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

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Short title: Cell surface-associated 82-kDa proMMP-9

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Abbreviations: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; MALDI-TOF MS, matrix-assisted laser desorption/ionization tunnel of flight mass spectra; PMCA-ATPase, plasma membrane-localized calcium dependent ATPase; APMA, 4-aminophenylmercuric acetate.

Keywords: matrix metalloproteinase (MMP), gelatinase B, glycosylation, tissue inhibitor of metalloproteinase (TIMP), acute myeloid leukemia, tumor cell invasion

SYNOPSIS

Matrix metalloproteinase 9 (MMP-9) plays a critical role in tumor progression. While 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 leukemic cells, featuring substantial differences when compared to 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 9 amino acid residues at its N-terminus, lacks O-linked oligosaccharides present in 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 to an active species of 35 kDa, which was also produced by autocatalytical processing in the absence of activating enzymes. The activated 35-kDa MMP-9 efficiently degraded gelatins, native collagen type IV, and fibronectin. The enzyme was less sensitive to TIMP-1 inhibition with IC_{50} values of 82 nM compared with 1 nM of the 82-kDa active MMP-9. The synthetic MMP inhibitor GM6001 blocked both enzymes with a similar IC_{50} value below 1 nM. The 82-kDa proMMP-9 is also produced in HL-60 and NB4 leukemic cell lines as well as ex vivo leukemic blast cells. It is, however, absent in neutrophils and mononuclear cells isolated from peripheral blood of healthy individuals. Thus, 82-kDa proMMP-9 expressed on the surface of malignant cells may escape inhibition by natural TIMP-1 facilitating cellular invasion in vivo.

(248 words)

INTRODUCTION

Matrix metalloproteinases (MMPs) 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 tumor growth and metastasis, arthrosis, arthritis, nephritis, neurodegenerative disease, and fibrosis [2,4]. Production and activity of MMPs in cells is precisely controlled at the level of gene expression modulated by a variety of stimuli (e.g. cytokines, hormones or extracellular matrix proteins), by activation of the latent proenzymes, and inhibition by endogenous tissue inhibitors of metalloproteinases (TIMPs) [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 potentials to process 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 is 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 hematopoietic 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 tumor with MMP-9 [12]. Moreover, in acute myeloid leukemia both constitutive and stimulated releases of MMP-9 are observed in various cell lines and *ex vivo* blast cells [13-17], indicating that this enzyme is also involved in leukemic cell dissemination [18]. Direct evidence for a crucial role of MMP-9 in the invasion and metastasis capacity of tumor 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 proenzyme and secreted from the cells in a glycosylated proenzyme form [21] with a molecular mass varying from 91 to 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].

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 tumor 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 α_2 chain of collagen IV [33]. MMP-9 colocalized with CD44 on the surface of neoplastic cells was demonstrated to activate latent transforming growth factor β thereby promoting tumor invasion, growth, and angiogenesis [34]. MMP-9 can also induce tumor-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 tumor 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 this study, we identified a novel non-secreted 82-kDa proMMP-9 species localized at the surface of leukemic cells. This particular zymogen form has not been recognized as an independent enzyme before, probably due to its high similarity to 'regular' proMMP-9 which after its secretion is able to re-associate to the cell membrane. Comparative analysis on purified enzymes clearly showed 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 leukemic cells.

EXPERIMENTAL

Cell culture.

The cell lines THP-1 (acute monocytic leukemia), HL-60 (acute myeloblastic leukemia), NB4 (acute promyelocytic leukemia), 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% heat inactivated fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/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% Nutridoma SP (Roche Applied Science, Mannheim, Germany). Mononuclear cells and polymorphonuclear cells/neutrophil granulocytes were isolated from peripheral blood of healthy volunteers and leukemic blast cells from peripheral blood of three patients with acute myeloid leukemia 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. Purified cell fractions were washed twice with buffer and resuspended in serum-free medium for subsequent experiments. Viability of cells was determined using the trypan-blue exclusion test.

Phorbol ester stimulation and preparation of crude cell extracts.

For stimulation experiments cells were cultivated at a density of 1×10^6 /ml either in serum-free medium alone or in medium containing 50 ng/ml phorbol 12-myristate 13-acetate (PMA) for 24 h. Thereafter, non-adherent cells were separated from culture supernatants by centrifugation. Adherent cells were scraped. The conditioned media were stored at -20°C. Cell pellets were washed three-times in cold Tris-buffered-saline (TBS) containing 50 mM Tris pH 7.4 and 150 mM NaCl. For lysis and protein extraction, cells were resuspended at a density of 1×10^7 /ml in TBS containing 1% 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 16,000 x g 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 two-times for 15 min in 2.7% Triton X-100 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% methanol/10% 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 according to a method described previously [39]. Zymograms were scanned using an Umax ImageScanner driven by the MagicScan software and analyzed 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% polyacrylamide minigels (Invitrogen, Groningen, The Netherlands). 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 polyvinyl difluoride (PVDF) membranes (Millipore, Bedford, MA, U.S.A.). The membranes were blocked in 10% non-fat milk in TBS for 1 h at room temperature. Thereafter, the blots were incubated with 0.5 µg/ml primary rabbit polyclonal antibody against proMMP-9 (M-5177; Sigma, Munich, Germany) for 1 h at room temperature, which allows detection of both latent and activated enzyme forms. Mouse monoclonal antibodies (IM09L, clone 6-6B, Calbiochem) that only recognize latent proMMP-9, but not the activated species under reducing conditions of immunoblotting were used at a concentration of 0.5 µg/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 enhanced chemiluminescence (ECL) system as recommended by the manufacturer (GE Healthcare Life Sciences). For molecular weight determination of proteins detected on the blots recombinant protein standards (Invitrogen; Biorad, Munich, Germany) were used. The developed blotting films were scanned and analyzed using the ImageMaster-1D Elite software (GE Healthcare Life Sciences).

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, fixed in 3.7% formaldehyde for 15 min at room temperature, subsequently blocked in 2% BSA for 15 min, and incubated with 50 µg/ml of monoclonal anti-MMP-9 antibodies (GE-213, NeoMarkers, Union City, CA, U.S.A.) or 50 µg/ml of mouse IgG₁ isotype control antibodies (DAKO, Hamburg, Germany) for 1 h at 37°C. The secondary fluorescence-conjugated antibody used was Alexa 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 505-530 nm on a Zeiss LSM 510 confocal scanning microscope equipped with a Plan-Neofluar 100x/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 cold phosphate buffered saline (PBS). All following procedures were carried out at 4°C according to a previously described method [26] with minor modifications. Cells were resuspended at 2×10^7 /ml in a buffer containing 25 mM Tris-HCl pH 7.5, 8.5% sucrose, 50 mM NaCl, and a mix of 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 x g. The pellet (nuclear fraction) was discarded and the supernatant (postnuclear fraction) was centrifuged at 110,000 x g 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 a buffer containing 25 mM Tris-HCl pH 7.5, 50 mM NaCl and proteinase

inhibitors (Complete-Mini). The sample was layered onto a discontinuous density gradient of 20/30/50/60% sucrose in water and once more centrifuged at 110,000 x g 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 110,000 x g for another 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 plasma membrane-localized calcium dependent (PMCA) 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 of 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, and washed twice with cold PBS (600 x g, 4°C). Viability of cells was $\geq 95\%$ as determined by trypan-blue staining. Cells were then diluted to 1×10^7 /ml in PBS and incubated with 40 μ l/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 two times in PBS and extracted with Triton X-100 as described above. For gelatinase enrichment cell extracts were passed through a gelatin-Sepharose affinity column (see below). Elution fractions containing gelatinolytic activity were pooled, diafiltrated with 50 mM Tris-HCl pH 7.5, 0.3% Triton X-100, 5 mM EDTA and concentrated using Microcon YM10 devices (Millipore). For immunoprecipitation, enriched gelatinase samples were then incubated with 9 μ g/ml of monoclonal anti-MMP-9 antibodies (GE-213, NeoMarkers) and protein G-Sepharose beads overnight at 4°C under constant shaking. The G-Sepharose beads were washed three-times with cold PBS, resuspended in reducing electrophoresis sample buffer, boiled, and centrifuged. The supernatant containing immunoprecipitated antigens was separated by 4-12% SDS-PAGE 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 (1:1500) and the ECL-chemiluminescence system (GE Healthcare Life Sciences). The specificity of detection was controlled by processing non-biotinylated cells in the same way as described for biotinylated cells.

Gelatin-Sepharose affinity chromatography and gel filtration.

Purification of MMP-9 was performed as previously described 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 autoproteolytic 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 (3000 x g, 15 min), the supernatant was applied to a gelatin-Sepharose column of 2 ml bed volume equilibrated with 50 mM Tris 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 with 5% DMSO in column buffer. Gelatinase containing fractions were assessed by zymography, pooled, and concentrated approximately 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 x 60 cm) (GE Healthcare Life Sciences) equilibrated with 50 mM Tris 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. 1 liter conditioned medium was adjusted to 5 mM EDTA and 0.02% Brij 35 and concentrated to a final volume of 50 ml by a Amicon Diaflo apparatus fitted with a YM-10 membrane (Amicon, Beverly, MA, U.S.A.). Thereafter, the material was applied to gelatin-Sepharose affinity chromatography and gel filtration as described above. Selective denaturation of copurifying TIMP molecules was performed according to a method published by J.F. Woessner and colleagues [42].

Microsequencing and MALDI-TOF mass spectrometry.

Samples of purified gelatinases were separated by SDS-PAGE in a 10% polyacrylamide gel and electroblotted onto glass fiber membranes (Glassybond, Biometra, Göttingen, Germany) [43]. Coomassie Blue stained bands were cut out and sequenced in an Applied Biosystems (Foster City, CA, U.S.A.) 492cLC gas phase sequencer. For mass spectrometry

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% SDS, 1 M β -mercaptoethanol) and heated at 100°C for 5 min. After cooling to room temperature, Triton X-100 solution was added 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 U) 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 mU), α 2-3,6,8,9-neuraminidase (1.25 mU), β 1,4-galactosidase (0.75 mU), and β -N-acetylglucosaminidase (2 mU) 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 analyzed 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 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 pretreated with or without MMP-3 (30 min/37°C) were reacted with an excess of α 2-macroglobulin

(Roche Applied Science) (500 µg/ml; 30 min/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 µM 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 for 2 h at 37°C as described previously [41]. Successful conversion to their active forms was controlled by zymography. Collagen type IV (Engelbreth-Holm-Swarm mouse sarcoma cells) (400 µg/ml) native or denatured (3 min, 100°C), collagen type I (rat tail) (300 µg/ml) denatured, and fibronectin (100 µg/ml) (all proteins purchased from Sigma, Munich, Germany) were incubated with or without equimolar amounts (1,5 µM) of activated enzymes for 48 h at 32°C. The reaction mixtures were then analyzed for degradation products by SDS-PAGE with subsequent silver staining of the proteins.

MMP-9 inhibition studies.

The active forms of 82- and 94-kDa proMMP-9 were assayed using a fluorescence-labeled gelatin substrate (Molecular Probes, Eugene, OR, U.S.A.). 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 µM ZnCl₂, and 0.02% Brij 35 (100 µl total volume). Complete conversion to 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 [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-labeled substrate (1.5 µg/ml) was added to a final volume of 200 µl. After a 1-h incubation period 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 phorbol 12-myristate 13-acetate (PMA). The culture supernatants and cell lysates were analyzed for gelatinase activity by zymography and Western blotting. The conditioned medium contained 94-kDa proMMP-9 whose amount was considerably enhanced upon PMA stimulation (Fig. 1 A,B). An additional band migrating on 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 (Fig. 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 forming a major zone of lysis between ~88 and 94 kDa (Fig. 1A). Under reducing conditions of Western blotting analysis using anti-MMP-9 antibodies two distinct bands were identified 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 (Fig. 1B). To assess whether the 82-kDa MMP-9 was generated from 94-kDa proMMP-9 by the Triton-X100 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 (data not shown), demonstrating that the 82-kDa form was not created by the extraction technique.

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 (Fig. 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 fraction contained the two MMP-9 species each with larger amounts of the 82-kDa compared to the 94-kDa form (Fig. 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 (Fig. 2C).

To verify that MMP-9 was bound to the outer periphery of the plasma membrane, cell surface proteins of THP-1 cells were labeled 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 detection system a strong band at 82 kDa together with a faint band of 94 kDa and additional weak protein bands of low molecular masses were identified (Fig. 2D). These results confirmed that the major MMP-9 detection on the surface of THP-1 cells was the 82-kDa form.

Purification of cell-associated and secreted forms of MMP-9.

To study the biochemical properties of the cell-associated 82-kDa variant in comparison to the secreted species of MMP-9, both enzyme forms were purified from cell extracts and culture medium of THP-1 cells treated with PMA. Gelatinases were first enriched from crude cell extracts or the conditioned medium by gelatin-Sepharose affinity chromatography and were further purified by gel filtration chromatography which separated the 82-kDa species and the 94-kDa form (Fig. 3A). Analysis of the pooled and concentrated fractions showed that the purified 82-kDa form migrated with an apparent mass of ~90 kDa in zymograms (Fig. 3B). This may be due to the absence of reducing agents in zymography since under reducing conditions of SDS-PAGE with subsequent silver staining of proteins it showed a single band with a molecular mass of 82 kDa (Fig 3C).

Microsequencing and MALDI-TOF MS analysis of purified MMP-9 forms.

As deduced from the molecular mass, we speculated that the cell-associated 82-kDa species could represent the activated form of mature MMP-9 lacking the N-terminal proenzyme domain. To address this question, the MMP-9 forms purified from cell extracts and conditioned media of PMA-stimulated THP-1 cells were analyzed by microsequencing and mass spectroscopy. Assessment of the N-terminal sequence of the cell-associated 82-kDa variant revealed a 9 amino acid truncation for this enzyme compared to regular proMMP-9 (Table I). In contrast, secreted 94-kDa proMMP-9 contained the complete N-terminus of the zymogen form (Table I). Microsequencing of intracellular 94-kDa proMMP-9 failed possibly due to N-terminal blockage. Further analysis by MALDI-TOF mass spectroscopy confirmed

each N-terminal sequence (Table I). The tryptic peptides of both the secreted and the cell-associated 94-kDa form of MMP-9 showed the N-terminal peptide mass of 1,345.7 Dalton, which agreed with the predicted mass of that of full-length proMMP-9. Mass mapping of the 82-kDa variant revealed a smaller fragment of 916.5 Dalton which matched the calculated molecular mass lacking 9 amino acids from the N-terminus of proMMP-9 (Table I). These results were in agreement with our findings obtained by microsequence analysis.

Studies on proMMP-9 glycosylation.

Since proMMP-9 is known to be highly glycosylated [21], we hypothesized that alterations in the pattern of glycosylation might also account for the difference in molecular mass for the 82- and 94-kDa species. Therefore, purified 82-kDa proMMP-9 and 94-kDa proMMP-9 were subjected to digestion with N-glycosidase F. Western blotting analysis demonstrated that both forms of proMMP-9 were reduced in size by 6 kDa (Fig. 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 (Fig. 4). Removal of all sugar residues from 82- and 94-kDa proMMP-9 using both N-linked and O-linked specific glycosidases yielded a 76- and a 80-kDa protein, respectively (Fig. 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 94-kDa proMMP-9 contains both types of glycans.

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 blotting analysis, treatment of the 94-kDa proMMP-9 with MMP-3 resulted in complete conversion to a single 82-kDa MMP-9 within 1 h with an intermediate form of about 86-kDa (Fig. 5A), as described by Ogata *et al.* [49]. In contrast, incubation of the 82-kDa proMMP-9 with MMP-3 initiated rapid conversion to a major gelatinase with an apparent molecular mass of ~35-kDa which migrated as a single protein of 35 kDa when analyzed by Western blotting

under reducing conditions (Fig. 5B). Due to the high sensitivity of zymography additional bands with lower intensity ranging between ~25-40 kDa were also detected which may represent minor activation products (Fig. 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 hours (Fig. 5C). These findings indicate, that 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 the activated 82-kDa species bound to α_2 -macroglobulin and the majority of activity shifted to the top of the gel (Fig. 5D). The 82-kDa proMMP-9 did not react with α_2 -macroglobulin (Fig. 5D), suggesting that it is proteolytically inactive like 94-kDa proMMP-9. The activated 35-kDa species bound to α_2 -macroglobulin (Fig. 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 (Tris-HCl pH 7.5, NaCl, CaCl₂, ZnCl₂, Brij 35) in the absence of activating agents for 1 to 6 h at 37°C gradually converted the enzyme into a major 35-kDa and a minor 25-kDa gelatinase as determined by zymography (Fig. 6A) and Western blotting analysis (Fig. 6B). The conversion was almost completed after 6 h of incubation (Fig. 6A). In contrast, the 94-kDa proMMP-9 remained stable and showed no change in molecular mass under these conditions (Fig. 6A). Autocatalytic processing of 82-kDa proMMP-9 was not affected by the addition of TIMP-1 even when the inhibitor was present in a 3-molar excess (Fig. 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 collagens type IV and type I (gelatins), native collagen type IV and fibronectin (Fig. 7). The two enzymes, however, showed different extent in degrading these substrates. While activated 94-kDa proMMP-9 prefers to cleave native type IV collagen to the denatured gelatins,

activated 82-kDa proMMP-9 digested denatured type I and type IV collagens more readily than the 94-kDa form (Fig. 7).

Inhibition of 82- 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 (Fig. 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 (Fig. 8A). Comparable results were obtained by TIMP-2 (data 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 activated 94-kDa and of 0.8 nM for activated 82-kDa proMMP-9 (Fig. 8B).

Expression of the 82-kDa proMMP-9 in other leukemic 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 blotting analysis with antibodies that specifically recognize the propeptide but not activated forms of MMP-9. As expected, both the 82-kDa proMMP-9 and the 94-kDa proMMP-9, but not their activated species were detectable by anti-proMMP-9-prodomain antibodies (Fig. 9A).

Western blotting 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 originated from patients with acute myeloid leukemia. 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 those in THP-1 cells (Fig. 9B). MMP-3 treatment of HL-60 and NB4 cell lysates also generated the 35-kDa activated MMP-9 species (data not shown) proving the presence of 82-kDa proMMP-9 in these cells. Western blotting analysis of extracts from PMA-treated *ex vivo* leukemic cells isolated from the peripheral blood of three patients with acute myeloid leukemia clearly displayed production of 82-kDa proMMP-9 in these cells (Fig. 9B). In contrast, proMMP-9 forms were absent in the lysates obtained from PMA-stimulated mononuclear cells and neutrophil granulocytes from peripheral blood of healthy donors (Fig. 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 tumor 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 our study, we have provided 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 leukemic cells. Moreover, this membrane associated proMMP-9 variant differs from the secreted zymogen form in several structural and functional aspects: It is truncated by 9 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 a 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 as 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 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 tumor cells has an apparent molecular mass of ~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 these 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. Our study showed that the 82-kDa form is a proMMP-9, but the N-terminus is truncated by 9 amino acids. Previously it has been reported that proMMP-9 isolated from culture supernatants of peripheral blood monocytes, neutrophil granulocytes, and HL-60 leukemic cells is also shortened by 8-9 amino acids at its N-terminus [9,10,41]. This indicates that N-terminal processing may occur both in 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 proprotein convertases which posttranslationally process precursor proteins during the secretory pathway.

In addition to the N-terminal lack of 9 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 (data not shown). Deglycosylation studies, however, revealed that the surface-bound 82-kDa proMMP-9 variant is lacking O-linked carbohydrates which amount to a molecular mass of ~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 detailed glycosylation analysis performed on proMMP-9 released from neutrophil granulocytes demonstrating that N-linked carbohydrates of ~5 kDa and O-linked carbohydrates of ~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 it is considered to influence the backbone conformation of the enzyme, its stability, and 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 matrix metalloproteinase are essential to bind to TIMP-2, but are not required for its collagenolytic activity [56]. Additionally, aberrant O-glycosylation found in tumor 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 structure in the endoplasmic reticulum and Golgi which results in the failure of O-glycosylation. Consequently, the lack of O-glycans may favor surface

binding and cause changes in 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. Here we could demonstrate that it converts the surface-associated 82-kDa proMMP-9 to 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 to 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 82-kDa proMMP-9 which may disrupt the cysteine-Zn²⁺ interaction ('cysteine switch') more readily. Although the autoproteolytic activation of 82-kDa proMMP-9 occurred at a slower rate compared to the MMP-3 catalyzed 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 secreted 94-kDa proMMP-9 by binding with its N-terminus into the catalytic site and with its C-terminus to the hemopexin domain of proMMP-9, thereby controlling its activation and activity [1,4,21,23,41,48]. Unexpectedly, coincubation of 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 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, 82-kDa proMMP-9 in its activated form required approximately 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 (data not shown),

indicating C-terminal truncation in the latter. The C-terminus of MMP-9 contains the hemopexin-like domain which represents the high affinity binding site for TIMP-1 [55,58]. Therefore, it is reasonable that extensive C-terminal processing of 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 which binds directly to the active site [59], blocked the active 35-kDa and 82-kDa MMP-9 species with a similar high 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.

Our data indicate that THP-1 leukemic 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 leukemic cell lines but not in normal white blood cells. Its detection in patient-derived leukemic blast cells indicates that 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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REFERENCES

- 1 Nagase, H. and Woessner, J. F. (1999) Matrix metalloproteinases. *J.Biol.Chem.* **274**, 21491-21494
- 2 Stetler-Stevenson, W. G., Aznavoorian, S., and Liotta, L. A. (1993) Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Annu.Rev.Cell Biol.* **9**, 541-573
- 3 Egeblad, M. and Werb, Z. (2002) New functions for the matrix metalloproteinases in cancer progression. *Nat.Rev.Cancer* **2**, 161-174
- 4 Woessner, J. F. and Nagase, H. (2000) *Matrix metalloproteinases and TIMPs*, Oxford University Press, New York
- 5 Ries, C. and Petrides, P. E. (1995) Cytokine Regulation of Matrix Metalloproteinase Activity and Its Regulatory Dysfunction in Disease. *Biol.Chem.* **376**, 345-355
- 6 Yu, Q. and Stamenkovic, I. (2000) Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev.* **14**, 163-176
- 7 Van den Steen, P. E., Proost, P., Wuyts, A., van Damme, J., and Opdenakker, G. (2000) Neutrophil gelatinase B potentiates interleukin-8 tenfold by aminoterminal processing, whereas it degrades CTAP-III, PF-4, and GRO-alpha and leaves RANTES and MCP-2 intact. *Blood* **96**, 2673-2681
- 8 Schonbeck, U., Mach, F., and Libby, P. (1998) Generation of biologically active IL-1 beta by matrix metalloproteinases: a novel caspase-1-independent pathway of IL-1 beta processing. *J.Immunol.* **161**, 3340-3346
- 9 Masure, S., Proost, P., van Damme, J., and Opdenakker, G. (1991) Purification and identification of 91-kDa neutrophil gelatinase. Release by the activating peptide interleukin-8. *Eur.J.Biochem.* **198**, 391-398
- 10 Opdenakker, G., Masure, S., Proost, P., Billiau, A., and van Damme, J. (1991) Natural human monocyte gelatinase and its inhibitor. *FEBS Lett.* **284**, 73-78
- 11 Welgus, H. G., Campbell, E. J., Cury, J. D., Eisen, A. Z., Senior, R. M., Wilhelm, S. M., and Goldberg, G. I. (1990) Neutral metalloproteinases produced by human mononuclear phagocytes. Enzyme profile, regulation, and expression during cellular development. *J.Clin.Invest.* **86**, 1496-1502
- 12 Coussens, L. M., Tinkle, C. L., Hanahan, D., and Werb, Z. (2000) MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell* **103**, 481-490
- 13 Van Ranst, M., Norga, K., Masure, S., Proost, P., Vandekerckhove, F., Auwerx, J., van Damme, J., and Opdenakker, G. (1991) The cytokine-protease connection: identification of a 96-kD THP- 1 gelatinase and regulation by interleukin-1 and cytokine inducers. *Cytokine* **3**, 231-239

- 14 Ries, C., Kolb, H., and Petrides, P. E. (1994) Regulation of 92-kD gelatinase release in HL-60 leukemia cells: tumor necrosis factor-alpha as an autocrine stimulus for basal- and phorbol ester-induced secretion. *Blood* **83**, 3638-3646
- 15 Ismail, M. G., Ries, C., Lottspeich, F., Zang, C., Kolb, H. J., and Petrides, P. E. (1998) Autocrine regulation of matrix metalloproteinase-9 gene expression and secretion by tumor necrosis factor-alpha (TNF-alpha) in NB4 leukemic cells: specific involvement of TNF receptor type 1. *Leukemia* **12**, 1136-1143
- 16 Ries, C., Loher, F., Zang, C., Ismail, M. G., and Petrides, P. E. (1999) Matrix metalloproteinase production by bone marrow mononuclear cells from normal individuals and patients with acute and chronic myeloid leukemia or myelodysplastic syndromes. *Clin.Cancer Res.* **5**, 1115-1124
- 17 Janowska-Wieczorek, A., Marquez, L. A., Matsuzaki, A., Hashmi, H. R., Larratt, L. M., Boshkov, L. M., Turner, A. R., Zhang, M. C., Edwards, D. R., and Kossakowska, A. E. (1999) Expression of matrix metalloproteinases (MMP-2 and -9) and tissue inhibitors of metalloproteinases (TIMP-1 and -2) in acute myelogenous leukaemia blasts: comparison with normal bone marrow cells. *Br.J.Haematol.* **105**, 402-411
- 18 Klein, G., Vellenga, E., Fraaije, M. W., Kamps, W. A., and de Bont, E. S. (2004) The possible role of matrix metalloproteinase (MMP)-2 and MMP-9 in cancer, e.g. acute leukemia. *Crit Rev.Oncol.Hematol.* **50**, 87-100
- 19 Bernhard, E. J., Gruber, S. B., and Muschel, R. J. (1994) Direct evidence linking expression of matrix metalloproteinase 9 (92-kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells. *Proc.Natl.Acad.Sci.U.S.A.* **91**, 4293-4297
- 20 Hua, J. and Muschel, R. J. (1996) Inhibition of matrix metalloproteinase 9 expression by a ribozyme blocks metastasis in a rat sarcoma model system. *Cancer Res.* **56**, 5279-5284
- 21 Wilhelm, S. M., Collier, I. E., Marmer, B. L., Eisen, A. Z., Grant, G. A., and Goldberg, G. I. (1989) SV40-transformed human lung fibroblasts secrete a 92-kDa type IV collagenase which is identical to that secreted by normal human macrophages. *J.Biol.Chem.* **264**, 17213-17221
- 22 Moll, U. M., Youngleib, G. L., Rosinski, K. B., and Quigley, J. P. (1990) Tumor promoter-stimulated Mr 92,000 gelatinase secreted by normal and malignant human cells: isolation and characterization of the enzyme from HT1080 tumor cells. *Cancer Res.* **50**, 6162-6170
- 23 Nagase, H. (1997) Activation mechanisms of matrix metalloproteinases. *Biol.Chem.* **378**, 151-160
- 24 Mazziere, R., Masiero, L., Zanetta, L., Monea, S., Onisto, M., Garbisa, S., and Mignatti, P. (1997) Control of type IV collagenase activity by components of the urokinase-plasmin system: a regulatory mechanism with cell-bound reactants. *EMBO J.* **16**, 2319-2332

- 25 Ramos-DeSimone, N., Hahn-Dantona, E., Siple, J., Nagase, H., French, D. L., and Quigley, J. P. (1999) Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion. *J.Biol.Chem.* **274**, 13066-13076
- 26 Toth, M., Gervasi, D. C., and Fridman, R. (1997) Phorbol ester-induced cell surface association of matrix metalloproteinase-9 in human MCF10A breast epithelial cells. *Cancer Res.* **57**, 3159-3167
- 27 Partridge, C. A., Phillips, P. G., Niedbala, M. J., and Jeffrey, J. J. (1997) Localization and activation of type IV collagenase/gelatinase at endothelial focal contacts. *Am J Physiol* **272**, L813-L822
- 28 Stefanidakis, M., Ruohtula, T., Borregaard, N., Gahmberg, C. G., and Koivunen, E. (2004) Intracellular and cell surface localization of a complex between alpha2beta2 integrin and promatrix metalloproteinase-9 progelatinase in neutrophils. *J.Immunol.* **172**, 7060-7068
- 29 Owen, C. A., Hu, Z., Barrick, B., and Shapiro, S. D. (2003) Inducible expression of tissue inhibitor of metalloproteinases-resistant matrix metalloproteinase-9 on the cell surface of neutrophils. *Am.J.Respir.Cell Mol.Biol.* **29**, 283-294
- 30 Rahat, M. A., Marom, B., Bitterman, H., Weiss-Cerem, L., Kinarty, A., and Lahat, N. (2006) Hypoxia reduces the output of matrix metalloproteinase-9 (MMP-9) in monocytes by inhibiting its secretion and elevating membranal association. *J.Leukoc.Biol.* **79**, 706-718
- 31 Bourguignon, L. Y., Gunja-Smith, Z., Iida, N., Zhu, H. B., Young, L. J., Muller, W. J., and Cardiff, R. D. (1998) CD44v(3,8-10) is involved in cytoskeleton-mediated tumor cell migration and matrix metalloproteinase (MMP-9) association in metastatic breast cancer cells. *J.Cell Physiol.* **176**, 206-215
- 32 Ellerbroek, S. M., Halbleib, J. M., Benavidez, M., Warmka, J. K., Wattenberg, E. V., Stack, M. S., and Hudson, L. G. (2001) Phosphatidylinositol 3-kinase activity in epidermal growth factor-stimulated matrix metalloproteinase-9 production and cell surface association. *Cancer Res.* **61**, 1855-1861
- 33 Olson, M. W., Toth, M., Gervasi, D. C., Sado, Y., Ninomiya, Y., and Fridman, R. (1998) High affinity binding of latent matrix metalloproteinase-9 to the alpha2(IV) chain of collagen IV. *J.Biol.Chem.* **273**, 10672-10681
- 34 Yu, Q. and Stamenkovic, I. (1999) Localization of matrix metalloproteinase 9 to the cell surface provides a mechanism for CD44-mediated tumor invasion. *Genes Dev.* **13**, 35-48
- 35 Fridman, R., Toth, M., Chvyrkova, I., Meroueh, S. O., and Mobashery, S. (2003) Cell surface association of matrix metalloproteinase-9 (gelatinase B). *Cancer Metastasis Rev.* **22**, 153-166
- 36 Stefanidakis, M. and Koivunen, E. (2006) Cell-surface association between matrix metalloproteinases and integrins: role of the complexes in leukocyte migration and

- cancer progression. *Blood* **108**, 1441-1450
- 37 Bergers, G., Brekken, R., McMahon, G., Vu, T. H., Itoh, T., Tamaki, K., Tanzawa, K., Thorpe, P., Itohara, S., Werb, Z., and Hanahan, D. (2000) Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat.Cell Biol.* **2**, 737-744
 - 38 Seiki, M. (2002) The cell surface: the stage for matrix metalloproteinase regulation of migration. *Curr.Opin.Cell Biol.* **14**, 624-632
 - 39 Kleiner, D. E. and Stetler-Stevenson, W. G. (1994) Quantitative zymography: detection of picogram quantities of gelatinases. *Anal.Biochem.* **218**, 325-329
 - 40 Heukeshoven, J. and Dernick, R. (1985) Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of the staining. *Electrophoresis* **6**, 103
 - 41 Ries, C., Lottspeich, F., Dittmann, K. H., and Petrides, P. E. (1996) HL-60 leukemia cells produce an autocatalytically truncated form of matrix metalloproteinase-9 with impaired sensitivity to inhibition by tissue inhibitors of metalloproteinases. *Leukemia* **10**, 1520-1526
 - 42 Dean, D. D., Martel Pelletier, J., Pelletier, J. P., Howell, D. S., and Woessner, J. F. J. (1989) Evidence for metalloproteinase and metalloproteinase inhibitor imbalance in human osteoarthritic cartilage. *J.Clin.Invest.* **84**, 678-685
 - 43 Eckerskorn, C., Mewes, W., Goretzki, H., and Lottspeich, F. (1988) A new siliconized-glass fiber as support for protein-chemical analysis of electroblotted proteins. *Eur.J.Biochem.* **176**, 509-519
 - 44 Eckerskorn, C. and Lottspeich, F. (2001) Internal amino acid sequence analysis of proteins separated by gel electrophoresis after tryptic digestion in polyacrylamide matrix. *Chromatographia* **28**, 92-94
 - 45 Meister, G., Buhler, D., Lagerbauer, B., Zobawa, M., Lottspeich, F., and Fischer, U. (2000) Characterization of a nuclear 20S complex containing the survival of motor neurons (SMN) protein and a specific subset of spliceosomal Sm proteins. *Hum.Mol.Genet.* **9**, 1977-1986
 - 46 Suzuki, K., Kan, C. C., Hung, W., Gehring, M. R., Brew, K., and Nagase, H. (1998) Expression of human pro-matrix metalloproteinase 3 that lacks the N-terminal 34 residues in *Escherichia coli*: autoactivation and interaction with tissue inhibitor of metalloproteinase 1 (TIMP-1). *Biol.Chem.* **379**, 185-191
 - 47 Huang, W., Meng, Q., Suzuki, K., Nagase, H., and Brew, K. (1997) Mutational study of the amino-terminal domain of human tissue inhibitor of metalloproteinases 1 (TIMP-1) locates an inhibitory region for matrix metalloproteinases. *J.Biol.Chem.* **272**, 22086-22091
 - 48 Olson, M. W., Bernardo, M. M., Pietila, M., Gervasi, D. C., Toth, M., Kotra, L. P., Massova, I., Mobashery, S., and Fridman, R. (2000) Characterization of the monomeric and dimeric forms of latent and active matrix metalloproteinase-9. Differential rates for activation by stromelysin 1. *J.Biol.Chem.* **275**, 2661-2668

- 49 Ogata, Y., Enghild, J. J., and Nagase, H. (1992) Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9. *J.Biol.Chem.* **267**, 3581-3584
- 50 Barrett, A. J. and Starkey, P. M. (1973) The interaction of alpha 2-macroglobulin with proteinases. Characteristics and specificity of the reaction, and a hypothesis concerning its molecular mechanism. *Biochem J* **133**, 709-724
- 51 Basbaum, C. B. and Werb, Z. (1996) Focalized proteolysis: spatial and temporal regulation of extracellular matrix degradation at the cell surface. *Curr.Opin.Cell Biol.* **8**, 731-738
- 52 Nguyen, M., Arkell, J., and Jackson, C. J. (1998) Active and tissue inhibitor of matrix metalloproteinase-free gelatinase B accumulates within human microvascular endothelial vesicles. *J.Biol.Chem.* **273**, 5400-5404
- 53 Mattu, T. S., Royle, L., Langridge, J., Wormald, M. R., Van-den-Steen, P. E., Van-Damme, J., Opdenakker, G., Harvey, D. J., Dwek, R. A., and Rudd, P. M. (2000) O-glycan analysis of natural human neutrophil gelatinase B using a combination of normal phase-HPLC and online tandem mass spectrometry: implications for the domain organization of the enzyme. *Biochemistry* **39**, 15695-15704
- 54 Rudd, P. M., Mattu, T. S., Masure, S., Bratt, T., Van den Steen, P. E., Wormald, M. R., Kuster, B., Harvey, D. J., Borregaard, N., Van-Damme, J., Dwek, R. A., and Opdenakker, G. (1999) Glycosylation of natural human neutrophil gelatinase B and neutrophil gelatinase B-associated lipocalin. *Biochemistry* **38**, 13937-13950
- 55 Van den Steen, P. E., Van, A., I, Hvidberg, V., Piccard, H., Fiten, P., Jacobsen, C., Moestrup, S. K., Fry, S., Royle, L., Wormald, M. R., Wallis, R., Rudd, P. M., Dwek, R. A., and Opdenakker, G. (2006) The hemopexin and O-glycosylated domains tune gelatinase B/MMP-9 bioavailability via inhibition and binding to cargo receptors. *J.Biol.Chem.* **281**, 18626-18637
- 56 Wu, Y. I., Munshi, H. G., Sen, R., Snipas, S. J., Salvesen, G. S., Fridman, R., and Stack, M. S. (2004) Glycosylation broadens the substrate profile of membrane type 1 matrix metalloproteinase. *J.Biol.Chem.* **279**, 8278-8289
- 57 Fry, S. A., Steen, P. E., Royle, L., Wormald, M. R., Leathem, A. J., Opdenakker, G., McDonnell, J. M., Dwek, R. A., and Rudd, P. M. (2006) Cancer-Associated Glycoforms of Gelatinase B Exhibit a Decreased Level of Binding to Galectin-3. *Biochemistry* **45**, 15249-15258
- 58 Olson, M. W., Gervasi, D. C., Mobashery, S., and Fridman, R. (1997) Kinetic analysis of the binding of human matrix metalloproteinase-2 and -9 to tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2. *J.Biol.Chem.* **272**, 29975-29983
- 59 Galardy, R. E., Grobelny, D., Foellmer, H. G., and Fernandez, L. A. (1994) Inhibition of angiogenesis by the matrix metalloprotease inhibitor N-[2R-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]- L-tryptophan methylamide. *Cancer Res.* **54**, 4715-4718

Table 1. Sequencing and MALDI-TOF mass spectroscopy analysis of purified MMP-9 forms.

THP-1 cells (4.6×10^9) were stimulated with 50 ng/ml of PMA for 24 hours. Cell-associated 82- and 94-kDa gelatinases were isolated from cell extracts (EX) and secreted 94-kDa gelatinase from conditioned medium (CM) by gelatin Sepharose affinity chromatography. The samples were then subjected to SDS-PAGE under reducing conditions. For N-terminal microsequence analysis, proteins were blotted and processed using a gas phase sequencer. The N-terminal amino acid sequences obtained were compared with that of proMMP-9 [21]. For mass spectroscopy analysis, proteins were excised from the SDS-gel, digested with trypsin and analyzed using a MALDI-TOF mass spectrometer. The molecular masses and the corresponding amino acid sequences of the N-terminal peptide fragment generated by trypsin digestion of purified MMP-9 forms are shown and compared to that of proMMP-9 [21]. *n.d.*, not detected.

Gelatinase	N-terminal amino acid sequence	N-terminal peptide fragment after trypsin digestion		
		Molecular mass [Da]		Amino acid sequence
		Determined	Calculated	
proMMP-9	1 10 20 A P R Q R Q S T L V L F P G D L R T N L –		1,345.7	6 17 Q S T L V L F P G D L R
82-kDa (EX)	V L F P G D –	916.5	916.5	V L F P G D L R
94-kDa (EX)	<i>n.d.</i>	1,345.7	1,345.7	Q S T L V L F P G D L R
94-kDa (CM)	A P R Q R Q S T L V L –	1,345.7	1,345.7	Q S T L V L F P G D L R

FIGURE LEGENDS

Figure 1. Detection of secreted and cell-associated MMP-9. THP-1 leukemic 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)** 3- μ l aliquots of THP-1 conditioned medium (CM) and 10- μ l aliquots of cell extract (EX) were analyzed by zymography. **(B)** For immunoblot detection, 15- μ l aliquots of gelatinases isolated from conditioned media (CM) and 30- μ l aliquots of gelatinases from cell extracts (EX) 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 weight standards (M) were used as a reference.

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 488-conjugated anti-mouse IgGs. 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. **(C)** Immunoblot detection of plasma membrane-localized calcium dependent-ATPase (PMCA) 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, subsequently immunoprecipitated using monoclonal antibodies against MMP-9, subjected to SDS-PAGE and then blotted. For detection of biotinylated proteins, blots were developed with streptavidin-POD. Recombinant marker proteins (M) were used for molecular mass determination

Figure 3. Purification of cell-associated and secreted MMP-9. Cell extracts and conditioned medium of PMA stimulated THP-1 cells were subjected to gelatin Sepharose affinity chromatography followed by gel filtration chromatography. **(A)** Zymogram showing a representative analysis of fractions obtained by gel filtration separation of gelatin Sepharose enriched material from cell extracts (G-Seph Pool). Fractions 9-13 containing 94-kDa

gelatinase (94k) and fractions 17-22 containing 82-kDa gelatinase (82k) were pooled and concentrated. **(B,C)** Purified 82-kDa MMP-9 from cell extracts (82k) and 94-kDa MMP-9 from conditioned medium (94k) were analyzed by zymography (200 pg per lane) **(B)** and SDS-PAGE with subsequent silver staining (120 ng per lane) **(C)**. HT1080 conditioned medium containing MMP-9 (94 kDa), MMP-2 (72 kDa) and activated MMP-2 (66 kDa) was used as a reference (M) in zymograms. Recombinant marker proteins (M) were applied for molecular mass determination in SDS-gels.

Figure 4. Glycosylation studies. Aliquots of purified secreted 94-kDa proMMP-9 (0.25 $\mu\text{g/ml}$) and cell-associated 82-kDa proMMP-9 (1 $\mu\text{g/ml}$) 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 electrophoretical mobility were monitored by 4-12% SDS-PAGE 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 weight assessment.

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 analyzed by zymography and immunoblotting using anti-MMP-9 polyclonal antibodies. **(C)** Enriched THP-1 plasma membranes containing 94-kDa proMMP-9 (pro-94k) and 82-kDa proMMP-9 (pro-82k) were incubated with MMP-3. Aliquots were taken at different time intervals and analyzed by zymography. **(D)** For examination of proteolytic activity purified 94-kDa proMMP-9 and 82-kDa proMMP-9 were treated with or without MMP-3 (30 min/37°C), then incubated with an excess of α_2 -macroglobulin (500 $\mu\text{g/ml}$) 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 ($\alpha_2\text{M}$) which binds and inhibits only active endopeptidases.

Figure 6. Autocatalytic activation of 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 analyzed by zymography (A) and Western blotting using polyclonal anti-MMP-9 antibodies (B).

Figure 7. Degradation of natural substrates. Collagen type IV (native or denatured) (400 µg/ml), collagen type I (denatured) (300 µg/ml) and fibronectin (FN) (100 µg/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 analyzed for degradation products (DP) by SDS-PAGE and silver staining of the proteins. Collagen type VI chains $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$. Collagen type I chains $\alpha 1(\text{I})$ and $\alpha 2(\text{I})$.

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-labeled gelatin as a substrate and expressed in percentage of activity without inhibitor. Results are given as mean of duplicate experiments representative for three independent determinations.

Figure 9. Specific detection of 82-kDa proMMP-9 in leukemic and non-leukemic cells. (A) The zymogen forms (Pro) and activated species (Act.) of 94-kDa proMMP-9 (94k) and 82-kDa proMMP-9 (82k) were analyzed 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 anti-proMMP-9 prodomain antibodies. Results are shown for the leukemic cell lines THP-1, HL-60 and NB4, for mononuclear cells (MNC) and neutrophil granulocytes (PMNC) from peripheral blood of healthy persons, and for leukemic blast cells from peripheral blood of patients with acute myeloid leukemia (Pat.#1-3).

Figure 1

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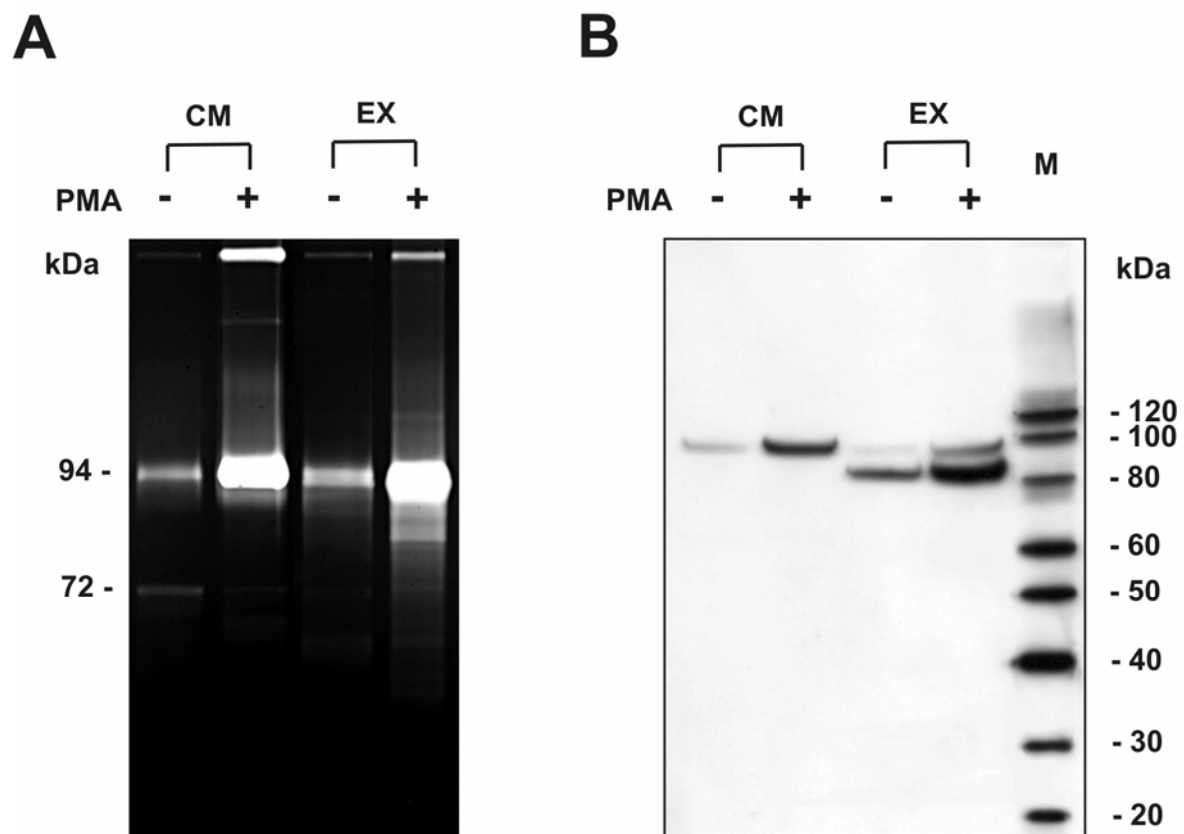
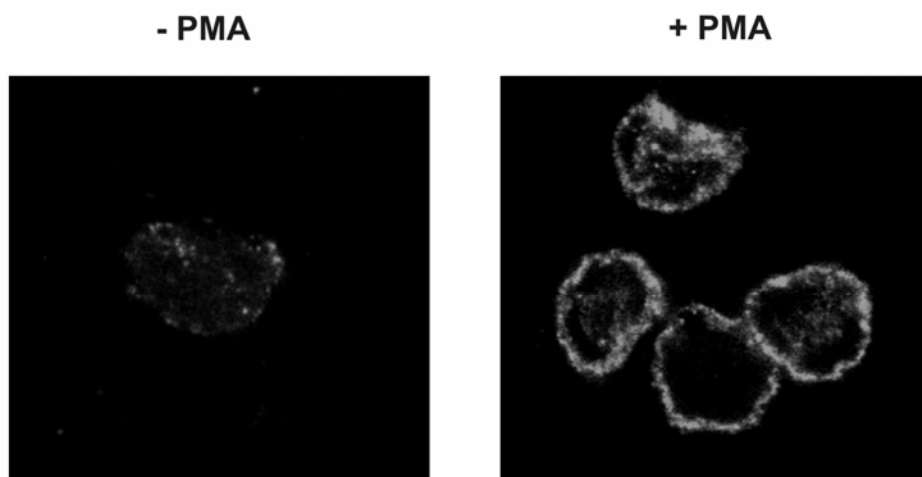


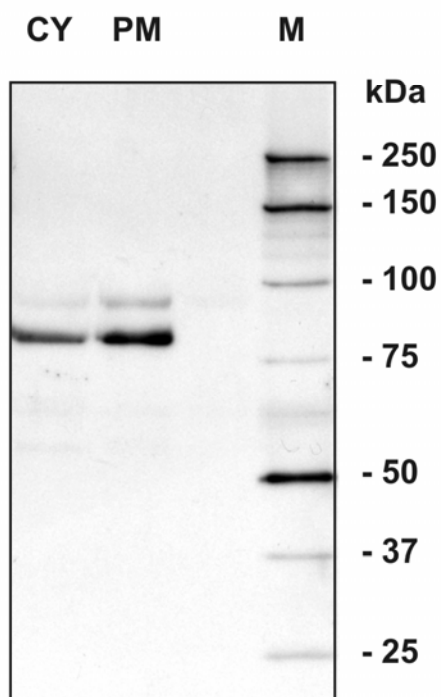
Figure 2

Ries et al.

A



B



C

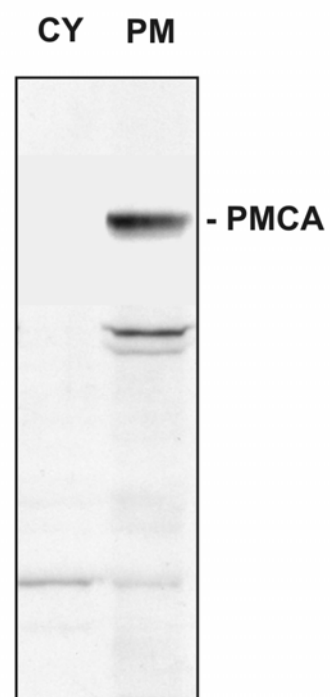


Figure 2

Ries et al.

D

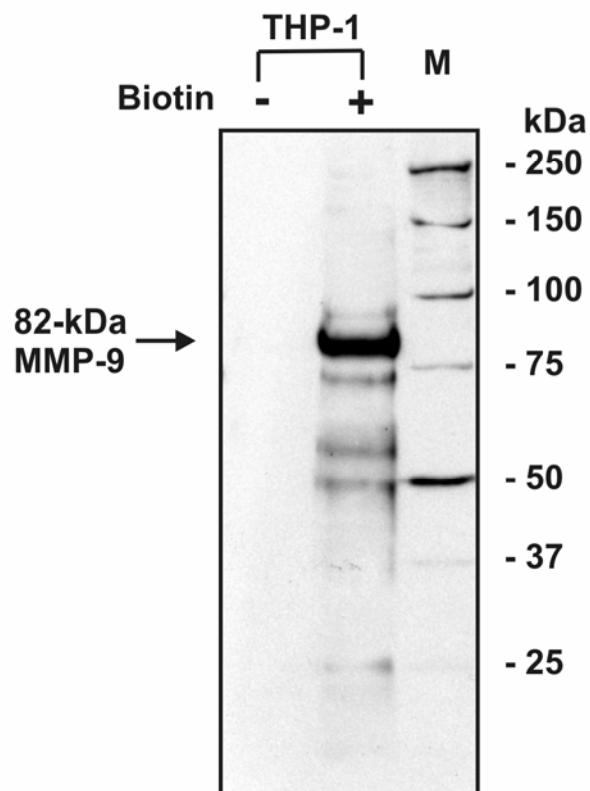
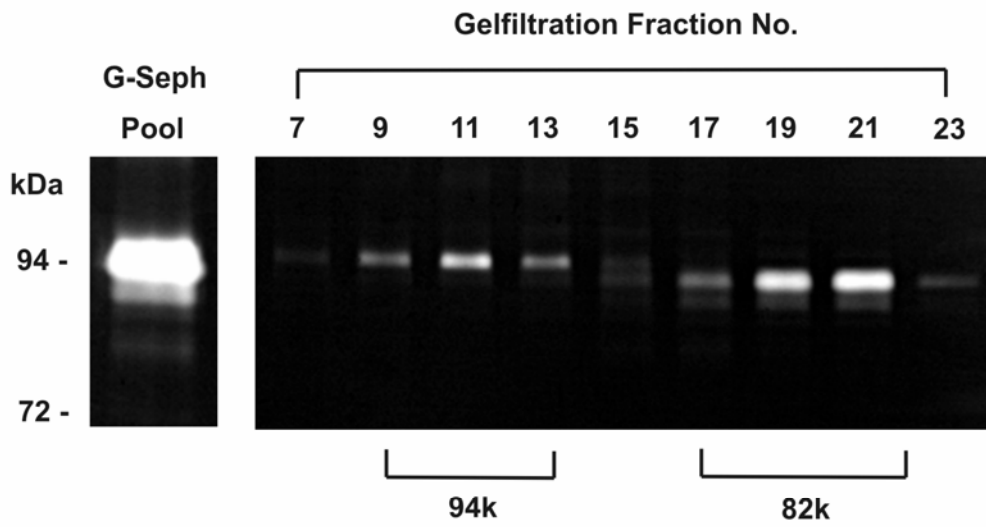


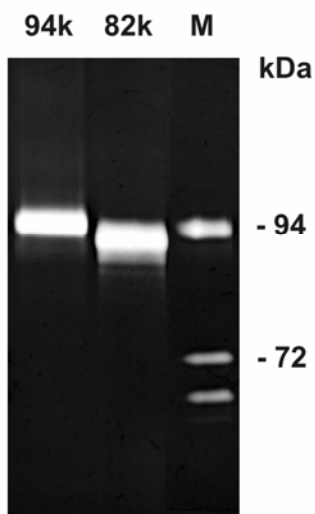
Figure 3

Ries et al.

A



B



C

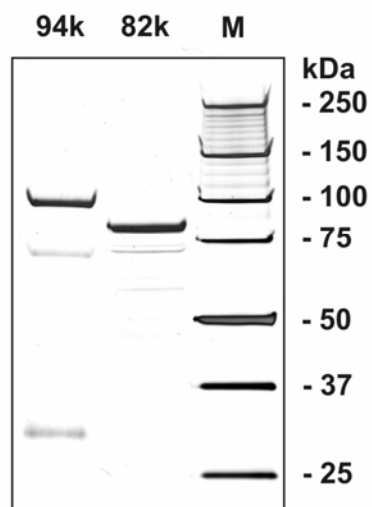


Figure 4

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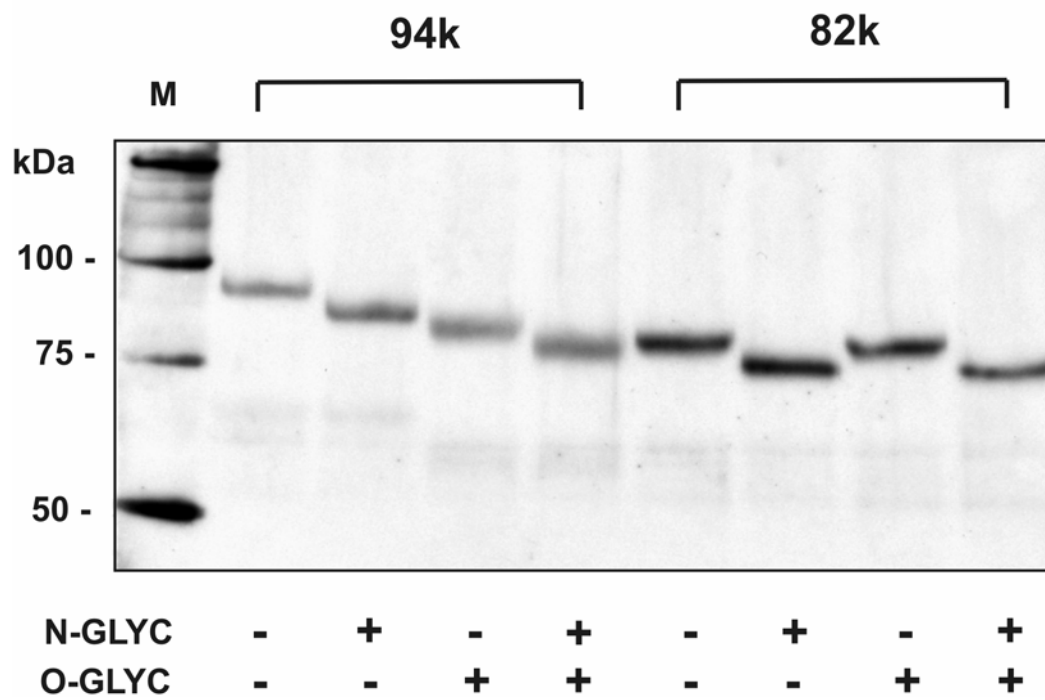


Figure 5

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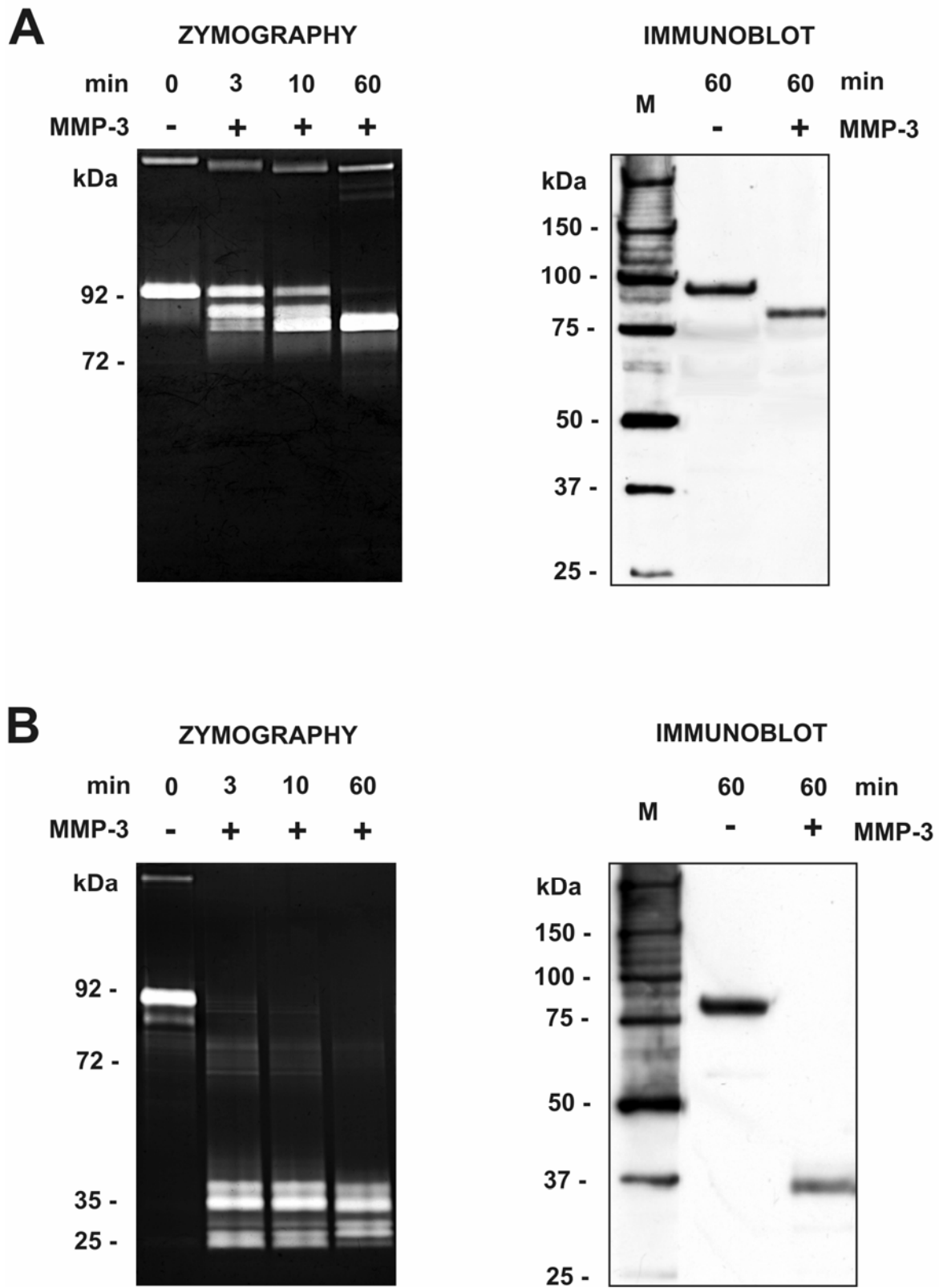
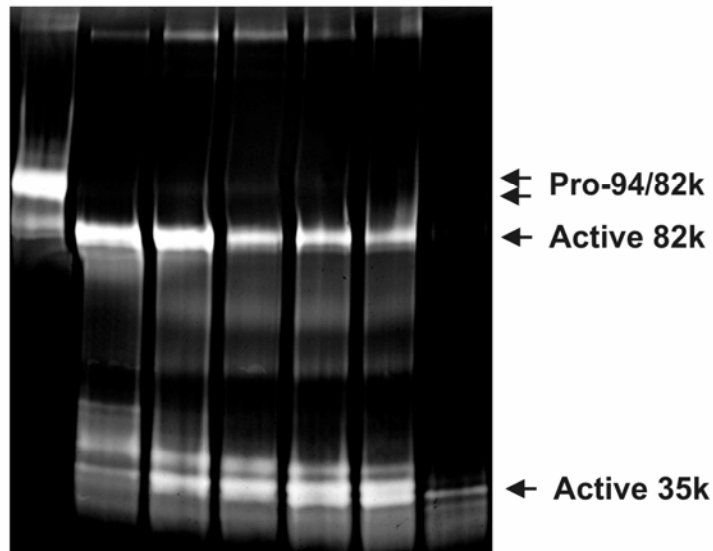


Figure 5

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C

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D

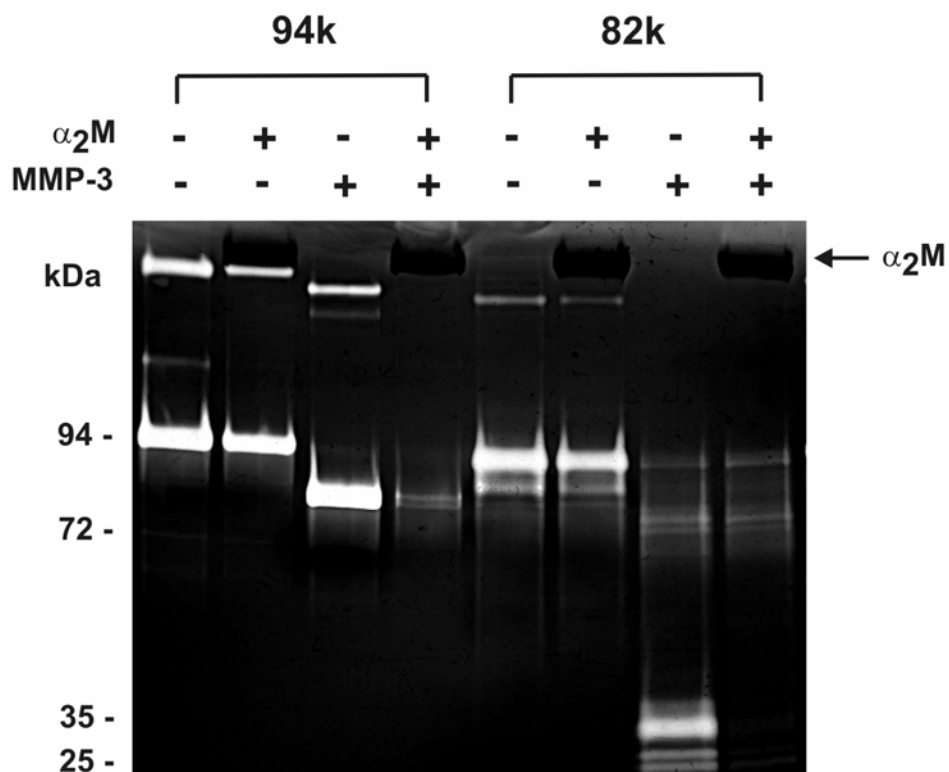
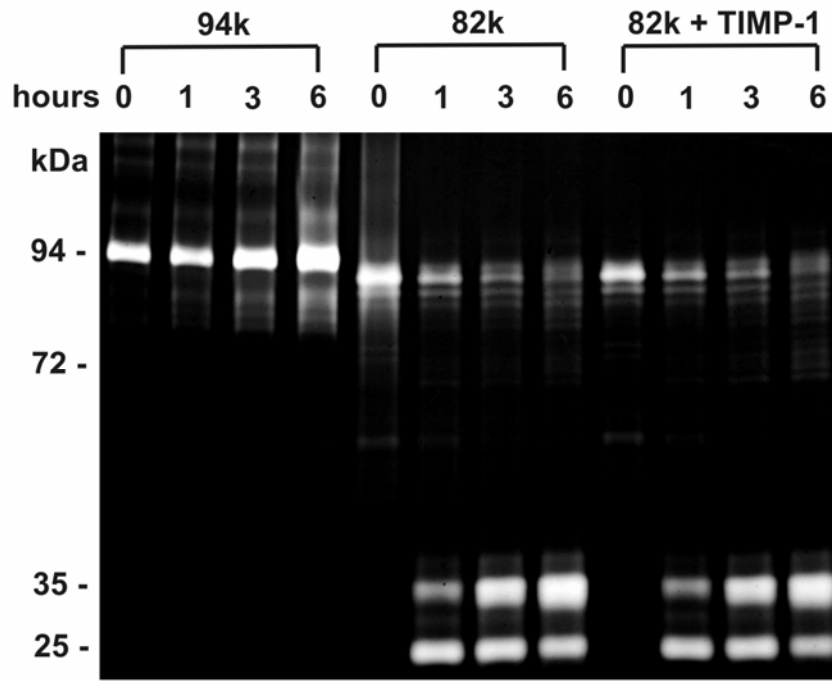


Figure 6

Ries et al.

A



B

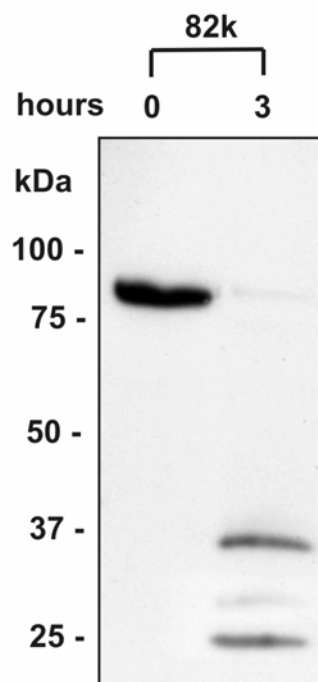


Figure 7

Ries et al.

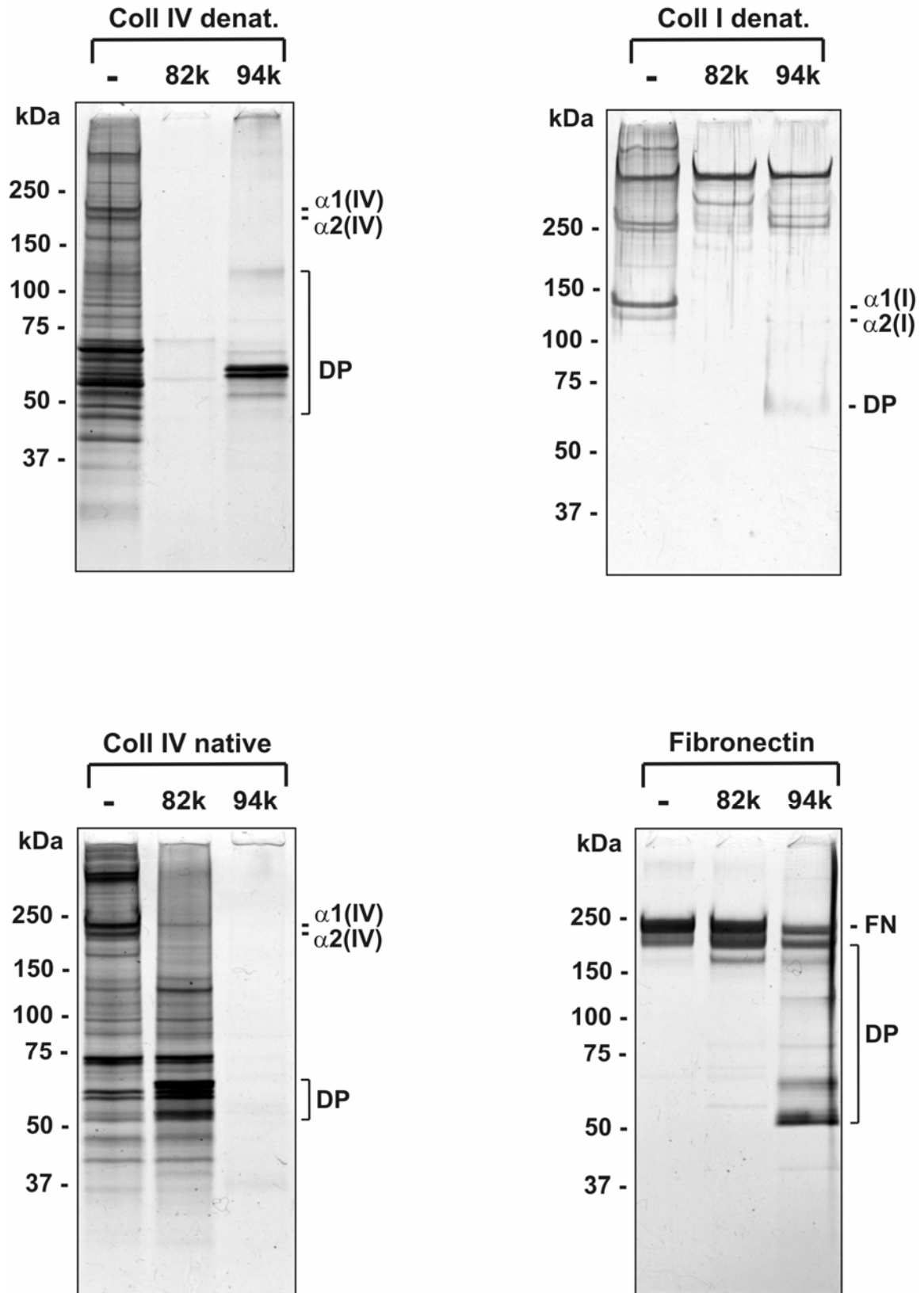
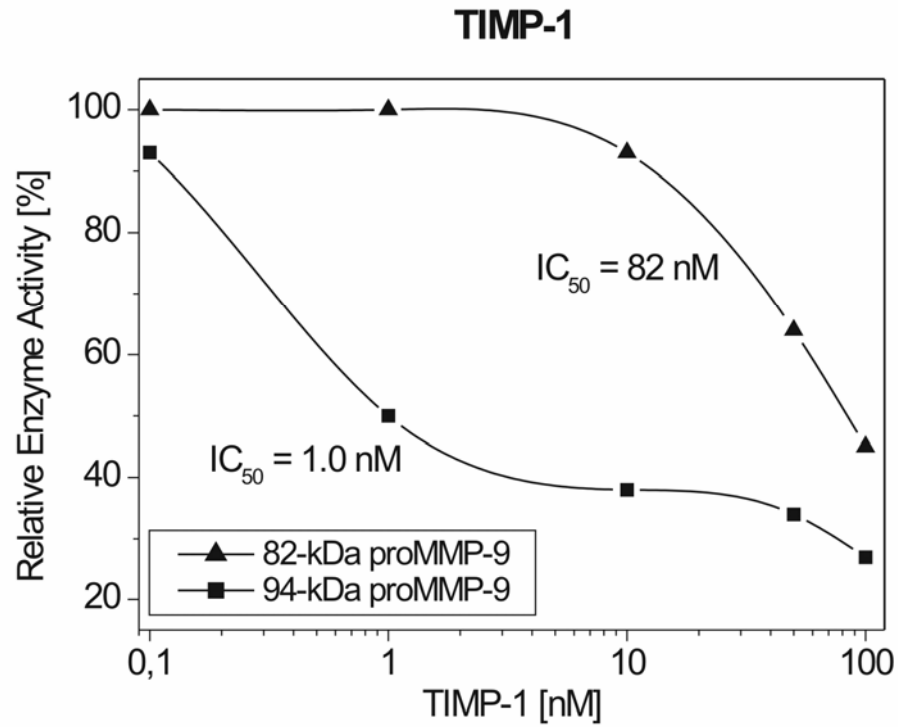


Figure 8

Ries et al.

A



B

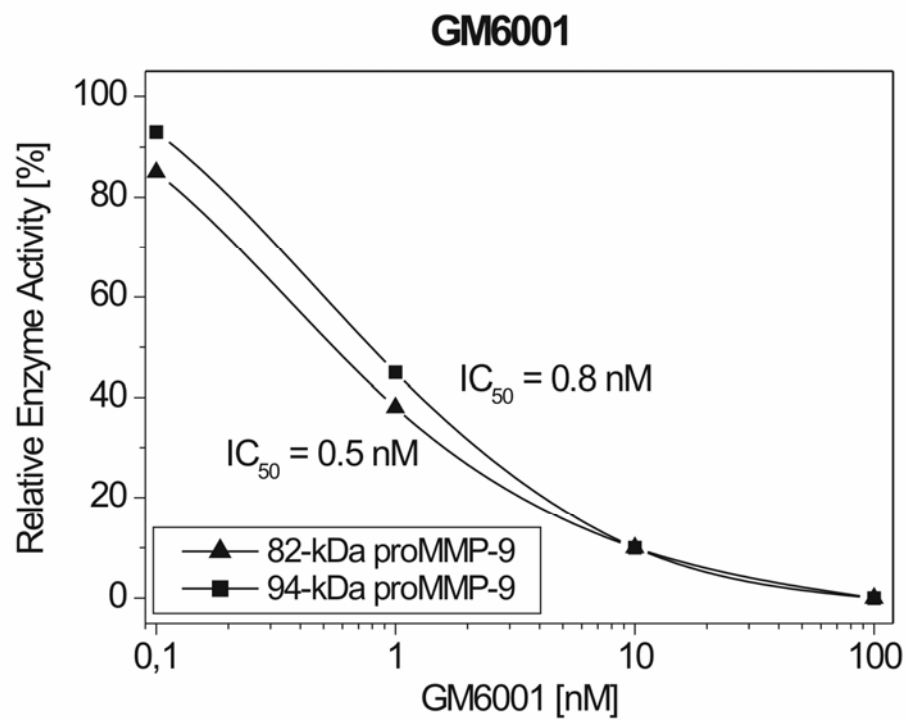


Figure 9

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