

Identification of a novel 82 kDa proMMP-9 species associated with the surface of leukaemic cells: (auto-)catalytic activation and resistance to inhibition by TIMP-1

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MMP-9 (matrix metalloproteinase 9) plays a critical role in tumour progression. Although the biochemical properties of the secreted form of proMMP-9 are well characterized, little is known about the function and activity of cell surface-associated proMMP-9. We purified a novel 82 kDa species of proMMP-9 from the plasma membrane of THP-1 leukaemic cells, which has substantial differences from the secreted 94 kDa proMMP-9. The 82 kDa form was not detected in the medium even upon stimulation with a phorbol ester. It is truncated by nine amino acid residues at its N-terminus, lacks O-linked oligosaccharides present in the 94 kDa proMMP-9, but retains N-linked carbohydrates. Incubation of 94 kDa proMMP-9 with MMP-3 generated the well-known 82 kDa active form, but the 82 kDa proMMP-9 was converted into an active species of 35 kDa, which was also produced by autocatalytic processing in the absence of activating enzymes. The activated 35 kDa MMP-9 effi-

ciently degraded gelatins, native collagen type IV and fibronectin. The enzyme was less sensitive to TIMP-1 (tissue inhibitor of metalloproteinase 1) inhibition with IC₅₀ values of 82 nM compared with 1 nM for the 82 kDa active MMP-9. The synthetic MMP inhibitor GM6001 blocked the activity of both enzymes, with similar IC₅₀ values below 1 nM. The 82 kDa proMMP-9 is also produced in HL-60 and NB4 leukaemic cell lines as well as *ex vivo* leukaemic blast cells. It is, however, absent from neutrophils and mononuclear cells isolated from peripheral blood of healthy individuals. Thus, the 82 kDa proMMP-9 expressed on the surface of malignant cells may escape inhibition by natural TIMP-1, thereby facilitating cellular invasion *in vivo*.

Key words: acute myeloid leukaemia, gelatinase B, glycosylation, matrix metalloproteinase (MMP), tissue inhibitor of metalloproteinase (TIMP), tumour cell invasion.

INTRODUCTION

MMPs (matrix metalloproteinases) represent a family of structurally and functionally related zinc-dependent endopeptidases capable of digesting extracellular matrix components [1–4]. In addition to their degradative function, they have abilities to process regulatory proteins, including cytokines, growth factors or their receptors [3]. Unbalanced MMP activities are involved in numerous pathological events, such as tumour growth and metastasis, arthrosis, arthritis, nephritis, neurodegenerative disease and fibrosis [2,4]. Production and activity of MMPs in cells is controlled precisely at the level of gene expression and is modulated by a variety of stimuli (e.g. cytokines, hormones or extracellular matrix proteins), by activation of the latent proenzymes, and by inhibition by endogenous TIMPs (tissue inhibitors of metalloproteinases) [5].

Two members of the MMP family, gelatinase A (MMP-2) and gelatinase B (MMP-9), are subclassified for their preferential abilities to degrade denatured collagens (gelatin) and collagen type IV, the main component of basement membranes. Moreover, these enzymes have the potential to process TGF- β (transforming growth factor β) [6], interleukin 8 [7], and interleukin 1 β [8] into their biologically active forms. In contrast to MMP-2, which is often expressed in a constitutive manner, MMP-9 synthesis and secretion are regulated, and in most cell types it occurs only

upon stimulation. Although MMP-9 production can be observed in diverse cell types, its synthesis in cells of haematopoietic origin, such as neutrophil granulocytes [9], monocytes [10] and macrophages [11], appears as major source of MMP-9 in humans. Leukocytes that infiltrate neoplastic tissue have been shown to act as co-conspirators of carcinogenesis by providing the tumour with MMP-9 [12]. Moreover, in acute myeloid leukaemia, both constitutive and stimulated release of MMP-9 is observed in various cell lines and *ex vivo* blast cells [13–17], indicating that this enzyme is also involved in leukaemic cell dissemination [18]. Direct evidence for a crucial role of MMP-9 in the invasion and metastatic capacity of tumour cells has been obtained by transfection and ribozyme-based approaches [19,20], suggesting MMP-9 as a preferential target in the development of anti-cancer drugs.

MMP-9 is synthesized as a pre-proenzyme and is secreted from the cells in a glycosylated proenzyme form [21] with a molecular mass varying from 91–96 kDa depending on the cell type [9,10,13,21,22]. ProMMP-9 may form a tight complex with TIMP-1 [21], which influences both activation and activity of this enzyme. *In vitro* activation of latent MMP-9 is achieved by incubation with organomercurials or several proteinases [23]. Under physiological conditions, a proteolytic cascade involving MMP-3 or the plasminogen activator/plasmin system is postulated to convert proMMP-9 into its active form [24,25].

Abbreviations used: APMA, 4-aminophenylmercuric acetate; ECL, enhanced chemiluminescence; MALDI-TOF-MS, matrix-assisted laser-desorption ionization-time-of-flight MS; MMP, matrix metalloproteinase; PMCA-ATPase, plasma membrane-localized calcium-dependent ATPase; TBS, Tris-buffered saline; TGF- β , transforming growth factor β ; TIMP, tissue inhibitor of metalloproteinase.

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Cell surface association of MMP-9 has been documented in various cell types, including epithelial cells [26], endothelial cells [27], neutrophil granulocytes [28,29], monocytes [30] and tumour cells [6,24,31–34]. Binding of MMP-9 to the plasma membrane is mediated by interaction of the enzyme with a distinct array of surface molecules [35,36], including CD44 [6,31,34], β_1 -integrins [27] and the $\alpha 2$ chain of collagen IV [33]. MMP-9 co-localized with CD44 on the surface of neoplastic cells was demonstrated to activate latent TGF- β , thereby promoting tumour invasion, growth and angiogenesis [34]. MMP-9 can also induce tumour-associated angiogenesis by release of vascular endothelial growth factor trapped in the extracellular matrix [37]. Thus, MMP-9 seems to be of relevance in carcinogenesis by activating and liberating tumour promoting factors from the cancer-cell surface and surrounding extracellular matrix [38]. Nevertheless, it is still unclear how the enzymatic activity of MMP-9 is controlled at the pericellular space.

In the present study, we identified a novel non-secreted 82 kDa proMMP-9 species localized at the surface of leukaemic cells. This particular zymogen form has not been recognized previously as an independent enzyme, probably due to its high similarity to 'regular' proMMP-9, which after secretion is able to re-associate to the cell membrane. Comparative analysis on purified enzymes showed clearly structural and functional differences between the two proMMP-9 species, suggesting a specific role for the 82 kDa proMMP-9 in surface-associated proteolysis of leukaemic cells.

EXPERIMENTAL

Cell culture

The cell lines THP-1 (acute monocytic leukaemia), HL-60 (acute myeloblastic leukaemia), NB4 (acute promyelocytic leukaemia) and HT1080 fibrosarcoma cells were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cells were grown in RPMI-1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 μ g/ml streptomycin. Incubation was performed at 37°C in a humidified air atmosphere in the presence of 5% CO₂. Cells were passaged twice a week. For all further experiments cells were washed two times with serum-free medium and maintained under serum-free conditions in RPMI-1640 supplemented with 1% (v/v) Nutridoma SP (Roche Applied Science, Mannheim, Germany). Mononuclear cells and polymorphonuclear cells/neutrophil granulocytes were isolated from peripheral blood of healthy volunteers, and leukaemic blast cells from peripheral blood of three patients with acute myeloid leukaemia by Percoll (Sigma, Munich, Germany) density gradient centrifugation as described previously [16]. All samples were collected at the University Hospital of Munich with consent of the persons involved. Purified cell fractions were washed twice and resuspended in serum-free medium for subsequent experiments. Cell viability was determined using the Trypan Blue exclusion test.

Phorbol ester stimulation and preparation of crude cell extracts

For stimulation experiments, cells were cultured at a density of 1×10^6 cells/ml either in serum-free medium alone or in medium containing 50 ng/ml PMA for 24 h. Thereafter, non-adherent cells were separated from culture supernatants by centrifugation at 400 g for 15 min at 4°C. Adherent cells were scraped to remove them from the culture dish. The conditioned media were stored at -20°C. Cell pellets were washed three-times in cold TBS (Tris-buffered saline; 50 mM Tris/HCl, pH 7.4, and 150 mM NaCl). For

lysis and protein extraction, cells were resuspended at a density of 1×10^7 cells/ml in lysis buffer [TBS containing 1% (v/v) Triton X-100]. To prevent (auto-)proteolytic degradation, 10 mM EDTA and a mixture of inhibitors of metallo-, serine- and cysteine-proteinases (Complete-Mini, Roche Applied Science) was added to the lysis buffer. The cell suspension was vortexed and incubated for 30 min at 4°C. Subsequently, the supernatants were collected by centrifugation at 16000 g for 15 min at 4°C and stored at -20°C. For zymography or immunoblot analysis, gelatinases present in the cell extracts were enriched by gelatin-Sepharose chromatography as described below.

Zymographic analysis and quantification of gelatinases in zymograms

Zymography was performed in precast 10% polyacrylamide minigels containing 0.1% gelatin as substrate (Invitrogen, Groningen, The Netherlands). Samples were run under non-reducing conditions without prior boiling. Conditioned medium from PMA-treated HT1080 fibrosarcoma cells containing proMMP-9 (94 kDa), proMMP-2 (72 kDa) and activated MMP-2 (66 kDa) [22] was used as marker for electrophoretic mobility of gelatinases in zymograms. After electrophoresis, gels were washed twice for 15 min in 2.7% (v/v) Triton X-100 (diluted in water) on a rotary shaker to remove SDS and to allow proteins to renature. The gels were then incubated in a buffer containing 50 mM Tris/HCl (pH 7.5), 200 mM NaCl, 5 mM CaCl₂ and 0.2% Brij35 (Invitrogen) for 18 h at 37°C. The zymograms were stained for 90 min with 0.02% Coomassie Blue R-350 in a 30% (v/v) methanol/10% (v/v) acetic acid solution by the use of PhastGel-Blue-R tablets (GE Healthcare Life Sciences, Freiburg, Germany). Quantification of gelatinolytic activity in zymograms was performed as described previously [39]. Zymograms were scanned using an Umax ImageScanner driven by the MagicScan software and analysed using the ImageMaster-1D Elite quantification software (GE Healthcare Life Sciences). The linear range of the assay was determined by the use of recombinant MMP-9 (Calbiochem, Schwalbach, Germany) as calibration standard.

SDS/PAGE and immunoblot analysis

SDS/PAGE was performed under reducing conditions in precast 4–12% (w/v) polyacrylamide minigels (Invitrogen). For electrophoresis and blotting the NuPAGE Bis/Tris system (Invitrogen) was used according to the manufacturer's instructions. After electrophoretic separation, proteins were either stained with silver according to a method described by Heukeshoven and Dernick [40] or were electroblotted on to PVDF membranes (Millipore, Bedford, MA, U.S.A.). The membranes were blocked in 10% (w/v) non-fat milk in TBS for 1 h at room temperature (21°C). Thereafter, the blots were incubated with 0.5 μ g/ml primary rabbit polyclonal antibody against proMMP-9 (M-5177; Sigma) for 1 h at room temperature, which allows detection of both latent and activated enzyme forms. A mouse monoclonal antibody (IM09L, clone 6-6B; Calbiochem) that only recognizes the latent proMMP-9 and not the activated species under reducing conditions for immunoblotting was used at a concentration of 0.5 μ g/ml. After washing, blots were incubated for 15 min with horseradish-peroxidase-conjugated anti-rabbit IgG (GE Healthcare Life Sciences) as secondary antibody at a dilution of 1:1000. The blots were then developed applying the ECL (enhanced chemiluminescence) system as recommended by the manufacturer (GE Healthcare Life Sciences). For molecular mass determination of proteins detected on the blots, recombinant protein standards (Invitrogen and Bio-Rad, Munich, Germany)

were used. The developed films were scanned and analysed using the ImageMaster-1D Elite software.

Confocal laser scanning microscopy

THP-1 cells were grown on 8-well CultureSlides (BD Falcon, Heidelberg, Germany) for 24 h under serum-free conditions in the presence of 50 ng/ml PMA. The cells were washed, and then fixed in 3.7% (v/v) formaldehyde in PBS for 15 min at room temperature. The fixed cells were blocked with 2% (w/v) BSA in PBS for 15 min, and then incubated with 50 µg/ml of monoclonal anti-MMP-9 antibody (GE-213, NeoMarkers, Union City, CA, U.S.A.) or with 50 µg/ml of mouse IgG₁ isotype control antibody (DAKO, Hamburg, Germany) for 1 h at 37°C. The secondary fluorescence-conjugated antibody used was Alexa Fluor® 488 anti-mouse IgG (Molecular Probes, Eugene, OR, U.S.A.). Detection was carried out at an excitation wavelength of 488 nm, beam splitter HFT 488, and emission at 505–530 nm on a Zeiss LSM 510 confocal scanning microscope equipped with a Plan-Neofluar 100×/1.3 oil immersion objective. Each image represents a single section with a field depth of 1 µm.

Subcellular fractionation and isolation of plasma membranes

THP-1 cells (1×10^6 /ml) were cultured for 24 h under serum-free conditions in the presence of 50 ng/ml PMA. Cells were harvested and washed three times with ice-cold PBS. All of the following procedures were carried out at 4°C according to a method described previously [26] with minor modifications. Cells were resuspended at 2×10^7 /ml in buffer containing 25 mM Tris/HCl (pH 7.5), 8.5% (w/v) sucrose, 50 mM NaCl and proteinase inhibitors (Complete-Mini, Roche Applied Science) at concentrations recommended by the manufacturer. The cell suspension was homogenized using a Dounce homogenizer and centrifuged for 10 min at 3000 g. The pellet (nuclear fraction) was discarded and the supernatant (postnuclear fraction) was centrifuged at 25 000 rev./min for 3 h in a Kontron ultracentrifuge with a TFT 28/38 rotor (Kendro, Hanau, Germany). The supernatant (cytosolic fraction) was collected and stored at –20°C. The pellet (crude membrane fraction) was resuspended in buffer containing 25 mM Tris/HCl (pH 7.5), 50 mM NaCl and proteinase inhibitors (Complete-Mini). The sample was layered on to a discontinuous density gradient of 20, 30, 50 and 60% sucrose in water and once more centrifuged at 25 000 rev./min for 3 h. The 30–50% interphase was carefully aspirated, resuspended in buffer containing 25 mM Tris/HCl (pH 7.5), 50 mM NaCl and 5 mM EDTA and centrifuged at 25 000 rev./min for a further 3 h. The pellet (enriched plasma membrane fraction) was resuspended in the same buffer and stored at –70°C. The successful enrichment of plasma membranes was verified by immunoblot detection of PMCA-ATPase (plasma membrane-localized calcium-dependent ATPase) using monoclonal antibodies against this enzyme (Affinity Bioreagents, Golden, CO, U.S.A.).

Cell surface protein biotinylation and immunoprecipitation

Labelling and detection of proteins localized on the surface of THP-1 cells was performed by using the ECL protein biotinylation module (GE Healthcare Life Sciences) according to the manufacturer's instructions. Cells (2×10^6 /ml) were grown under serum-free conditions in the presence of 50 ng/ml PMA for 24 h. Cells were harvested by centrifugation at 600 g at 4°C, and then washed twice with ice-cold PBS. The cell viability was $\geq 95\%$, as determined by Trypan Blue staining. Cells were then diluted to 1×10^7 /ml in PBS and incubated with 40 µl/ml of the water-soluble and cell impermeable biotinylation

reagent (biotinamidocaproate *N*-hydroxysuccinamide ester) for 15 min at 4°C on a roller mixer. Control cells received vehicle only. After incubation, cells were washed twice in PBS and extracted with Triton X-100 as described above. For gelatinase enrichment, cell extracts were passed through a gelatin–Sephacryl affinity column (see below). Elution fractions containing gelatinolytic activity were pooled, dialysed against 50 mM Tris/HCl (pH 7.5), 0.3% Triton X-100 and 5 mM EDTA, and concentrated using Microcon YM10 devices (Millipore). For immunoprecipitation, enriched gelatinase samples were then incubated with 9 µg/ml of monoclonal anti-MMP-9 antibody (GE-213, NeoMarkers) and Protein G–Sephacryl beads overnight at 4°C under constant shaking. The Protein G–Sephacryl beads were washed three times with ice-cold PBS, resuspended in reducing electrophoresis sample buffer (141 mM Tris/HCl, pH 8.5, 1 M glycerol, 73 mM lithium dodecyl sulfate, 0.5 mM EDTA and 20 mg/ml dithiothreitol), boiled, and centrifuged at 16 000 g for 1 min at 4°C. The supernatant containing immunoprecipitated antigens was separated by SDS/PAGE (4–12% gels) under reducing conditions, and blotted as described above. After blocking and washing of the membrane, detection of biotinylated proteins was achieved by incubation with streptavidin-conjugated horseradish peroxidase (diluted 1:1500) and detection with ECL. The specificity of detection was controlled by processing non-biotinylated cells in the same way as described for biotinylated cells.

Gelatin–Sephacryl affinity chromatography and gel filtration

Purification of MMP-9 was performed as described previously by us [41] with some modifications. All procedures were carried out at 4°C with buffers containing proteinase inhibitors (Complete-Mini) and 5 mM EDTA to avoid autolytic processing.

THP-1 cell-associated MMP-9 forms were purified from crude cell extracts (0.1–0.5 ml) or postnuclear fractions (40 ml) by Triton X-100 extraction with vigorous vortexing and incubation on ice for 30 min. After centrifugation at 3000 g for 15 min, the supernatant was applied to a gelatin–Sephacryl column of 2 ml bed vol. equilibrated with 50 mM Tris/HCl (pH 7.5), 400 mM NaCl, 5 mM EDTA and 0.1% Brij 35 at a flow rate of 0.03 ml/min using the FPLC system (GE Healthcare Life Sciences). After the column had been washed in the same buffer, gelatin-binding proteins were eluted by 5% (v/v) DMSO in column buffer. Gelatinase containing fractions were assessed by zymography, pooled and concentrated by approx. 10-fold (Centricon YM10, Millipore). For separation of 82 kDa and 94 kDa MMP-9 forms, the sample (0.5 ml) was then applied to a Superdex G-200 gel filtration column (1.6 cm × 60 cm; GE Healthcare Life Sciences) equilibrated with 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA and 0.02% Brij 35 at a constant flow of 0.4 ml/min. Fractions (1 ml) containing 94 kDa MMP-9 or 82 kDa MMP-9 respectively, were pooled and concentrated. Protein concentrations were determined using the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL, U.S.A.). The enzyme preparations were stored at –20°C.

The secreted 94-kDa species of proMMP-9 was purified from culture supernatants of PMA-treated THP-1 cells. Conditioned medium (1 litre) was adjusted to 5 mM EDTA and 0.02% Brij 35, and concentrated to a final volume of 50 ml using an Amicon Diaflo apparatus fitted with a YM-10 membrane (Amicon, Beverly, MA, U.S.A.). Thereafter, the material was processed by gelatin–Sephacryl affinity chromatography and gel filtration as described above. Selective denaturation of co-purifying TIMP molecules was performed according to a method published previously [42].

Microsequencing and MALDI-TOF-MS (matrix-assisted laser-desorption ionization-time-of-flight MS)

Samples of purified gelatinases were separated by SDS/PAGE (10% gel) and electroblotted on to glass fibre membranes (Glassybond, Biometra, Göttingen, Germany) [43]. Coomassie Blue stained bands were cut out and sequenced in a 492cLC gas phase sequencer (Applied Biosystems, Foster City, CA, U.S.A.). For MS analysis, samples were separated by SDS/PAGE and the protein bands were excised and digested directly in the gel with trypsin as outlined by Eckerskorn and Lottspeich [44]. Peptide mass fingerprinting was performed according to Meister et al. [45] using a Bruker reflex III MALDI-TOF mass spectrometer equipped with a 337 nm nitrogen laser (Bruker-Franzen, Bremen, Germany).

Deglycosylation of purified proMMP-9 forms

Purified proMMP-9 preparations were digested by enzymes provided by the Glycoprotein Deglycosylation Kit from Calbiochem (San Diego, CA, U.S.A.) following the denaturing or non-denaturing standard protocols of the manufacturer. For Western blot detection of deglycosylated proteins, samples (30 μ l) were mixed with reaction buffer (250 mM PBS, pH 7.0) and denaturation solution [2% (w/v) SDS and 1 M 2-mercaptoethanol] and heated at 100°C for 5 min. After cooling to room temperature, Triton X-100 solution [15% (v/v) Triton X-100 diluted in water] was added at a final concentration of 1% to remove free SDS. Subsequently, samples were incubated with different glycosidases alone or in combination in a final reaction volume of 47.5 μ l for 3 h at 37°C. N-glycosidase F (2.5 units) was added to remove asparagine-linked (N-linked) oligosaccharides. Cleavage of serine/threonine-linked (O-linked) sugar residues was achieved by simultaneous incubation with a combination of endo- α -N-acetylgalactosaminidase (0.6 milliunits), α 2-3,6,8,9-neuraminidase (1.25 milliunits), β -1,4-galactosidase (0.75 milliunits) and β -N-acetylglucosaminidase (2 milliunits) according to the manufacturers instructions. Mobility shift analysis of digested samples was performed by SDS/PAGE and immunoblotting using anti-MMP-9 antibodies as described above. To preserve enzymatic activity of gelatinases during the deglycosylation reaction, samples were incubated with glycosidase for 4 days at 37°C under non-reducing conditions and subsequently analysed by zymography.

Activation of purified proMMP-9 forms and reaction with α 2-macroglobulin

Purified 82 kDa proMMP-9 from THP-1 cell extracts and 94 kDa proMMP-9 from culture supernatants were diluted to a final concentration of 50 ng/ml in enzyme buffer containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM CaCl₂, 1 μ M ZnCl₂ and 0.02% Brij 35, and were treated with or without equimolar amounts of active recombinant MMP-3 prepared according to the method of Suzuki et al. [46] at 37°C in a total volume of 250 μ l. Aliquots were taken at different time intervals and stored on ice prior to analysis for gelatinolytic activity in zymograms. For identification of catalytically active gelatinase species, samples pre-treated with or without MMP-3 for 30 min at 37°C were reacted with an excess (500 μ g/ml) of α ₂-macroglobulin (Roche Applied Science) for 30 min at 37°C and subsequently applied to zymographic analysis.

Cleavage of biological substrates

Purified 82 kDa proMMP-9 was autocatalytically activated by incubation in buffer containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM CaCl₂, 1 μ M ZnCl₂ and 0.02% Brij 35

for 3 h at 37°C. Activation of the secreted 94 kDa proMMP-9 species was performed by exposure to 1 mM APMA (4-aminophenylmercuric acetate) for 2 h at 37°C as described previously [41]. Successful conversion into their active forms was controlled by zymography. Native or denatured (by heating to 100°C for 3 min) collagen type IV (400 μ g/ml) from Engelbreth-Holm-Swarm mouse sarcoma cells, 300 μ g/ml denatured collagen type I (rat tail) and 100 μ g/ml fibronectin (all from Sigma) were incubated with or without equimolar amounts (1.5 μ M) of activated enzymes for 48 h at 32°C. The reaction mixtures were then analysed for degradation products by SDS/PAGE followed by silver staining of the proteins.

MMP-9 inhibition studies

The active forms of 82 and 94 kDa proMMP-9 were assayed using a fluorescence-labelled gelatin substrate (Molecular Probes). For activation 20 nM of purified enzymes were incubated with 1–5 nM of active recombinant MMP-3 for 1–3 h at 37°C in a buffer containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 30 mM CaCl₂, 10 μ M ZnCl₂, and 0.02% Brij 35 (100 μ l total volume). Complete conversion into active MMP-9 forms was controlled by zymography. For inhibition studies 1 nM of each activated enzyme was pre-incubated for 30 min at 37°C with or without 0.1–100 nM of purified TIMP-1 (prepared as described previously [47]), 0.1–100 nM GM6001 (a broad-spectrum hydroxamic acid inhibitor of MMPs) (Calbiochem) or 1 mM 1,10-phenanthroline (Sigma) before the fluorescence-labelled substrate (1.5 μ g/ml) was added to a final volume of 200 μ l. After 1 h incubation at 37°C, substrate hydrolysis was measured using a Molecular Dynamics Biolumin 960 (GE Healthcare Life Sciences) fluorescence spectrophotometer with excitation and emission wavelengths set at 485 and 520 nm respectively.

RESULTS

Detection of secreted and cell-associated forms of MMP-9

THP-1 cells were cultivated in the absence and presence of PMA. The culture supernatants and cell lysates were analysed for gelatinase activity by zymography and Western blotting. The conditioned medium contained 94 kDa proMMP-9, expression of which was enhanced considerably upon PMA stimulation (Figure 1). An additional band migrating at the top of the zymogram gels represented the homodimer of proMMP-9 as observed under non-reducing conditions of electrophoresis [48]. The production of 72-kDa proMMP-2 was negligible in these cells (Figure 1A).

Zymographic analysis of cell lysates from untreated cells revealed the presence of gelatinolytic activity at 92–94 kDa consisting of two single merging bands that were significantly augmented upon PMA treatment, which formed a major zone of lysis between approx. 88 and 94 kDa (Figure 1A). Under reducing conditions, Western blot analysis using anti-MMP-9 antibodies detected two distinct bands in cell lysates: a minor protein with a molecular mass of 94 kDa, which presumably represents intracellular proMMP-9 prior to secretion, and a major 82 kDa form. Both MMP-9 species were augmented by PMA treatment (Figure 1B). To assess whether the 82-kDa MMP-9 was generated from 94-kDa proMMP-9 by the Triton X-100 extraction procedure, conditioned medium comprising only the 94-kDa enzyme was treated with extraction buffer for 18 h at 37°C, but no effect on 94 kDa proMMP-9 was observed (results not shown), demonstrating that the 82 kDa form was not created by the extraction technique.

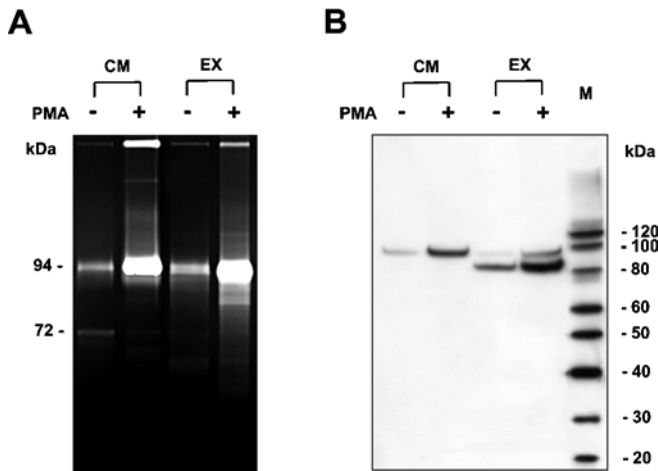


Figure 1 Detection of secreted and cell-associated MMP-9

THP-1 leukaemic cells (1×10^6 cells/ml) were incubated under serum-free conditions for 24 h in the presence (+) or absence (-) of 50 ng/ml PMA. **(A)** Aliquots ($3 \mu\text{l}$) of THP-1 conditioned medium (CM) and $10 \mu\text{l}$ aliquots of cell extract (EX) were analysed by zymography. **(B)** For immunoblot detection, $15 \mu\text{l}$ aliquots of gelatinases isolated from conditioned medium and $30 \mu\text{l}$ aliquots of gelatinases from cell extracts containing 5–10 ng of protein were separated by SDS/PAGE under reducing conditions, blotted and probed with polyclonal antibodies directed against MMP-9. Molecular mass standards (M) were used as a reference, and their sizes are given in kDa to the left-hand and right-hand sides of **(A)** and **(B)** respectively.

Cell surface localization of MMP-9

Confocal fluorescence microscopy analysis of THP-1 cells stimulated with PMA indicated a zonular array of fluorescence on the plasma membrane, which was weaker in untreated cells (Figure 2A). With isotype matched primary control antibodies, instead of anti-MMP-9 antibodies, no fluorescence signals were obtained.

To analyse in more detail which MMP-9 forms were associated with the cell surface, plasma membranes were separated from cytosolic components of PMA-stimulated cells by sucrose density gradient centrifugation. As determined by immunoblotting, both plasma membrane and cytosolic fractions contained the two MMP-9 species each with larger amounts of the 82 kDa form compared with the 94 kDa form (Figure 2B). PMCA-ATPase, an integral protein of cellular plasma membranes, was detected in the membrane fraction, but not in the cytosolic fraction, demonstrating successful separation and purification of plasma membranes (Figure 2C).

To verify that MMP-9 was bound to the outer periphery of the plasma membrane, cell surface proteins of THP-1 cells were labelled with biotin. Proteins were then extracted from the cells and MMP-9 was enriched from the extracts by gelatin–Sepharose chromatography and subsequently immunoprecipitated using antibodies against MMP-9. The precipitates were then subjected to SDS/PAGE and blotted. Using the streptavidin–POD (peroxidase) detection system a strong band at 82 kDa together

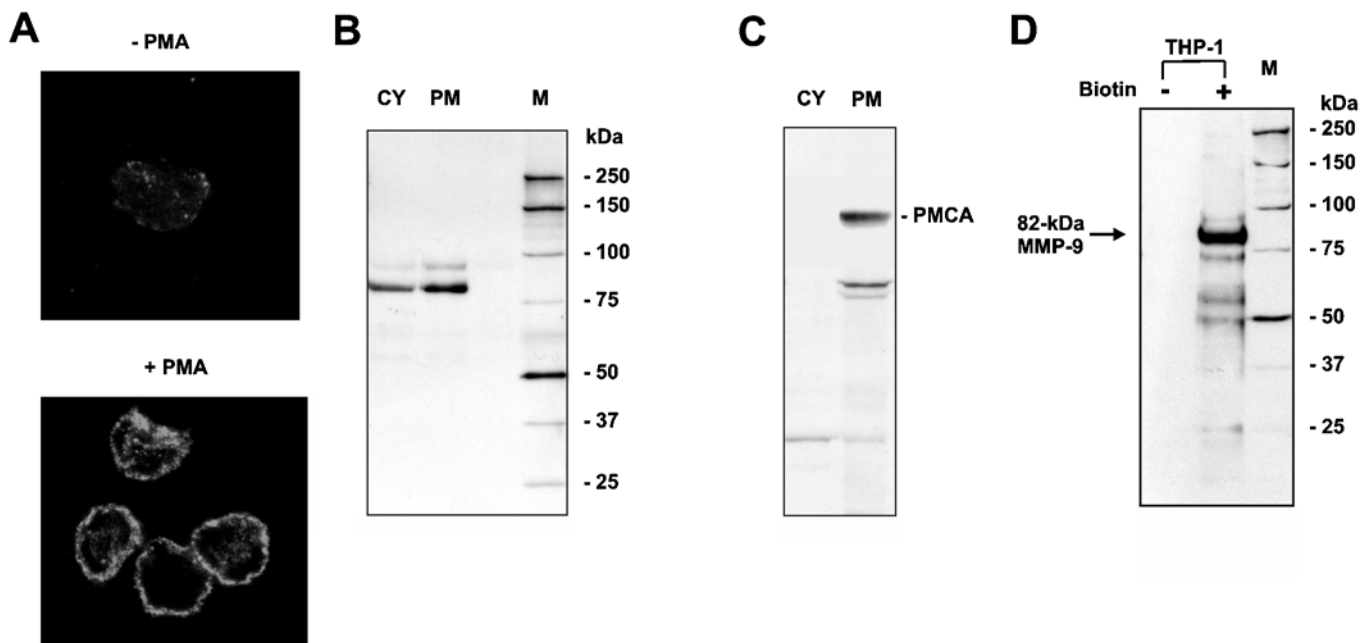


Figure 2 Plasma membrane localization of MMP-9

THP-1 cells were cultured in serum-free medium for 24 h in the absence and presence of 50 ng/ml PMA. **(A)** Cells grown on culture-slides were fixed without permeabilization, then incubated with monoclonal anti-MMP-9 antibody and stained with Alexa Fluor[®] 488-conjugated anti-mouse IgG. Confocal laser scanning microscopy images taken under fluorescence conditions are shown for cells incubated with (+PMA) or without PMA (-PMA). **(B)** PMA-treated cells were homogenized and fractionated by sequential sucrose density ultracentrifugation. Cytosolic (CY) fractions and the enriched plasma membrane (PM) were examined for the presence of MMP-9 by immunoblot analysis using polyclonal antibodies. Recombinant marker proteins (M) were used as a reference and the sizes are given in kDa to the right-hand side of the Figure. **(C)** Immunoblot detection of PMCA-ATPase was performed to verify the successful enrichment of plasma membranes from the cells. **(D)** PMA-treated cells were incubated with cell surface protein biotinylation reagent (+) or vehicle alone (-) for 15 min at 4°C. Proteins were extracted from the cells and MMP-9 was enriched by gelatin–Sepharose affinity chromatography, and then immunoprecipitated using monoclonal antibodies against MMP-9, subjected to SDS/PAGE and blotted on to PVDF membranes. For detection of biotinylated proteins, blots were developed with streptavidin–POD. Recombinant marker proteins (M) were used for molecular mass determination, and the sizes are given in kDa to the right-hand side of the Figure.

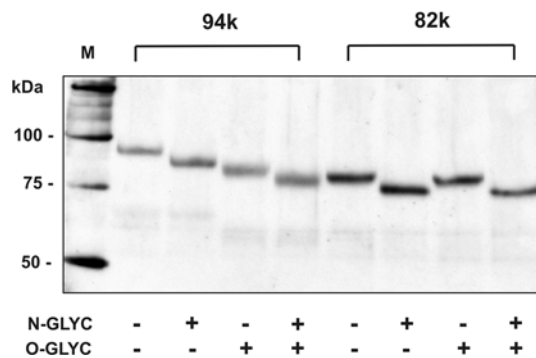


Figure 4 Glycosylation studies

Aliquots of the purified secreted 94 kDa proMMP-9 (0.25 μ g/ml) and the cell-associated 82 kDa proMMP-9 (1 μ g/ml) were reduced, denatured and then incubated for 3 h at 37 °C with either N-glycosidase F (N-GLYC) to remove N-linked oligosaccharides, with glycosidases (O-GLYC) that digest O-linked carbohydrates or with a combination of these enzymes. Shifts in electrophoretic mobility were monitored by SDS/PAGE (4–12% gels) under reducing conditions and subsequent immunoblotting using polyclonal antibodies against MMP-9. Results from a representative experiment of three independent determinations are shown. Recombinant marker proteins (M) were used for molecular mass assessment and the sizes are shown in kDa to the left-hand side of the Figure.

also account for the difference in molecular mass for the 82 and 94 kDa species. Therefore the purified 82 and 94 kDa proMMP-9 forms were subjected to digestion with N-glycosidase F. Western blot analysis demonstrated that both forms of proMMP-9 were reduced in size by 6 kDa (Figure 4), indicating a similar extent of N-linked glycosylation for the two proenzymes. When the proMMP-9 forms were incubated with glycosidases which remove O-linked carbohydrates, the molecular mass of secreted 94 kDa proMMP-9 was decreased by 9 kDa, whereas that of the 82 kDa variant remained unchanged (Figure 4). Removal of all sugar residues from the 82 and 94 kDa proMMP-9 using both N-linked and O-linked specific glycosidases yielded 76 and 80 kDa proteins respectively (Figure 4). The molecular mass of the 76 kDa protein is in accordance to that calculated from the amino acid sequence of proMMP-9 [21], confirming that the 82 kDa proMMP-9 retains N-linked carbohydrates, but lacks O-linked sugars, whereas the 94 kDa proMMP-9 contains both types of glycan molecules.

Proteolytic activation of the 82 kDa proMMP-9

To investigate whether N-terminal truncation and lack of O-glycosylation influence activation of the 82-kDa proMMP-9, the purified zymogen was incubated with stromelysin 1 (MMP-3), a potent physiological activator of proMMP-9, and the mode of activation was compared with that of the 94 kDa proMMP-9. As determined by zymography and Western blot analysis, treatment of the 94 kDa proMMP-9 with MMP-3 resulted in complete conversion into a single 82 kDa MMP-9 within 1 h, with an intermediate form of approx. 86 kDa (Figure 5A), as described by Ogata et al. [49]. In contrast, incubation of the 82 kDa proMMP-9 with MMP-3 initiated rapid conversion into a major gelatinase with an apparent molecular mass of approx. 35 kDa, which migrated as a single protein of 35 kDa when analysed by Western blotting under reducing conditions (Figure 5B). Owing to the high sensitivity of zymography additional bands with lower intensity ranging between approx. 25 and 40 kDa were also detected, which may represent minor activation products (Figure 5B). Similar to the purified enzyme, 82 kDa proMMP-9 present in plasma membrane fractions from THP-1 cells treated with MMP-3 led to the formation of 35 kDa gelatinase that was

stable for at least 6 h (Figure 5C). These findings indicate that the 82 kDa proMMP-9, located in the plasma membrane, is different from the regular secreted 94 kDa proMMP-9 in its susceptibility to MMP-3 activation.

To examine the activity status of the different MMP-9 species, we utilized the property of the inhibitor α_2 -macroglobulin that binds only to active endopeptidases [50]. The 94 kDa proMMP-9 pre-incubated with α_2 -macroglobulin and subsequently subjected to zymography showed no significant decrease in gelatinolytic activity, whereas for the activated 82 kDa species bound to α_2 -macroglobulin the majority of the activity had shifted to the top of the gel (Figure 5D). The 82 kDa proMMP-9 did not react with α_2 -macroglobulin (Figure 5D), suggesting that it is proteolytically inactive, like the 94 kDa proMMP-9. The activated 35 kDa species bound to α_2 -macroglobulin (Figure 5D), indicating that it is an active enzyme.

Autocatalytic activation of 82 kDa proMMP-9

Incubation of the purified 82 kDa proMMP-9 in enzyme buffer in the absence of activating agents for 1–6 h at 37 °C gradually converted the enzyme into a major 35 kDa and a minor 25 kDa gelatinase as determined by zymography (Figure 6A) and Western blot analysis (Figure 6B). The conversion was almost complete after 6 h of incubation (Figure 6A). In contrast, the 94 kDa proMMP-9 remained stable and showed no change in molecular mass under these conditions (Figure 6A). Autocatalytic processing of the 82 kDa proMMP-9 was not affected by the addition of TIMP-1 even when the inhibitor was present in a 3-fold molar excess (Figure 6A). These findings indicate that the 82 kDa proMMP-9 is readily auto-activated compared with the inert secreted 94 kDa proMMP-9.

Substrate specificities

Like the 94 kDa proMMP-9, the 82 kDa proMMP-9, when activated, digested denatured collagen type IV and type I (gelatins), native collagen type IV and fibronectin (Figure 7). The two enzymes, however, showed different extent in degrading these substrates. Although activated 94 kDa proMMP-9 prefers to cleave native type IV collagen to the denatured gelatins, the activated 82 kDa proMMP-9 digested denatured type I and type IV collagens more readily than the 94 kDa form (Figure 7).

Inhibition of the 82 kDa and 94 kDa proMMP-9

To examine their susceptibility to inhibition, the activated 82 kDa and 94 kDa proenzymes were incubated with increasing concentrations of TIMP-1, as well as a synthetic MMP inhibitor, and inhibition of their gelatinolytic activity was monitored. TIMP-1 efficiently blocked the activated 94 kDa proMMP-9 with an IC_{50} of 1 nM (Figure 8A). In contrast, inhibition of the activated 82 kDa proMMP-9 by TIMP-1 was significantly weaker with an IC_{50} value of 82 nM (Figure 8A). Comparable results were obtained by TIMP-2 (results not shown). The synthetic MMP inhibitor, GM6001, blocked both forms of MMP-9 with a similar efficiency, indicated by the IC_{50} value of 0.5 nM for the activated 94 kDa form and of 0.8 nM for the activated 82 kDa proMMP-9 (Figure 8B).

Expression of the 82 kDa proMMP-9 in other leukaemic cells

The molecular mass of the 82 kDa proMMP-9 is identical to that of the activated form (82 kDa) of 94 kDa proMMP-9. To distinguish these two enzyme species, we used Western blot analysis with antibodies that specifically recognize the propeptide, but not the activated form of MMP-9. As expected, both the 82 kDa proMMP-9 and the 94 kDa proMMP-9, but not their

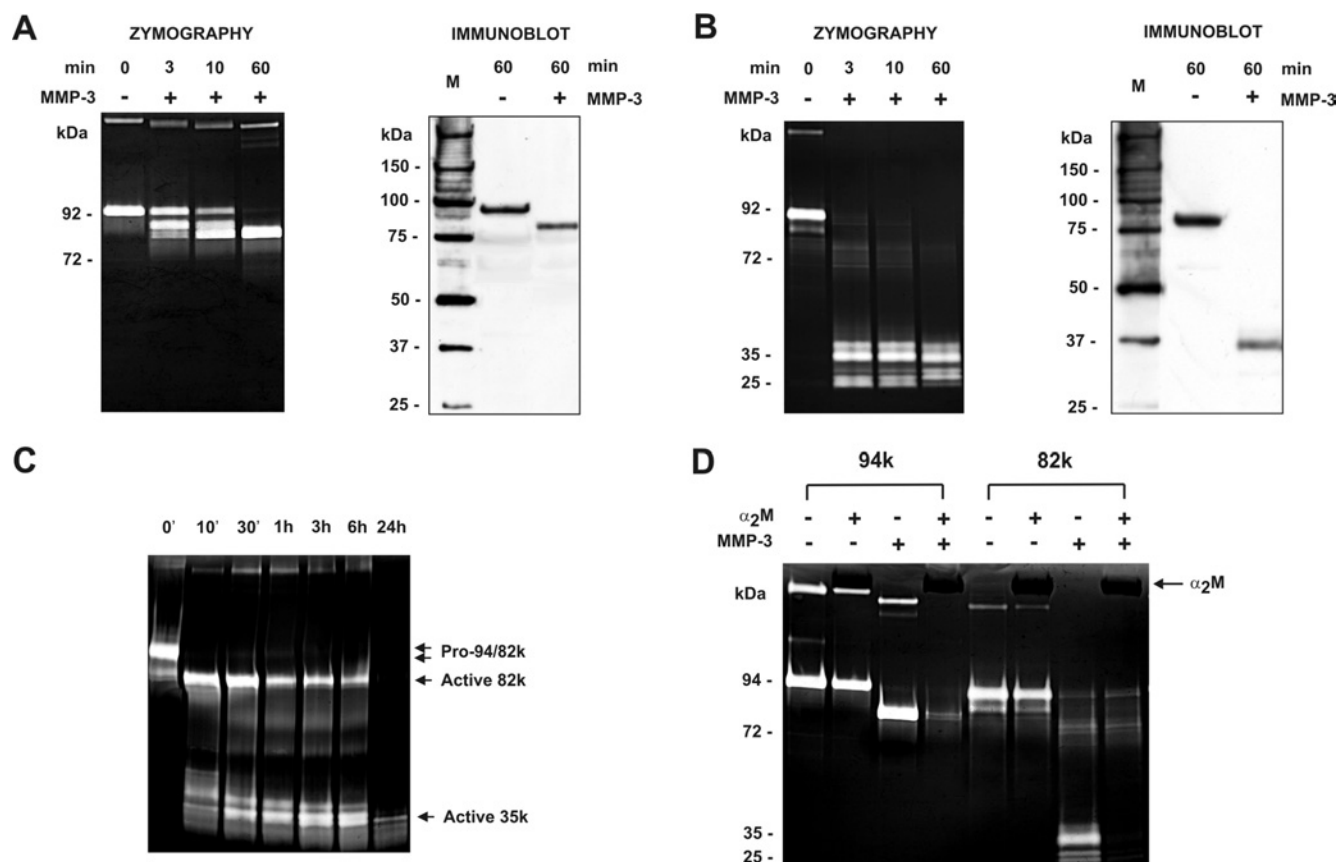


Figure 5 Proteolytic activation of proMMP-9 forms and binding to α_2 -macroglobulin

Purified 94 kDa proMMP-9 from culture supernatants (**A**) and 82 kDa proMMP-9 from cell extracts (**B**) were diluted to a final concentration of 50 ng/ml in enzyme buffer. The samples were then incubated at 37°C in the presence or absence of equimolar amounts of active MMP-3. Aliquots were taken at different time intervals and analysed by zymography and immunoblotting using anti-MMP-9 polyclonal antibodies. (**C**) Enriched THP-1 plasma membranes containing the 94 kDa proMMP-9 (pro-94k) and the 82 kDa proMMP-9 (pro-82k) were incubated with MMP-3. Aliquots were taken at different time intervals and analysed by zymography. (**D**) For examination of proteolytic activity the purified 94 kDa proMMP-9 and the 82 kDa proMMP-9 were treated with or without MMP-3 for 30 min at 37°C, then incubated with an excess of α_2 -macroglobulin (500 μ g/ml) for 30 min at 37°C, and subsequently subjected to zymography. The dark protein band migrating on top of the gel represents α_2 -macroglobulin (α_2 M), which binds and inhibits only active endopeptidases.

activated species, were detected by anti-(proMMP-9-prodomain) antibodies (Figure 9A).

Western blot analysis using the MMP-9 propeptide-directed antibodies was then applied to investigate whether the 82 kDa proMMP-9 was also present in other cell types. We first examined HL-60 and NB4 cells, which originate from patients with acute myeloid leukaemia. The immunoblotting procedure clearly showed prominent amounts of the 82 kDa proMMP-9 and a weak band of 94 kDa proMMP-9 in extracts from PMA-stimulated HL-60 and NB4 cells, consistent with the observations in THP-1 cells (Figure 9B). MMP-3 treatment of HL-60 and NB4 cell lysates also generated the 35 kDa activated MMP-9 species (results not shown), proving the presence of 82 kDa proMMP-9 in these cells. Western blot analysis of extracts from PMA-treated *ex vivo* leukaemic cells isolated from the peripheral blood of three patients with acute myeloid leukemia clearly displayed production of the 82 kDa proMMP-9 in these cells (Figure 9B). In contrast, proMMP-9 forms were absent from the lysates obtained from PMA-stimulated mononuclear cells and neutrophil granulocytes from peripheral blood of healthy donors (Figure 9B).

DISCUSSION

Cell surface-associated proteinases concentrate proteolytic events at the sites of cell–matrix contact and mediate responses very

rapidly in contrast to secreted enzymes, which diffuse into the pericellular space after their release [51]. Previous studies have demonstrated localization of MMP-9 on the plasma membrane of various tumour cells [6,24,26,31,33–36]. So far, it has been assumed that surface-bound MMP-9 represents secreted enzyme molecules that re-associate with the plasma membrane. In the present study, we provide evidence for the existence of an unique variant of MMP-9 zymogen with a molecular mass of 82 kDa that is not detectable in the culture medium, but is present as the prominent proMMP-9 on the surface of leukaemic cells. Moreover, this membrane associated proMMP-9 variant differs from the secreted zymogen form in several structural and functional aspects: it is truncated by nine amino acids at its N-terminus, it lacks O-linked glycosylation and it exhibits a distinct activation process which produces an unusually small active MMP-9 species with less susceptibility to TIMP-1 inhibition.

Previous studies in immortalized MCF10A breast epithelial cells demonstrated that secreted proMMP-9 was localized at the plasma membrane together with an 85 kDa species of MMP-9, which was not released from the cells even upon its stimulation with PMA [26]. Thus, the latter form of MMP-9 appears to have similar trafficking properties to the 82 kDa proMMP-9 described in our THP-1 cell system. This 82 kDa species was absent in medium, but readily detected in cell lysates and the plasma membrane, suggesting that this enzyme is retained at the cell

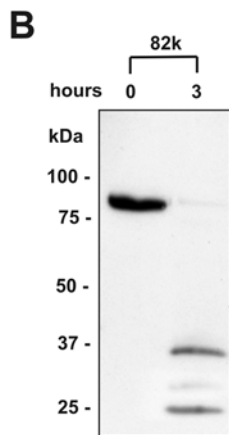
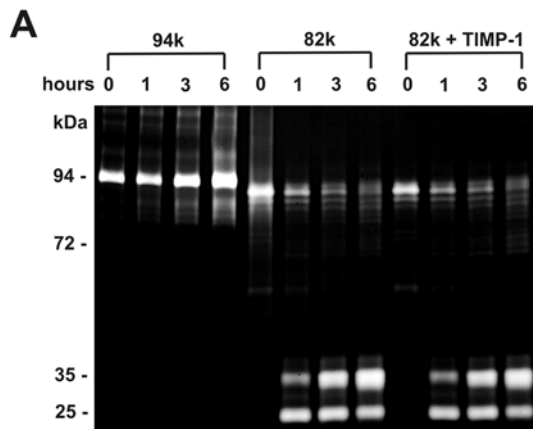


Figure 6 Autocatalytic activation of the 82 kDa proMMP-9

Purified 94 kDa proMMP-9 (94k) and 82 kDa proMMP-9 (82k) were diluted to a final concentration of 0.5 nM in enzyme buffer and incubated at 37°C with or without addition of 1.5 nM TIMP-1. Aliquots were taken at different time intervals and analysed by zymography (A) and Western blotting using a polyclonal anti-MMP-9 antibody (B).

surface by a unique interaction. Although the specific surface receptors of 82 kDa proMMP-9 remain to be identified, proteins known to attach secreted proMMP-9 to the plasma membrane, such as CD44, collagen chains and various integrins [33–36], might also act as binding partners for the 82 kDa zymogen. Direct translocation of proMMP-9 from the intracellular pool to the cell surface of activated neutrophils has been demonstrated to involve complex formation with β_2 integrins [28]. However, the mechanisms of trafficking and cell surface attachment for the 82 kDa proMMP-9 variant remain to be determined.

Because MMP-9 bound to certain tumour cells has an apparent molecular mass of approx. 83 kDa, it was suggested to represent an activated species of secreted proMMP-9 [6,34]. Similarly, an 82 kDa gelatinase detected in lysates of PMA-treated endothelial cells was claimed to represent the active form of MMP-9 [52]. However, a biochemical characterization of this cell-associated low molecular mass MMP-9 was not reported. To elucidate the molecular properties of the THP-1 cell-associated MMP-9, we purified the 82 kDa species from the cell lysates and compared it with the 94 kDa proMMP-9 released into the culture medium. The results of the present study showed that the 82 kDa form is a proMMP-9, but that the N-terminus is truncated by nine amino acids. Previously it has been reported that proMMP-9 isolated from culture supernatants of peripheral blood monocytes,

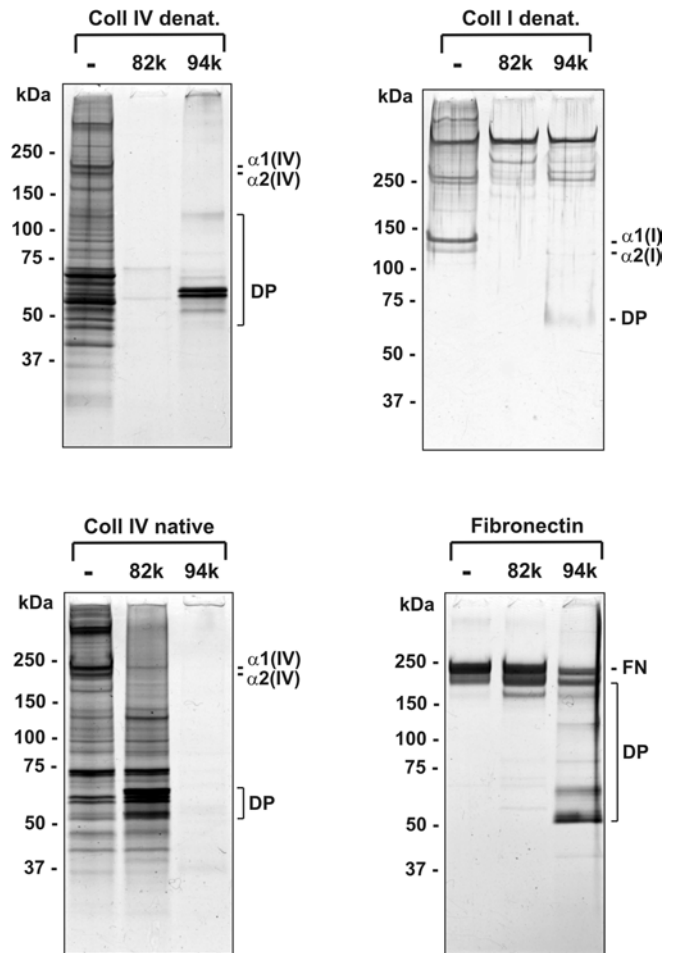


Figure 7 Degradation of natural substrates

Collagen type IV (native or denatured; 400 μ g/ml), collagen type I (denatured; 300 μ g/ml) and fibronectin (FN; 100 μ g/ml) were incubated with or without equimolar amounts of auto-activated 82 kDa proMMP-9 (82k) and APMA-activated 94 kDa proMMP-9 (94k) for 48 h at 32°C. The reaction mixtures were then analysed for degradation products (DP) by SDS/PAGE and silver staining of the proteins. $\alpha 1(I)$, collagen type I chain $\alpha 1(I)$; $\alpha 2(I)$, collagen type I chain $\alpha 2(I)$; $\alpha 1(IV)$, collagen type VI chain $\alpha 1(IV)$; $\alpha 2(IV)$, collagen type VI chain $\alpha 2(IV)$.

neutrophil granulocytes and HL-60 leukaemic cells is also shortened by eight to nine amino acids at its N-terminus [9,10,41]. This indicates that N-terminal processing may occur in both surface-bound and secreted proMMP-9 forms, but the underlying procedure is unclear. It can be speculated that intracellular or membrane-associated proteinases might be involved in the cleavage of the N-terminal peptide from 82 kDa proMMP-9, such as pro-protein convertases, which post-translationally process precursor proteins during the secretory pathway.

In addition to the lack of the nine N-terminal amino acids, other structural modifications had to be assumed for the novel 82 kDa proMMP-9 to explain its reduced molecular mass. Therefore we searched for alternatively spliced MMP-9 mRNAs, but they were not detected in THP-1 cells (results not shown). Deglycosylation studies, however, revealed that the surface-bound 82 kDa proMMP-9 variant lacks O-linked carbohydrates, which amount to a molecular mass of approx. 9 kDa in the 94 kDa proMMP-9. This finding is consistent with previous data reporting that an epithelial cell membrane associated 85 kDa variant of MMP-9 is not fully glycosylated [26]. Our results on secreted proMMP-9 are in accordance with those of a

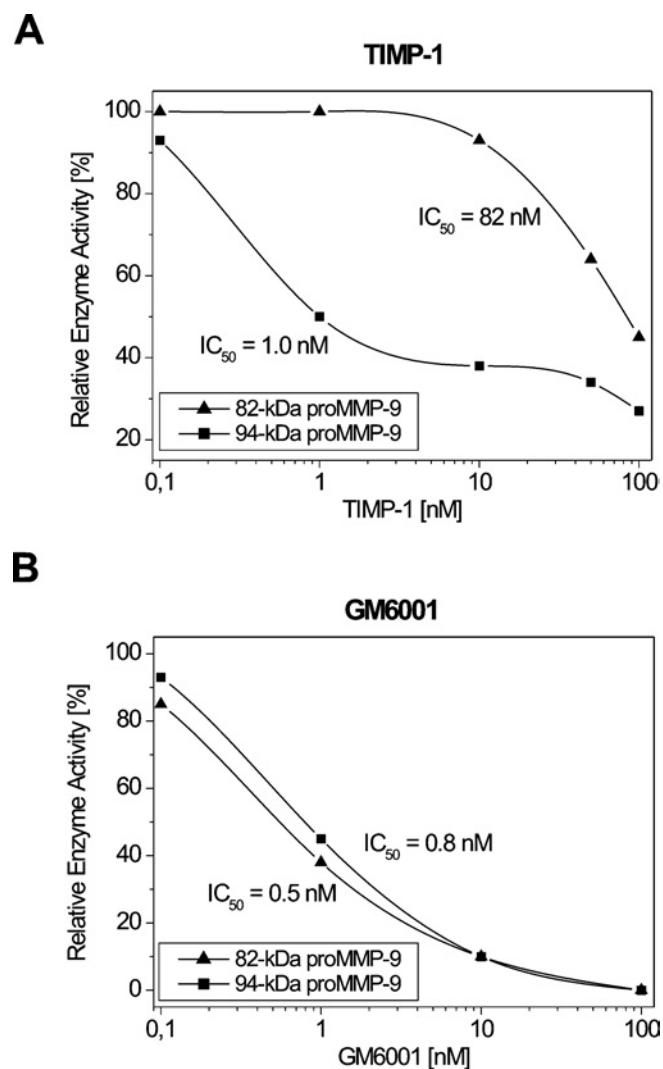


Figure 8 Inhibition studies

Activated 82 kDa proMMP-9 and 94 kDa proMMP-9 (1 nM) were assayed with increasing concentrations of the physiological MMP-9 inhibitor TIMP-1 (**A**) and the synthetic MMP-inhibitor GM6001 (**B**). Residual enzyme activity was quantified using fluorescence-labelled gelatin as a substrate and expressed in percentage of activity without inhibitor. Results are given as means of duplicate experiments representative for three independent determinations.

detailed glycosylation analysis performed on proMMP-9 released from neutrophil granulocytes, demonstrating that N-linked carbohydrates of approx. 5 kDa and O-linked carbohydrates of approx. 10 kDa are attached to the 76 kDa zymogen core protein [53,54]. The biological significance of oligosaccharides for proMMP-9 function is far from clear, but they are considered to influence the backbone conformation of the enzyme, its stability and its interaction with other molecules. Recent studies using recombinant variants of proMMP-9 indicate that the abundant O-linked glycans present in this enzyme are important for its interaction with TIMP-1, but dispensable for catalytic activity [55]. Likewise, O-glycans present in membrane type 1 MMP are essential for binding to TIMP-2, but are not required for its collagenolytic activity [56]. Additionally, aberrant O-glycosylation found in tumour cell-derived proMMP-9 compared with that from neutrophil granulocytes was shown to contribute to impaired interaction with galectin-3 [57]. We hypothesize that N-terminal processing of the 82 kDa proMMP-9 alters its protein

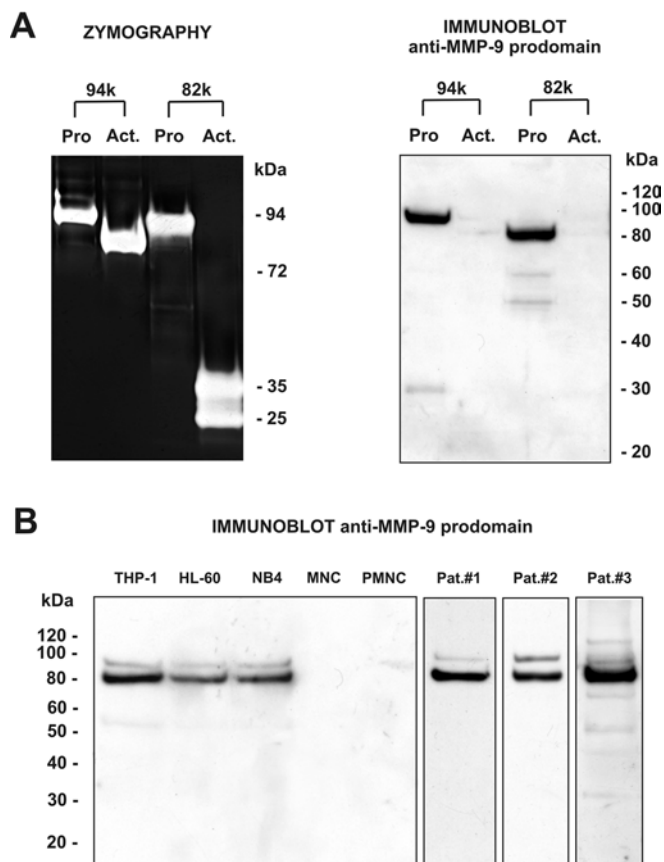


Figure 9 Specific detection of 82 kDa proMMP-9 in leukaemic and non-leukaemic cells

(**A**) The zymogen forms (Pro) and activated species (Act.) of the 94 kDa proMMP-9 (94k) and the 82 kDa proMMP-9 (82k) were analysed by zymography. Aliquots were subjected to SDS/PAGE under reducing condition, blotted and incubated with monoclonal antibodies directed against the propeptide of proMMP-9 to allow specific detection of the 82 kDa proMMP-9 in distinction to 82 kDa active MMP-9. (**B**) Cells were incubated in serum-free medium (1×10^6 cells/ml) for 24 h in the presence of 50 ng/ml PMA. Proteins were extracted and 10 ng were subjected to Western blotting analysis using an anti-(proMMP-9 prodomain) antibody. Results are shown for the leukaemic cell lines THP-1, HL-60 and NB4, for mononuclear cells (MNC) and neutrophil granulocytes (PMNC) from peripheral blood of healthy persons, and for leukaemic blast cells from peripheral blood of patients with acute myeloid leukemia (Pat.#1-3).

structure in the endoplasmic reticulum and Golgi, which results in the failure of O-glycosylation. Consequently, the lack of O-glycans may favour surface binding and cause changes in its interaction with substrates, inhibition by TIMPs and accessibility to (auto-)proteolytic cleavage.

MMP-3 is an effective proteolytic activator for secreted proMMP-9 [23,25,48,49] generating the active 82 kDa species. In the present study, we could demonstrate that it converts the surface-associated 82 kDa proMMP-9 into an unusually small and so far undescribed 35 kDa active form. This species was found in cell lysates and purified plasma membranes, but was absent in the culture supernatants. This failure may be due to a strong binding to the cell surface or caused by dilution and limited stability in the medium. The 35 kDa MMP-9 has a substrate spectrum similar to the well-known 82 kDa MMP-9, but preferentially cleaves denatured collagens, suggesting a biological role differing from that of secreted MMP-9. Moreover, autocatalytic conversion into its active species occurred in the surface-bound 82 kDa proMMP-9, but not in the secreted 94 kDa zymogen. This auto-activation may be promoted by the lack of a nonapeptide at the N-terminus

of the 82 kDa proMMP-9, which may disrupt the cysteine–Zn²⁺ interaction ('cysteine switch') more readily. Although the autoproteolytic activation of the 82 kDa proMMP-9 occurred at a slower rate compared with the MMP-3 catalysed reaction, both conditions led to the appearance of a major 35 kDa active species and a minor 25 kDa form detectable in zymograms, indicating that similar processing mechanisms may apply for autocatalytic and MMP-3-mediated activation. TIMP-1 is known to build a tight complex with the secreted 94 kDa proMMP-9 by binding with its N-terminus to the catalytic site and with its C-terminus to the haemopexin domain of proMMP-9, thereby controlling its activation and activity [1,4,21,23,41,48]. Unexpectedly, co-incubation of the 82 kDa proMMP-9 with an excess of TIMP-1 could not prevent or slow down the auto-activation, suggesting limited reaction between the proenzyme variant and TIMP-1. This may be explained by the lack of O-glycans in 82 kDa proMMP-9, which were shown to be essential for optimal binding of proMMP-9 to TIMP-1 [55].

To gain further insight into the structure/function relationship of the 82 kDa proMMP-9, we performed comparative inhibition studies. The activated 94 kDa proMMP-9 was efficiently blocked by equimolar concentrations of TIMP-1, consistent with the results of previous studies on secreted proMMP-9 [58]. In contrast, the 82 kDa proMMP-9 in its activated form required approx. 80-fold higher amounts of TIMP-1 to abrogate gelatinolytic activity. It seems obvious that this dramatically reduced inhibition is related to the extensive truncations characterizing active 35-kDa MMP-9. In fact, antibodies directed against a C-terminal peptide of MMP-9 recognized 82 kDa proMMP-9, but not its activated 35 kDa form (results not shown), indicating C-terminal truncation in the latter. The C-terminus of MMP-9 contains the haemopexin-like domain, which represents the high-affinity binding site for TIMP-1 [55,58]. Therefore it is reasonable that extensive C-terminal processing of the 82 kDa proMMP-9 during activation reduces its susceptibility to TIMP-1 inhibition and thereby increases its bioavailability. Presumably, the remaining 35 kDa mini MMP-9 essentially consists of the catalytic domain of MMP-9. This area contains the fibronectin-type II repeats, which are important for gelatin binding, and includes the zinc-binding sequence that is required for proteolytic activity [1,4]. GM6001, a small synthetic metalloproteinase inhibitor that binds directly to the enzyme's active site [59], blocked the active 35 kDa and 82 kDa MMP-9 species with a similar efficiency. This indicates the suitability of low molecular mass inhibitors in targeting both surface-associated and secreted MMP-9 activity, e.g. in the treatment of cancer.

The results of the present study indicate that THP-1 leukaemic cells endogenously produce a unique proMMP-9 variant exposed on the cell surface with reduced susceptibility to inhibition by TIMP-1. Adopting an immunological method specifically detecting this novel 82 kDa proMMP-9, we found the enzyme to be expressed in different leukaemic cell lines, but not in normal white blood cells. Its detection in patient-derived leukaemic blast cells indicates that the 82 kDa proMMP-9 also occurs *in vivo*, supporting the importance of this enzyme species. Physiological stimuli such as cytokines, chemokines or extracellular matrix components may influence its synthesis and exposition on the plasma membrane. Therefore overexpression of the TIMP-1-insensitive MMP-9 variant on the surface of malignantly transformed cells may increase pericellular proteolysis and thereby promote cancer progression *in vivo*.

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