mM 4-aminophenylmercuric acetate (APMA) for 16 h at 35 °C. The autolysed gelatinase was dialysed against 2 × 2 l of 25 mM Tris/HCl (pH 7.4)/5 mM CaCl₂/0.02 % NaN₃ (w/v)/0.04 % Brij 35 (v/v) (TCAB buffer)/1 M NaCl at 4 °C. Following dialysis the sample was applied to a 1 ml bed volume column of gelatin-Sepharose, and eluted with TCAB buffer containing 10 % dimethyl sulphoxide (DMSO) and 1 M NaCl. The non-gelatin binding fractions from this step were pooled and concentrated to 4 ml (Amicon Centricon microconcentrator). A further gel filtration step on the non-gelatin binding pool, using an Ultrogel AcA54 (0.6 cm × 54 cm column) equilibrated in TCAB buffer with 1 M NaCl, yielded an homogeneous 31 kDa protein (SDS/PAGE, reducing conditions).

Electrophoresis

Samples were analysed by electrophoresis on 7–12 % (w/v) polyacrylamide gels (Laemmli and Favre, 1973). Proteins were detected by silver staining (Merril et al., 1981). Gelatin degrading activity was assessed by electrophoresis on non-reducing SDS/polyacrylamide gels (Laemmli and Favre, 1973), incorporating 0.5 mg of gelatin/ml (Heussen and Dowdle, 1980).

Gelatinase assays

Assays for gelatinase activity were performed as described by Murphy et al. (1981). One unit of gelatinase degraded 1 µg of gelatin in 1 min at 37 °C.

¹²⁵I labelling of progelatinase A

Human recombinant progelatinase A purified from a mouse myeloma expression system (Murphy et al., 1991) was radio-labelled with ¹²⁵I by the method of Bolton and Hunter (1973), to

a specific activity of 7 × 10⁵ c.p.m./µg or 1 × 10⁶ c.p.m./µg. ¹²⁵I-labelled progelatinase was separated from free ¹²⁵I by the use of a 0.5 ml bed volume spin column of Sephadex G-15. ¹²⁵I-labelled progelatinase A (¹²⁵I-proGLA) was stored at 4 °C prior to use.

Autoradiographic detection of ¹²⁵I-proGLA binding to fibroblasts

Human foreskin fibroblasts were cultured on Lab-Tek tissue culture chamber slides until they were approximately 50 % confluent. The cell layer was then washed with Dulbecco's Modified Eagle's Medium (DMEM) and cultured in DMEM with or without 50 µg/ml Con-A for 18 h. Following Con-A stimulation the cell layer was washed three times with DMEM and then chilled to 4 °C for 30 min. Aliquots of ¹²⁵I-proGLA (7 × 10⁵ c.p.m./µg) were then added to the cell layer at a concentration of 0.7 µg/ml (total of 100 ng/well) and incubated for 30 min at 4 °C. For the investigation of the effect of TIMPs on binding, equimolar recombinant human TIMP-1 (a gift from Peter Koklitis and Saroj Angal, Celltech, Slough, Berks, U.K.) or TIMP-2 (purified by the method of Willenbrock et al., 1993) was incubated with the ¹²⁵I-proGLA at 25 °C for 1 h prior to addition to the cell layer. ¹²⁵I-proGLA ligand was then removed from the chamber slide wells and the cell layer washed three times with ice-cold PBS followed by ice-cold distilled water and air dried. Slides were dipped in k2 photographic emulsion in gel form (Ilford Scientific Products) and left for between 2 and 21 days prior to development. Slides were stained with haematoxylin and eosin and examined with a Zeiss photomicroscope loaded with Ektachrome 64 film.

Quantitative analysis of ¹²⁵I-proGLA binding to fibroblast monolayers

Human infant foreskin fibroblast monolayers for binding experiments were cultured in 24-well Linbro cell culture plates with DMEM plus 10 % foetal calf serum until monolayers were approximately 90 % confluent (approx. 2 × 10⁵ cells/well). The monolayers were then washed with serum-free DMEM and cultured for 18 h with either serum-free DMEM or 50 µg/ml Con-A in DMEM. Monolayers were then chilled to 4 °C for 30 min and washed three times with ice-cold DMEM containing 1 mg/ml BSA (binding buffer). ¹²⁵I-proGLA (300 µl per well at concentrations between 1 × 10⁻¹¹ M and 2 × 10⁻⁷ M) was added to the cell monolayers in the presence or absence of competing ligands. Unlabelled competing ligands were added to the labelled ligand as a 25-fold excess (except in the case of TIMPs for which up to 10 mol of TIMP were added per mole of ligand) relative to the concentration of labelled ligand and incubated at 25 °C for 1 h prior to addition to fibroblast monolayers. Ligand was incubated with prepared fibroblast monolayers for 30 min at 4 °C followed by three 1 ml washes with ice-cold binding buffer. Bound ¹²⁵I-proGLA was determined by extracting the monolayer with 0.1 % Triton X-100/0.1 M NaOH and quantitating the solubilized ¹²⁵I in a Packard Multi Prias gamma counter. Scatchard plots were derived by analysis of the binding data on an IBM PC using the Biosoft radioligand binding analysis programme 'LIGAND' (McPherson, 1985), which accounts for non-specific binding and obtains final parameter estimates using weighted non-linear curve fitting techniques.

RESULTS

Preparation and purification of the C-terminal domain of progelatinase A

The C-terminal domain of progelatinase A was prepared by autolysing progelatinase A in the presence of the organomercurial

Figure 1 Purification of the C-terminal domain of progelatinase A

The C-terminal domain of progelatinase A was prepared by performing a prolonged 16 h auto-activation of progelatinase A at 35 °C with 2 mM APMA. The products of this reaction which were analysed on a silver-stained 10% polyacrylamide gel are shown in lane 1. The C-terminal domain was found not to bind to gelatin–Sephacrose; the unbound material from this step is shown in lane 2. A further gel filtration chromatography step yielded a homogeneous 31 kDa band (lane 3). Molecular mass standards are indicated.

Figure 2 Activation of progelatinase A by a fibroblast membrane preparation in the presence of the C-terminal gelatinase preparation and TIMP-2

Aliquots (23 ng) of progelatinase A were incubated for 18 h at 35 °C in the presence of 25 μ g aliquots of a Con-A-stimulated fibroblast membrane preparation. The aliquots of membrane preparation had previously been pre-incubated for 30 min at 35 °C: (lane 1) with 2 mol of TIMP-2 per mol of progelatinase; (lane 2) 1.5 mol of C-terminal domain per mol of progelatinase; (lane 3) 15 mol of C-terminal domain per mol of progelatinase; and (lane 4) in the absence of TIMP-2 or C-terminal domain. An aliquot of the progelatinase (lane 5), C-terminal domain (lane 6) and membrane preparation (lane 7), were incubated alone for comparison purposes. Samples were electrophoresed on a 7.5% SDS/polyacrylamide gel containing gelatin. Molecular mass standards are indicated.

APMA for a prolonged period. This treatment led to the generation of at least seven products (Figure 1, lane 1). The autolysed sample was applied to a 1 ml bed volume of gelatin–Sephacrose and eluted with 10% DMSO (see the Materials and methods section). SDS/PAGE analysis of the non-gelatin binding pool identified a major species of 31 kDa (Figure 1, lane 2). A further gel filtration step on the non-gelatin binding pool using an Ultrogel AcA54 yielded a homogeneous 31 kDa protein (Figure 1, lane 3).

Figure 3 Effect of the C-terminal domain on the activation of progelatinase A initiated by the fibroblast membrane preparation

Pregelatinase A (40 ng) was incubated for 8 h with 20 μ g of fibroblast membrane preparation. The membrane preparation had been pre-incubated for 30 min at 35 °C either in the absence of C-terminal gelatinase preparation or in the presence of up to 10 mol of C-terminal domain per mol of gelatinase. Following the 8 h incubation of progelatinase with membrane preparation in the presence of increasing amounts of the C-terminal domain, samples were assayed for gelatinolytic activity.

Properties of the C-terminal domain

The purified C-terminal fragment had a molecular mass of 31 kDa under reducing SDS/PAGE conditions and 25 kDa under non-reducing conditions, suggesting that the C-terminal fragment had an intact disulphide bond. Activity analysis by gelatin zymography demonstrated that this gelatinase fragment had no gelatinolytic activity (Figure 2, lane 6). N-terminal sequence analysis of the 31 kDa non-gelatin binding protein after reduction and pyridylethylation (Amons, 1987) yielded the sequence LYGASPDIDLGXGPTXXLGP (where X is an undetermined residue), enabling this protein to be identified as the complete C-terminal domain plus the last three residues of the catalytic domain.

The C-terminal domain of progelatinase A inhibits activation of progelatinase A by fibroblast membranes

Addition of aliquots of plasma membrane preparation from Con-A-stimulated fibroblasts to progelatinase A resulted in the processing of the 66 kDa proenzyme into two main zones of lysis of molecular mass 59 kDa and 43 kDa and a minor 62 kDa form (Figure 2, lane 4). Pre-incubation of the membrane preparation with 1.5 mol of the C-terminal gelatinase preparation per mol of progelatinase A prior to its addition to progelatinase A resulted in a significant reduction in the intensity of the 59 kDa and 43 kDa zones of lysis and an increase in the intensity of the 62 kDa intermediate form (Figure 2, lane 2). Pre-incubation of the fibroblast membrane preparation with 15 mol of the C-terminal gelatinase preparation per mol of progelatinase A resulted in minor zones of lysis of 62 kDa and 59 kDa with the major zone of lysis corresponding to the 66 kDa proenzyme (Figure 2, lane 3). Figure 2 also demonstrates that processing of the proenzyme to the 62 kDa and 59 kDa forms could be fully inhibited by 2 mol of TIMP-2 per mol of progelatinase (Figure 2, lane 1). The ability of the C-terminal preparation to inhibit the processing by the fibroblast membrane preparation of the proenzyme to 62 kDa and 59 kDa forms also resulted in inhibition of the appearance of gelatin-degrading activity (Figure 3), measured in a heat-denatured type I collagen assay. This inhibition was dose-dependent and required at least 10 mol of C-

Figure 4 SDS/PAGE analysis of the effect of the C-terminal gelatinase domain on the APMA-initiated auto-activation of progelatinase A

Aliquots (0.4 μ g) of progelatinase A (lane 1) were incubated for 3 h with 2 mM APMA at 25 °C either in the absence of the C-terminal domain (lanes 2 and 6) or in the presence of 1 mol of C-terminal gelatinase per mol of progelatinase (lane 3); or 5 mol of C-terminal gelatinase per mol of progelatinase (lane 4); or 10 mol of C-terminal gelatinase per mol of progelatinase (lane 5). Samples were analysed under reducing conditions on a silver stained 10% SDS/polyacrylamide gel. Molecular mass standards are indicated.

terminal domain per mol of full-length progelatinase in order to inhibit completely the appearance of gelatin degrading activity. The C-terminal gelatinase A preparation was also found to inhibit the processing of progelatinase A to the 59 kDa form by Con-A-stimulated cell monolayers but not as fully as the processing initiated by stimulated fibroblast membrane preparations (results not shown).

The C-terminal domain of progelatinase A inhibits membrane-mediated activation but not organomercurial-initiated auto-activation of progelatinase A

In an attempt to discover whether the C-terminal preparation was inhibiting autoprocessing of the progelatinase to lower molecular mass forms, progelatinase A was activated with the organomercurial APMA in the presence or absence of up to 10 mol of C-terminal domain per mol of progelatinase A. Figure 4 demonstrates that addition of the C-terminal domain did not inhibit the APMA-initiated autocatalytic processing of the 72 kDa proenzyme to its 62 kDa form via a 66 kDa intermediate (molecular masses determined under reducing SDS/PAGE conditions). Gelatinase activity assays measuring [14 C]gelatin degradation established that up to a 10-fold molar excess of the C-terminal fragment had no effect on the activation of gelatinase A by APMA, nor did it affect the gelatinolytic activity of APMA-activated gelatinase A (results not shown).

Autoradiography of 125 I-proGLA binding to human fibroblasts

The appearance of gelatinase A activating capacity following stimulation of fibroblasts with 50 μ g/ml Con-A was found to lead to an increased ability of fibroblasts to bind 125 I-proGLA at 4 °C. Figure 5, panel A demonstrates the sparse silver grain distribution following exposure of non-stimulated fibroblasts to 125 I-proGLA for 30 min followed by autoradiography. These grains were uniformly distributed with no increased intensity associated with the fibroblasts. When this procedure was per-

Figure 5 Autoradiographic analysis of the effects of TIMP-1 and TIMP-2 on the binding of 125 I-proGLA to human fibroblasts

Sixth passage human infant foreskin fibroblasts were cultured on Lab Tek tissue culture chamber slides in the presence or absence of 50 μ g/ml Con-A. 125 I-proGLA (100 ng/well) was applied to fibroblasts cultured in the absence of Con-A (panel A) or the presence of Con-A (panel B). 125 I-proGLA was incubated (1 h, 25 °C) with equimolar TIMP-2 prior to incubation with non-Con-A-stimulated (panel C) and Con-A-stimulated (panel D) fibroblasts or with equimolar TIMP-1 prior to incubation with non-stimulated (panel E) or Con-A-stimulated (panel F) fibroblasts. Cell-bound 125 I-proGLA was detected by the use of an autoradiographic emulsion.

formed with Con-A-stimulated fibroblasts (Figure 5, panel B) a significant increase in the number of cell-associated silver grains was detected whilst the background non-cell-associated level of silver grains was not significantly increased. It was found that the intensity of silver grains observed when 125 I-proGLA was incubated with stimulated fibroblasts could be significantly reduced by pre-incubation of the progelatinase with equimolar TIMP-2 for 1 h at 25 °C prior to addition to cell monolayers (Figure 5, panel D). Within the same experiment it was observed that a similar pre-incubation of the progelatinase with TIMP-1 instead of TIMP-2 resulted in no such reduction in the intensity of silver grain position (Figure 5, panel F). TIMP-1 and TIMP-2 were found to have no detectable effect on the binding of progelatinase A to non-stimulated fibroblasts (Figure 5, panels C and E).

Fibroblasts stimulated to activate progelatinase A demonstrate an increased capacity to bind progelatinase A, which can be blocked by either TIMP-2 or the C-terminal domain of the proenzyme

The use of human infant foreskin fibroblasts grown as monolayers in multi-well culture dishes and 125 I-proGLA enabled a number of quantitative progelatinase binding studies to be performed. Figure 6 demonstrates the time course of the binding of 125 I-proGLA to stimulated and non-stimulated fibroblasts. Stimulated fibroblasts were found to bind the 125 I-proGLA rapidly with half-maximal binding observed after 1 min at 4 °C; non-stimulated fibroblasts demonstrated a similar binding profile but maximal binding reached was approx. 30% of

Figure 6 Time course of the binding of ^{125}I -proGLA to stimulated and non-stimulated human fibroblasts

Following an 18 h incubation in serum-free medium with or without 50 $\mu\text{g}/\text{ml}$ Con-A, stimulated (Δ) and non-stimulated ($\circ$) fibroblasts were washed three times with binding buffer. Aliquots (300 μl) of ^{125}I -proGLA at a concentration of 2×10^{-7} M were applied to fibroblast monolayers and incubated for between 0.5 and 60 min. Cell monolayers were washed three times and cell-bound ^{125}I -proGLA determined.

Figure 7 Effects of TIMP-1 and TIMP-2 on the binding of ^{125}I -proGLA to stimulated and non-stimulated human fibroblasts

Following an 18 h incubation in serum-free medium with or without 50 $\mu\text{g}/\text{ml}$ Con-A, stimulated and non-stimulated fibroblasts were washed 3 times with binding buffer. Fibroblast monolayers were incubated with 250 μl aliquots of ^{125}I -proGLA ligand at a concentration of 6×10^{-9} M. Prior to incubation with cell monolayers ^{125}I -proGLA had been pre-incubated for 1 h at 25 $^{\circ}\text{C}$ with up to 10 mol of TIMP-2 per mol of ^{125}I -proGLA or up to 10 mol of TIMP-1 per mol of ^{125}I -proGLA. Fibroblast monolayers were incubated for 30 min at 4 $^{\circ}\text{C}$ with ligand as follows: ^{125}I -proGLA pre-incubated with TIMP-1 followed by incubation with stimulated fibroblasts ($\bullet$) and non-stimulated fibroblasts ($\circ$); ^{125}I -proGLA pre-incubated with TIMP-2 followed by incubation with stimulated fibroblasts ($\blacktriangle$) and non-stimulated fibroblasts ($\triangle$). Cell monolayers were washed three times and cell-bound ^{125}I -proGLA determined.

that observed with stimulated fibroblasts. Figure 7 demonstrates the effect of TIMP-1 and TIMP-2 on the binding of ^{125}I -proGLA to stimulated and non-stimulated fibroblasts. Pre-incubation of ^{125}I -proGLA ligand with increasing amounts of TIMP-2 prior to addition to stimulated fibroblast monolayers resulted in a substantial reduction in ^{125}I -proGLA bound to the fibroblasts. The

Figure 8 Effects of excess C-terminal, N-terminal and full-length progelatinase A on the binding of ^{125}I -proGLA to Con-A-stimulated (b) and non-stimulated (a) human fibroblasts

Following an 18 h incubation in the presence (stimulated) or absence (unstimulated) of 50 $\mu\text{g}/\text{ml}$ Con-A, fibroblasts were washed three times with binding buffer. Ligand binding was performed at 4 $^{\circ}\text{C}$ for 30 min with increasing concentrations of ^{125}I -proGLA alone on stimulated (Δ) and non-stimulated fibroblasts ($\blacktriangle$). Ligand binding was also performed in the presence of (i) 25 mol of full-length progelatinase A per mol of ^{125}I -proGLA on stimulated ($\circ$) and non-stimulated ($\bullet$) fibroblasts, (ii) 25 mol of N-terminal progelatinase A per mol of ^{125}I -proGLA on stimulated ($\square$) and non-stimulated ($\blacksquare$) fibroblasts, and (iii) 25 mol of C-terminal progelatinase A per mol of ^{125}I -proGLA on stimulated ($\diamond$) and non-stimulated ($\blacklozenge$) fibroblasts. Following ligand binding, monolayers were washed three times and cell-bound ^{125}I -proGLA determined.

quantity of ^{125}I -proGLA bound to stimulated fibroblasts was reduced to the level of binding to non-stimulated fibroblasts by pre-incubation of the ligand with between 2 and 5 mol of TIMP-2 per mol of ^{125}I -proGLA. Pre-incubation of ^{125}I -proGLA with increasing amounts of TIMP-1 prior to addition to stimulated fibroblasts led to a small dose-dependent increase in the cell-associated binding of ^{125}I -proGLA. When these experiments were performed with non-stimulated fibroblasts the binding profile was very similar but the extent of ^{125}I -proGLA binding was much reduced when compared with stimulated fibroblasts. The effect of TIMP-1 and TIMP-2 on the binding of ^{125}I -proGLA to non-stimulated fibroblasts was most pronounced at TIMP concentrations of up to 1 mol of TIMP per mol of ^{125}I -proGLA. Little further effect was observed over the range of 1–10 mol of TIMP per mol of progelatinase. Differences in levels of bound gelatinase between the time course experiments and the TIMP inhibition experiments can be explained by the necessity of using a higher concentration of ligand in the former experiments. The limits of ^{125}I labelling of the enzyme required the use of an approx. 30-fold increase in ligand concentration to facilitate the detection of differences in bound radioactivity at very early time points compared with later time points. Raising the concentration of ligand led to an approximately 10-fold increase in bound ligand overall.

Stimulated and non-stimulated fibroblasts were found to differ with respect to the ability of the isolated C-terminal domain to compete with ^{125}I -proGLA binding. This result was further supported by the use of a truncated progelatinase lacking the C-terminal domain ($\Delta 418$ –631 gelatinase, proN-GL; Murphy et al., 1992). With non-stimulated fibroblasts, proN-GL was found to be as effective a competitor of ^{125}I -proGLA binding as unlabelled progelatinase A (Figure 8, panel a). However in the case of stimulated fibroblasts, whereas unlabelled proGLA was an effective competitor of ^{125}I -proGLA binding, proN-GL was a

Figure 9 Scatchard analysis of the binding of progelatinase A to Con-A-stimulated fibroblasts

Scatchard plot for the binding of progelatinase A ligand to Con-A-stimulated fibroblasts. The dissociation constant for a single site is shown in the top right-hand corner. Each data point is the mean of a triplicate determination.

poor competitor of binding. Figure 8, panel B demonstrates that the enhancement in the binding of ^{125}I -proGLA to fibroblasts following Con-A stimulation can be abolished by the presence of excess C-terminal gelatinase. In contrast the C-terminal domain resulted in no significant reduction in ^{125}I -proGLA binding to non-stimulated fibroblasts.

Scatchard analysis

Scatchard analysis of the binding of ^{125}I -proGLA to Con-A-stimulated fibroblasts identified 950 000 gelatinase binding sites per cell with a K_d of 1.3×10^{-8} M (Figure 9). Analysis of non-stimulated fibroblasts identified 500 000 sites per cell with a K_d of 2.6×10^{-8} M.

DISCUSSION

We previously showed that unlike the full-length proenzyme, a C-terminally truncated progelatinase A (proN-GL) cannot be activated by membrane preparations from normal primary fibroblasts stimulated with Con-A. Treatment of proN-GL with APMA, however, demonstrated that in the absence of the C-terminal domain, proN-GL is able to undergo auto-activation with similar kinetics to the wild-type enzyme (Murphy et al., 1992). In this paper we have investigated the hypothesis that the C-terminal domain of progelatinase A is essential for the binding/interaction of progelatinase A with a membrane-bound activator. Our data demonstrate that the progelatinase A-activating capacity of isolated cell membranes is inhibited by pre-incubation of the membrane preparation with the C-terminal domain. The results are in agreement with the experiments of Strongin et al. (1993) reported whilst this paper was in preparation. These workers used the fibrosarcoma cell line HT1080 stimulated with phorbol 12-myristate 13-acetate to demonstrate membrane-associated activation which could be inhibited by both TIMP-2 and a 26 kDa peptide derived from the C-terminal domain. Our work indicates that a similar inhibition of activation can be demonstrated in normal primary fibroblasts stimulated with Con-A. We propose that pre-incubation of the membrane preparation with the C-terminal domain enables it to bind non-productively to membrane-associated binding sites and in so doing act as a competitive inhibitor of the binding and activation

of progelatinase A. The C-terminal domain was not inhibitory to APMA-initiated auto-activation of progelatinase A, even when present as a 10-fold molar excess. Thus the possibility that the C-terminal domain inhibits the auto-activation of progelatinase, a process proposed as a mechanism for the physiological activation of progelatinase A (Ward et al., 1991a; Strongin et al., 1993), seems to be ruled out by the data presented here.

In order to support the proposed role of the C-terminal domain in progelatinase A activation, the ability of the C- and N-terminal domains and full-length progelatinase to compete with ^{125}I -proGLA for cell surface binding sites was investigated. The conclusions drawn from these experiments, of which Figure 8 is representative, are that there are at least two potential modes of binding of progelatinase A to fibroblasts. With non-stimulated fibroblasts, gelatinase binding is predominantly mediated via the N-terminal domain of the proenzyme, possibly through the gelatin binding domain. In this case an excess of C-terminal domain is not an inhibitor of ^{125}I -proGLA binding, whereas the proN-GL and full-length gelatinase A are effective competitive inhibitors. The stimulated fibroblasts also demonstrate an N-terminal-mediated gelatinase binding capacity which competes for ^{125}I -proGLA binding to a similar extent as the non-stimulated cells. In addition they have a significantly larger number of C-terminal binding sites, which are not competed off with proN-GL. These sites are, however, competed off with the isolated C-terminal domain.

Scatchard analysis of the ^{125}I -proGLA binding sites revealed approximately twice the number of binding sites on the Con-A-stimulated cells compared with sites on the unstimulated cells. However our data suggest that the affinity of gelatinase A for binding to matrix (occurring in both unstimulated and stimulated cells) and to the Con-A-induced cell membrane sites is of a comparable order of magnitude. Gelatinase A binding in unstimulated cell cultures is largely via the extracellular matrix; thus the majority of binding sites estimated for unstimulated cells are not cell surface sites.

The autoradiography studies also showed an enhanced level of progelatinase A bound to stimulated fibroblasts and further support the proposal that activation occurs at the cell surface (Ward et al., 1991a; Brown et al., 1993; Strongin et al., 1993). Silver grain distribution in unstimulated cells (Figure 5, panels A, C and E) demonstrates widely distributed binding compared with the intensely cell-associated binding seen in the stimulated cells (Figure 5, panel B) where all the Con-A-induced sites are on the cell surface. We have previously reported that TIMP-2 is a more potent inhibitor of membrane-mediated progelatinase A activation than TIMP-1 (Ward et al., 1991a) and conclude that by binding to progelatinase A, TIMP-2 is able to block the interaction of the proenzyme with the cell surface and inhibit activation (Ward et al., 1991a; Murphy et al., 1992). These observations have also been made by Strongin et al. (1993). Figure 5 also demonstrates that TIMP-2 inhibits binding of labelled progelatinase A to the plasma membrane of Con-A-stimulated fibroblasts. The direct binding studies (Figure 7) confirm that the enhanced level of progelatinase A bound to gelatinase activating-fibroblasts can be abolished by pre-incubation of the progelatinase with TIMP-2. At approximately equimolar concentrations of progelatinase and inhibitor, binding drops to 65 % of that bound to stimulated cells in the absence of TIMP-2, whereas 5 mol of TIMP-2 per mol of enzyme reduces the binding to the level of control binding to unstimulated cells. In contrast, TIMP-1 appears to cause a marginal enhancement of proGLA binding. Howard and Banda (1991) and Fridman et al. (1992) have reported that IMP-2 binds to the C-terminal domain of progelatinase A and our data suggest that TIMP-2 may play

a key role in the regulation of its cell surface activation. In the absence of TIMP-2, progelatinase A is able to bind through its C-terminal domain to cell surface activation sites; however, this binding is blocked when TIMP-2 binds to the C-terminal domain of progelatinase A and cell surface-associated activation is decreased.

Our results suggest that the appearance of progelatinase A activating capacity can be correlated with the appearance of a cell surface gelatinase binding site. Interaction of progelatinase with this site leads to the binding and activation of progelatinase A at the cell surface. Strongin et al. (1993) have proposed that binding to the cell membrane may alter the conformation of proGLA such that it is capable of autoproteolytic processing. A role for the C-terminal domain in enhancing auto-activation rates has been identified by the experiments of Crabbe et al. (1993), who conclude that interaction of the C-terminal domain of progelatinase A with ionic cell surface macromolecules may trigger bimolecular autoprocessing of progelatinase A. Similar autoprocessing of progelatinase A can be shown in the presence of dextran sulphate (G. Murphy, unpublished work). We have, however, ruled out the possibility that heparan sulphate proteoglycans on the cell surface may be responsible for progelatinase A binding and processing: the presence of heparin or protamine or heparitinase treatment of cells has had no effect upon the processing of progelatinase A by Con-A fibroblasts. Contact activation reactions have been described for many other zymogens. Procathepsin L, secreted as an inactive zymogen, can undergo activation in the presence of negatively charged surfaces (Mason and Massey, 1992). Additionally, auto-activation of Factor XII by dextran sulphate is described by Samuel et al. (1992), who propose an *in vivo* contact activation mechanism in which initial conformational change on binding of Factor XII is followed by efficient proteolytic cleavage when molecules are aggregated together on the cell surface. Crabbe et al. (1993) also show that at sufficiently high concentrations autoprocessing of progelatinase A can occur spontaneously. It is therefore possible that the binding of progelatinase A to activated cell surfaces also concentrates the enzyme locally to allow initiation of the autoproteolytic process. However, we cannot completely rule out the action of a membrane-bound proteinase that cleaves progelatinase A in a manner analogous to the plasmin initiation of prostromelysin and procollagenase autoprocessing. Further studies on the nature of the cellular binding site should shed further light on the means by which this enzyme is activated.

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