Propeptide glycosylation and galectin-3 binding decrease proteolytic activation of human proMMP-9/progelatinase B

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Keywords
galectin-3; matrix metalloproteinase-9; N-linked glycosylation; propeptide; proteolytic activation

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(Received 5 July 2018, revised 21 September 2018, accepted 9 November 2018)

doi:10.1111/febs.14698

Matrix metalloproteinases (MMPs) are secreted as proenzymes, containing propeptides that interact with the catalytic zinc, thereby controlling MMP activation. The MMP-9 propeptide is unique in the MMP family because of its post-translational modification with an N-linked oligosaccharide. ProMMP-9 activation by MMP-3 occurs stepwise by cleavage of the propeptide in an aminoterminal (pro-AT) and carboxyterminal (pro-CT) peptide. We chemically synthesized aglycosyl pro-AT and pro-CT and purified recombinant glycosylated pro-AT\textsuperscript{Sf-9}. First, we report new cleavage sites in the MMP-9 propeptide by MMP-3 and neutrophil elastase. Additionally, we demonstrated with the use of western blot analysis a higher resistance of glycosylated versus aglycosyl pro-AT against proteolysis by MMP-3, MMP-9, meprin \(\alpha\), neutrophil elastase and by protease-rich synovial fluids from rheumatoid arthritis patients. Moreover, we investigated the effect of glycosylation on proteolytic activation of human proMMP-9 with the use of zymography and dye-quenched gelatin cleavage analysis. Compared to recombinant Sf-9 proMMP-9 glycoforms, larger oligosaccharides of human neutrophil proMMP-9 increased resistance against proteolytic activation. Additionally, proMMP-9 from Congenital Disorder of Glycosylation patients, compared to healthy controls, showed a higher activation rate by MMP-3. Finally, we demonstrated that glycan-galectin-3 interactions reduced proMMP-9 activation. In conclusion, modification of MMP-9 propeptide glycosylation is a fine-tuning mechanism and co-determines the specific activity of MMP-9 in physiology and pathology.

Enzymes
MMP-9 EC 3.4.24.35, MMP-3 EC 3.4.24.17, meprin \(\alpha\) EC 3.4.24.18, neutrophil elastase EC 3.4.21.37, trypsin EC 3.4.21.4 and PNGase F EC 3.5.1.52.

Abbreviations
actMMP-9, activated form of MMP-9; CDG, congenital disorder of glycosylation; cdMMP-3, catalytic domain of MMP-3; DQ-gelatin, Dye-Quenched gelatin; MMP, matrix metalloproteinase; NE, neutrophil elastase; PMM2, phosphomannomutase 2; PNGase F, protein N-glycosidase F; pro-AT\textsuperscript{Sf-9}, recombinant Sf-9-derived aminoterminal peptide of MMP-9 propeptide; pro-AT\textsuperscript{Synthetic}, synthetic aminoterminal peptide of MMP-9 propeptide; pro-CT, synthetic carboxyterminal peptide of MMP-9 propeptide; TIMP-1, tissue inhibitor of metalloproteinases-1; tKK, tissue kallikrein; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.
Introduction

ProMMP-9 is presently one of the most studied enzymes in biology and medicine [1]. It plays a fundamental role in physiological processes, such as reproduction [2,3], tissue reorganization [4] and angiogenesis [5,6]. In addition, it is viewed as a marker and possible target in pathological conditions, including cancer [7,8], acute and chronic inflammation and vascular diseases [9,10]. Detection methods with sensitivities in the picogram range [11] and probes for in situ demonstration of gelatinase activity have been developed for preclinical mouse models and clinical applications in humans [12,13].

Molecular modelling studies based on sedimentation [14] and small-angle X-ray scattering data [15] have led to a reasonable understanding of the structure of proMMP-9 and insights about the functions of its structural domains. For instance, the hemopexin domain provides the molecule with various non-catalytic activities [16,17], whereas the fibronectin repeats enhance gelatin affinity [18]. The propeptide is the least studied domain of proMMP-9 in terms of its modifications and functions. The human propeptide consists of 87 amino acids and is glycosylated at Asn38 (all amino acid numberings in this report include the signal peptide) [1,14]. The propeptide keeps proMMP-9 in a latent state by coordinating the catalytic zinc ion with a sulfhydryl group, the so-called cysteine switch mechanism [19]. Several proenzymes in biology and medicine [1].

As a new approach of MMP-9 inhibition with possible therapeutic applications, zymogen activation can be blocked with a compound binding in proximity to this secondary cleavage site [36].

A recent molecular dynamics simulation study based on the crystal structure of an N-terminal proMMP-9 segment by Elkins and colleagues [37] suggests that the glycosylation of the propeptide may regulate this order of stepwise cleavage [38]. However, molecular biology data do not yet exist.

Glycans attached to proMMP-9 generate several glycoforms, increasing structural complexity and generating cell- and tissue-specificities. Recombinant expression in Sf-9 insect cells yields simple oligomannose structures on proMMP-9 [14], while glycans on human neutrophil proMMP-9 are complex [39]. Cancer cells produce proMMP-9 glycoforms that differ from those of normal cells, suggesting a potential role for specific glycosylation of proMMP-9 in cancer. For example, proMMP-9 from MCF-7 breast cancer cells or THP-1 myeloid leukaemia cells contains glycans that show significantly reduced binding to galectin-3, compared to those from neutrophil proMMP-9 [40]. Glycoforms of human neutrophil, MCF-7, THP-1 and recombinant (Sf-9) proMMP-9 are compared in Fig. 1. In general, insights into the biological roles of sugars attached to MMPs are limited [41]. One study refers to the role of the propeptide glycan in secretion of proMMP-9 [42]. However, oligosaccharides attached to glycoproteins also protect against proteolysis, as has been demonstrated for ribonuclease B [43], CD59 [44] and suggested for β-interferon [45].

The role of glycosylation stretches further than the post-translational modification itself. Glycans also create an interface for molecular interactions with lectins, including galectins. Galectins are a protein family with binding specificity for β-galactoside sugars (such as GalβGlcNAc), which can be found on both N-linked and O-linked glycans [46]. Unlike the majority of lectins, galectins are not membrane-bound, but soluble proteins with both intra- and extracellular functions. Galectin-3 is unique among other galectins because of its chimeric structure, having a carbohydrate recognition domain and a long non-lectin domain, through which it can associate into a pentameric form [47]. We discovered that both the glycosylation of the propeptide and the galectin-3 binding to proMMP-9 inhibit the processing of the propeptide, thereby fine-tuning proMMP-9 activation.

Results

Production and detection of the MMP-9 propeptide

Pro-AT and pro-CT, as cleaved from proMMP-9 by MMP-3 during the activation process [25], were
chemically synthesized or produced from natural sources. The synthetic peptides were used to immunize rabbits for the production of polyclonal antibodies for detection by western blot analysis (Fig. 2A,B).

To investigate the function of the oligosaccharide attached to Asn38, glycosylated pro-AT was purified from recombinant proMMP-9, produced in Sf-9 insect cells, after incubation with the catalytic domain of MMP-3 (cdMMP-3) and further referred to as pro-AT\textsuperscript{Sf-9} (Fig. 2C,D). As demonstrated with the use of protein staining, the glycosylated pro-AT\textsuperscript{Sf-9} displays slower electrophoretic mobility, compared to the synthetic aglycosyl pro-AT (Fig. 2D), further referred to as pro-AT\textsupersynth. Treatment of pro-AT\textsuperscript{Sf-9} with protein N-glycosidase F (PNGase F) to remove N-linked sugars from proteins, resulted in a similar electrophoretic mobility as pro-AT\textsupersynth, confirming N-linked glycosylation as a post-translational modification (Fig. 2E). Several types of validation experiments were performed to exclude cross-reactivity of the generated antibodies. First, the reactivities of pro-AT and pro-CT were assessed by ELISAs (Fig. 3A) and no cross-reactivity was observed. Secondly, we used western blot analysis to exclude cross-reactivity between pro-AT and pro-CT (Fig. 3B) and to prove that the polyclonal antiserum against pro-AT recognized both the synthetic pro-AT\textsupersynth and the recombinant human propeptide (pro-AT\textsuperscript{Sf-9}) with similar sensitivities (Fig. 3C,D).

### Cleavage of the MMP-9 propeptide by MMP-3 and NE

Stepwise cleavage of proMMP-9 behind Glu\textsubscript{59} and Arg\textsubscript{106} in the propeptide by MMP-3 is a well-described...
We investigated the cleavage of the synthetic propeptide parts by cdMMP-3 and discovered two additional cleavage sites, one in pro-AT and one in pro-CT (Fig. 4A). The exact cleavage sites in the propeptide by cdMMP-3 were determined by N-terminal Edman sequencing to be located behind Asp34 and Leu71 in pro-AT and pro-CT, respectively. So far, the only two described cleavage sites in the MMP-9 propeptide by NE are behind Val57 and Ala58 [27]. However, we discovered additional cleavage sites, more specifically behind Val29 in pro-AT and at several sites in pro-CT (Fig. 4B). All

**Fig. 2.** Detection of pro-AT and pro-CT and purification of pro-AT\[^{St-9}\] from human MMP-9. (A) Synthetic MMP-9 pro-AT was used as antigen to produce rabbit polyclonal antibodies. UV Absorption (in mAU) for rabbit serum. (a) After immunization with pro-AT, the serum was diluted, filtered and loaded on a recombinant protein A-Sepharose fast flow column (GE Healthcare). Rabbit IgGs were eluted in 0.1 M Glycine pH 2.8 as indicated (vertical arrow). (b) Affinity purification of antibodies on pro-AT-coupled NHS Sepharose (2 mg peptide/mL Sepharose, GE Healthcare) of pooled rabbit IgG fractions (from graph a). (c) Immunoblot detection of pro-AT with the affinity-purified polyclonal antisera. (B) Similar experimental set-up for detection with affinity-purified polyclonal antisera of pro-CT. (C) Purification of pro-AT from recombinant human Stf-9 proMMP-9, further referred to as pro-AT\[^{St-9}\], after incubation with the cdMMP-3 (E/S = 1/100). Pro-AT\[^{St-9}\] was separated from the activated form of MMP-9 by performing gel filtration chromatography in 6 M ureum. UV Absorption (in mAU) is shown for each fraction of 0.5 mL. (D) Coomassie blue staining of proteins in fractions 13 (F13), F24 and F25 separated in a 16% SDS-polyacrylamide gel. F13 and F25 contain, respectively, actMMP-9 and the glycosylated pro-AT\[^{St-9}\] and are shown next to synthetic pro-AT and pro-CT. (E) Detection of the pro-AT by immunoblotting after incubation with the cdMMP-3 at time 0 (lane 1) and after 3 h (lanes 2–3) incubated at 37 °C, with additional PNGase F treatment for 1 h at 37 °C (lane 3).
known and newly discovered cleavage sites in the MMP-9 propeptide by MMP-9 and NE are indicated in Fig. 4C.

**Glycosylation of pro-AT reduces cleavage by proMMP-9-activating enzymes**

Known and newly discovered cleavage sites in pro-AT are in close proximity to the glycosylated asparagine. Therefore, we tested the hypothesis that glycosylation might affect propeptide cleavage. Comparing the cleavage of pro-ATsynth (lacking N-linked glycosylation), with the cleavage of pro-ATSf–9 (with oligosaccharide) by cdMMP-3, we showed a significantly faster decrease in intact peptide levels of pro-ATsynth (Fig. 5A). This higher cleavage velocity of the synthetic versus the recombinant pro-AT by cdMMP-3 was confirmed with different substrate concentrations (Fig. 5B,C). These results indicated that glycosylation of the propeptide reduces proteolysis by cdMMP-3. Additionally, cleavage of pro-ATsynth by active MMP-9 and meprin α was significantly faster than cleavage of pro-ATSf–9, confirming our hypothesis that glycosylation of the propeptide decreases proteolysis kinetics (Fig. 6A,B, respectively). Furthermore, while NE cleaves close to the glycosylation site, trypsin cleavage is at a more distant position. In line with our expectations, cleavage of pro-ATf–9 by NE was slower, compared to cleavage of non-glycosylated pro-ATsynth (Fig. 6C), whereas no difference was observed after cleavage by trypsin (Fig. 6D).

Next, we evaluated if glycosylation also plays a role in MMP-9 propeptide cleavage by complex biological fluids, which contain many proteases additionally to those tested *in vitro*. The non-glycosylated pro-ATsynth and the glycosylated pro-ATf–9 (Fig. 6E) were incubated with serum from healthy control subjects. Upon immunoblotting, almost no cleavage of the peptides was detected. However, incubation of the pro-AT peptides with synovial fluids of patients with rheumatoid arthritis showed a trend towards faster degradation of pro-ATsynth compared to pro-ATf–9, with a significant difference after 24 h (Fig. 6F). In conclusion,
glycosylation of the propeptide decreases proteolysis kinetics of the propeptide.

**Neutrophil proMMP-9 is more resistant against stepwise activation by MMP-3 than recombinant proMMP-9**

To test if glycosylation of proMMP-9 affects its activation in human, we used zymography (picogram sensitivity [11]) and DQ-gelatin degradation assays, since these are more sensitive methods and require less material than western blot analysis (nanogram sensitivity). We compared the stepwise activation of equimolar amounts of intact human recombinant Sf-9 and neutrophil proMMP-9 by MMP-3 with the use of gelatin zymography analysis. This was done by inclusion of several protein forms of recombinant MMP-9 and by standardization to quantify specific MMP-9 forms: proMMP-9 (Ala\textsubscript{20} form, arrow 1), intermediate MMP-9 (Met\textsubscript{60} form, arrow 2) and the activated MMP-9 form (Phe\textsubscript{107} form, arrow 3) (Fig. 7A–C). ProMMP-9 from neutrophils was more resistant to stepwise processing by cdMMP-3 compared to recombinant proMMP-9. The decrease of proMMP-9 levels (Fig. 7D), in combination with the increase of the activated form of MMP-9 (actMMP-9, Fig. 7E) indicated that activation levels of neutrophil proMMP-9 were lower compared to those of recombinant Sf-9 proMMP-9 with a smaller sized N-glycosylation. Together, these data suggested that higher sugar complexity interfered more with the activation of proMMP-9 by MMP-3 in vitro.

**ProMMP-9 from CDG patients versus healthy controls has a higher activation rate**

Genetic defects in glycosylation have been first described in 1980 [48]. The most common disease is phosphomannomutase 2 deficiency, also named PMM2-Congenital Disorder of Glycosylation (CDG) [49]. We detected more efficient conversion of proMMP-9 into its activated form by MMP-3 in PMM2-CDG patients (n = 5) compared to healthy controls (n = 7), as analysed with zymography (Fig. 8A). Quantification of the percentage of proMMP-9 (Fig. 8B) and its activated form (Fig. 8C) showed a significantly faster activation of proMMP-9 from CDG patients compared to controls.

**Galectin-3 interaction with proMMP-9 inhibits its activation by MMP-3**

An important function of protein glycosylation is to mediate glycan–lectin interactions. Such interactions may be relevant in cancer biology [40]. We evaluated the role of galectin-3, a galactoside-binding lectin, on the activation of human neutrophil proMMP-9 by MMP-3. Interestingly, we discovered that stepwise activation of neutrophil proMMP-9 by MMP-3 into...
its activated form is reduced by co-incubation with galectin-3 (Fig. 9A). Consequently, gelatinolytic activity of MMP-9 after incubation with MMP-3, quantified by the velocity of Dye-Quenched (DQ) gelatin cleavage, was also significantly reduced (Fig. 9C). Overnight incubation of neutrophil proMMP-9 with galectin-3 (1/10) prevented activation by MMP-3, while recombinant proMMP-9 was fully converted into its active form (Fig. 9B). Interestingly, a galectin-3 mutant, defective in the formation of pentameric structures, lacking the non-lectin domain failed to inhibit proMMP-9 activation by MMP-3 (Fig. 9D). Although addition of homodimeric galectin-1 slightly reduced MMP-3-induced activation of proMMP-9 (Fig. 9E), this effect was much less compared to inhibition by galectin-3 and was not statistically significant.

**Discussion**

The two-step proteolytic activation of proMMP-9 by MMP-3 is known since long [25], but the role of propeptide glycosylation in this process remained enigmatic. Here, we investigated the role of MMP-9 propeptide glycosylation. First, we report new cleavage sites in the MMP-9 propeptide by MMP-3 and NE. All known and newly discovered cleavage sites in the MMP-9 propeptide are indicated in Fig. 10A. Additionally, we discovered a role of the propeptide glycosylation in decreasing its proteolytic cleavage by MMP-3, MMP-9, meprin α, NE and by proteases in synovial fluids of rheumatoid arthritis patients. Interestingly, larger glycan structures, such as those occurring in neutrophil proMMP-9, increased the resistance against proteolysis,
compared to smaller oligosaccharides of recombinant Sf-9 proMMP-9 and proMMP-9 from CDG patients. Besides this direct effect on altering proteolysis kinetics, glycosylation of proMMP-9 decreased the activation rate and enzyme activity through binding with galectin-3 in an indirect way.

A three-dimensional model of full-length proMMP-9 with the N-linked sugar from Sf-9 cells and the more complex N-linked sugar from neutrophils on the propeptide illustrates the importance of investigating the role of sugars, given their relative sizes in comparison with protein domains (Fig. 10B). Our data document a significant role of glycosylation in protection of the MMP-9 propeptide against proteolysis. This finding is reminiscent of the protective function of oligosaccharides against proteases, as was originally described by Rudd and co-workers for pancreatic ribonuclease B as a protease substrate [43, 50].

However, these five amino acids were unlikely to induce any difference in proteolysis of the peptide since the cleavage by trypsin, which cleaves in the closest proximity to Arg₁, is similar for pro-ATsynth and pro-ATSf⁻⁹. Furthermore, in the comparison of recombinant and neutrophil proMMP-9 cleavage, this was not an issue, since we observed more resistance against cleavage of neutrophil proMMP-9, lacking these amino acids.

Glycans of the O-glycosylated domain of proMMP-9 have been suggested to be important for lectin binding, enzyme elongation [14], domain organization [51] and interdomain flexibility of proMMP-9 [52]. Terminal sialylation of sugars is essential for the interaction of MMP-9 with its inhibitor TIMP-1 [53] and, as recently shown, with staphylococcal superantigen-like protein 5 [54]. Out of three potential N-linked glycosylation sites in proMMP-9, only Asn₃₈ in the propeptide and Asn₁₂₀ in the active site are glycosylated [14]. Interestingly, while the N-linked glycosylation of the active site of MMP-9 is also present in collagenases MMP-8, -13, -17 and MMP-23, the N-linked glycan in the propeptide is unique for MMP-9 [41]. By site-directed mutagenesis, a role of both N-glycosylation sites for the secretion of
proMMP-9 has been proven. Both Asn$_{38}$ and Asn$_{120}$ glycosylation-deficient mutants showed retention in the endoplasmic reticulum (ER), through a strong N-glycosylation-independent interaction with ER-resident calreticulin [42]. Additionally, Duellman et al. showed a loss of proMMP-9 expression due to the degradation of N-glycosylation-deficient proMMP-9 by the proteasome [55], suggesting protection by N-glycosylation against intracellular proteolysis. Recently, a molecular dynamics simulation study suggested that glycosylation might induce a conformation of the propeptide that facilitates the temporal order of the first and second cleavage events [38]. The authors speculated that glycosylation does not hinder the activation process directly but that glycosylation of proMMP-9 decreases the flexibility of the protein backbone, having a stabilizing effect, as described previously [50]. However, we demonstrated decreased proteolysis due to glycosylation.

While investigating galectin-8 interactions, Nishi et al. [56] showed increased proMMP-9 activation by MMP-3 when adding galectin-8. This effect was not observed when adding galectin-1 or -3 [56], which is in agreement with our results proving an inhibitory effect of galectin-3 on proMMP-9 activation. Interestingly, galectin-3 is efficiently cleaved by MMP-9 and MMP-2 in the non-lectin domain [57], which is necessary for multimerization. Our results indicate that a multimerization-deficient mutant of galectin-3 is less capable of inhibiting proMMP-9 activation, suggesting a loop, that once MMP-9 is active, it promotes activation of other proMMP-9 molecules through galectin-3 cleavage.

MMP-9 plays a role in many organ systems, including the endocrine, nervous, reproductive and immune systems [1] and in CDGs several organ systems are affected [49]. The present findings, illustrating diminished fine-tuning of proMMP-9 activation by altered propeptide glycosylation, may be relevant in these contexts.

In conclusion, we document the functional role of glycosylation of the propeptide of MMP-9 in controlling proteolysis kinetics, directly and indirectly by galectin-3 binding, thereby regulating proMMP-9 activation, in vitro and ex vivo.
Materials and methods

Proteins and antibodies

Synthetic peptides (pro-AT: APRQRQSTLVLFGDLRTNLTDQQLAEYLRYGYTRVAE and pro-CT: MRGESKSLGPALLLQLKQLSLPETGELDSATLKMRTPRCG VPDGLGR) were synthesized by Thermo Scientific (Waltham, MA, USA). Recombinant full-length human proMMP-9 was expressed in Sf-9 insect cells and purified as described previously [14]. The human recombinant catalytic domain of MMP-3 (cdMMP-3) was purchased from Calbiochem (San Diego, CA, USA, cat# 444217). For the purification of recombinant pro-AT\textsuperscript{SF-9}, human recombinant proMMP-9 was incubated with cdMMP-3 at an enzyme substrate ratio of 1/100 for 3 h at 37 °C in 50 mM Tris/HCl pH 7.4, 150 mM NaCl, 5 mM CaCl\textsubscript{2} and 0.01% Tween-20 (Tris buffer). The recombinant peptide, pro-AT\textsuperscript{SF-9}, was separated from the activated form of MMP-9 by gel filtration chromatography in 6 M urea (Superdex 200 10/300 GL, GE Healthcare, Chicago, IL, USA; Fig. 2C). Afterwards, pro-AT was concentrated and dialysed using Tris buffer and 3 kDa centrifugal filters (Amicon, Merck Millipore, Burlington, MA, USA).

Fig. 8. Congenital disorder of glycosylation patients, having defective N-glycosylation, show higher proMMP-9 activation rate compared to healthy controls. (A) Zymography analysis of gelatin-Sepharose purified serum samples of healthy controls (n = 7) and CDG patients (n = 5) after 3-h incubation with 0-14.2 nM cdMMP-3. One zymography gel is shown for both groups. Recombinant proMMP-9 standard (s) was added. Dotted white line marks splicing of image. Quantification of zymolytic bands of (B) proMMP-9 and (C) actMMP-9 in the serum of healthy controls (black) and CDG patients (red) are shown as a percentage of both forms at different concentrations of cdMMP-3. Lines connect the mean of all data points. Significance, assessed with one-way ANOVA, is indicated with ** (P < 0.01).
Coomassie Blue-stained proteins within SDS/PAGE gels (of protein peak fractions after gel filtration chromatography) confirmed the separation of activated MMP-9 and the pro-ATSf/CO9 (Fig. 2D). Recombinant human active meprin α was produced in insect cells and purified and activated as described previously [58,59]. Human recombinant active neutrophil elastase (NE) was from Abcam (Cambridge, UK, ab91099) and trypsin was obtained from Sigma-Aldrich (Saint-Louis, MO, USA). cDNAs coding for the human galectin-1 and galectin-3 were generated by PCR with reverse transcription on total RNA extracted from a melanoma cell line.

Galectin-3 cDNA was used as template to amplify by PCR the sequence coding for galectin-3 (amino acids Met1-Ile250) fused to the C-terminal tag: SG(H)6SC. The galectin-3 mutant, lacking the non-lectin domain corresponds to Gly112-Ile250. The PCR products were cloned into a derivative of plasmid pET9 (Novagen, Merck Millipore, Burlington, MA, USA). The soluble proteins were expressed in E. coli BL21-AI (Invitrogen, Thermo Fisher Scientific) and purified by affinity chromatography on α-lactose agarose (Sigma-Aldrich, St. Louis, MO, USA). Peroxidase-conjugated affinity-purified donkey anti-rabbit IgG was purchased from Jackson ImmunoResearch Laboratories, West Grove, PA, USA.

**Fig. 9.** Galectin-3 binding with proMMP-9 inhibits its activation and gelatinolytic activity. (A) Zymography analysis of neutrophil proMMP-9 cleavage into actMMP-9 by cdMMP-3 (1/20), alone or co-incubated with galectin-3 (with proMMP-9/galectin-3: 1/10) for 0, 0.16, 0.5, 1, 2 and 3 h. Recombinant proMMP-9 standard (s) was loaded as a reference. (B) Overnight incubation of neutrophil (neutro) and recombinant (recomb) proMMP-9 and cdMMP-3, with or without galectin-3 (1/5 and 1/10). Standard (s) is as in panel A. (C) Gelatinolytic activity of neutrophil proMMP-9/galectin-3 (1/0 in black, 1/5 in red and 1/10 in blue) after incubation with cdMMP-3 was assessed by DQ-gelatin cleavage. Significance, assessed with one-way ANOVA, is indicated with * (P < 0.05). (D) Similar experimental set-up with an oligomerization-deficient mutant of galectin-3 and with (E) galectin-1.
ImmunoResearch (Cambridgeshire, UK, cat# 711-035-152). Synthetic pro-AT and pro-CT were used to immunize New Zealand White rabbits for the production of polyclonal antibodies, in the presence of complete Freund’s adjuvant. A boost injection of the antigen with incomplete Freund’s adjuvant was given monthly and 14 days prior to serum collection. Hyperimmune rabbit serum was diluted (1/5 with 20 mM NaH$_2$PO$_4$ pH 7.5, 0.2 mM NaCl) and filtered before loading on a recombinant protein A-Sepharose fast flow column (GE Healthcare). Rabbit IgGs were eluted with 0.1 M Glycine pH 2.8 followed by a neutralization step with the use of 1 M Tris pH 8.0. Affinity purification of pooled rabbit IgG fractions was performed on N-hydroxysuccinimide (NHS)-Sepharose (GE Healthcare), coupled with either pro-AT or pro-CT (2 mg mL$^{-1}$) according to the manufacturer’s protocol, with similar buffers to load, elute and neutralize, as indicated above. These affinity-

Fig. 10. Domain organization, post-translational modifications and cleavage sites in the MMP-9 propeptide. Human proMMP-9 is composed of an aminoterminal signal peptide (grey), a propeptide (green), an active site (yellow), three fibronectin repeats (blue), a Zn$^{2+}$ binding domain (orange), an O-glycosylated domain (brown) and a carboxyterminal hemopexin domain (red). (A) The propeptide (Ala$_{20}$-Arg$_{106}$) contains the cysteine switch consensus sequence PRCXXPD (bold) of which Cys$_{99}$ interacts with the Zn$^{2+}$ ion coordinated in the catalytic site. Numbered arrows indicate cleavage sites in the propeptide by meprin $\alpha$, $\alpha$-chymase, meprin $\beta$, $\beta$-hematin, hemin (italic=interaction induces autocatalytic activation of proMMP-9), MMP-1, -2, -3, -7, -10, -13 and -26, neutrophil elastase (NE), tissue kallikrein (tKK), trypsin and urokinase type plasminogen activator (uPA). For MMP-3 and NE, newly discovered cleavage sites are indicated in orange and blue respectively. At Asn$_{38}$ (in the NLT sequon, indicated in purple), the propeptide contains an N-linked oligosaccharide. (B) Three-dimensional model of proMMP-9 and the N-linked oligosaccharide (purple) at Asn$_{38}$ on human recombinant Sf-9 proMMP-9 (left), which is smaller compared to oligosaccharides on human neutrophil proMMP-9 (middle, glycoform A3G3F2) and a model of activated MMP-9 (right) (model based on references 14, 15, 37 and 61) and figure adapted from (1). The N-linked oligosaccharides at Asn$_{120}$ are not included.
purified rabbit polyclonal antibodies were used to optimize immunoblot analysis and ELISA for the detection of pro-AT and pro-CT (Figs 2Ac,Bc and 3A–D).

**Immunoblot analysis**

Peptides were separated in 16% acrylamide (Biorad, Hercules, CA, USA) gels, transferred to polyvinylidene difluoride (PVDF) membranes (Biorad) and probed with indicated polyclonal rabbit antibodies (0.5 μg·mL⁻¹), recognizing either pro-AT or pro-CT of human proMMP-9. As secondary reactant, we used peroxidase-conjugated polyclonal donkey anti-rabbit antibodies. Detection was performed after addition of chemiluminescent substrate (Super Signal West Pico #34080 and Femto #34095, ThermoFisher, Waltham, MA, USA).

**Patient samples**

Blood of healthy controls and synovial fluid samples from rheumatoid arthritis patients were collected in accordance with the Declaration of Helsinki and rules of the local Ethics Committee (KU Leuven, Belgium). Serum samples of PMM2-CDG patients and healthy controls were obtained at the University Hospitals of Leuven under the supervision of J. Jaeken. Written informed consent from healthy controls and patients was obtained.

**DQ-gelatin or fluorogenic peptide degradation assay**

A dye-quenched (DQ) gelatin degradation assay was performed as previously described [60] with fluorescence detection (Clariostar, MBG Labtech, ex 485 nm/em 530 nm). All data were corrected by subtraction of negative control values.

**Proteolytic processing of propeptide**

The synthetic pro-AT and pro-CT (10 μM) were incubated with cdMMP-3 (enzyme substrate ratio of 1/50) in Tris buffer or with NE (1/100) in 200 mM Tris/HCl pH 8.8 at 37 °C to determine cleavage sites. Pro-AT<sup>synth</sup> and pro-AT<sup>Sn–9</sup> were incubated at different concentrations (20 μM, 15 μM, 10 μM, 5 μM and 1 μM) with the cdMMP-3 (1/50) at 37 °C in Tris buffer. ProMMP-9 was activated with cdMMP-3 (1/100) for 3 h at 37 °C in Tris buffer. Active MMP-9 (1/200), active meprin α (1/5000) were used to cleave pro-AT<sup>synth</sup> and pro-AT<sup>Sn–9</sup> (10 μM) at 37 °C in Tris buffer. Active NE (1/500) was used to cleave pro-AT<sup>synth</sup> and pro-AT<sup>Sn–9</sup> (10 μM) at 37 °C in 200 mM Tris/HCl pH 8.8. Trypsin (1/10 000) was used to cleave pro-AT<sup>synth</sup> and pro-AT<sup>Sn–9</sup> (10 μM) at 37 °C in Tris buffer (without CaCl<sub>2</sub>). Finally, pro-AT<sup>synth</sup> and pro-AT<sup>Sn–9</sup> (5 μM) were incubated in the presence of serum or synovial fluid of patients with rheumatoid arthritis (1/5 diluted in PBS) for several time points.

**Peptide sequencing to identify cleavage sites**

Peptide fragments of pro-AT or pro-CT were transferred onto a PVDF membrane of a ProSorb cartridge (Applied Biosystems, Foster City, CA, USA) followed by Edman degradation (Procise 491cLC, Applied Biosystems).

**Gelatin zymography analysis**

Samples of MMP-9 were analysed on 7.5% polyacrylamide gels containing 0.1% gelatin, as previously described [11]. Multimeric and monomeric proMMP-9 and a specific domain deletion mutant of human proMMP-9 (lacking the O-glycosylated and the hemopexin domains) were included as molecular size marker and standardization of the gelatin zymography method (Figs 7–9).

**Gelatin-Sepharose bound MMP-9 activation**

Five microlitre of serum was incubated for 30 min with gelatin-Sepharose (GE Healthcare) in 50 mM Tris/HCl pH 7.4, 10 mM CaCl<sub>2</sub> and 0.01% Tween<sub>20</sub> under agitation. After washing, the gel was incubated with cdMMP-3 (from 0 to 14.2 nm) for 3 h at 37 °C while shaking and the residual gelatin-bound MMPs were quantified by zymography.

**Statistical analysis**

Statistical analyses were performed using GRAPHPAD PRISM 6 software (La Jolla, CA, USA). Data points were assessed for normality with the use of the Shapiro–Wilk test. Depending on the distribution of the data points, unpaired t-test (for normal distribution) or Mann–Whitney test (for non-normal distribution) were used to calculate P values. All P values <0.05 were considered significant.

**Molecular modelling**

For molecular modelling of proMMP-9 with the glycan attached to Asn<sub>38</sub>, we used a previous model of proMMP-9 created by Rosenblum et al. [15], which is based on SAXS data and crystal structures obtained by Elkins et al. [37] and Cha et al. [61]. N-linked glycans, Fc and FcA<sub>3</sub>G<sub>3</sub>F<sub>2</sub> (Fig. 1) for recombinant Sf-9 and natural neutrophil proMMP-9, respectively, were built using the Glycam webserver (http://www.glycam.org) and then attached to Asn<sub>38</sub> in PyMOL (Fig. 10).

**Acknowledgements**

The present study was funded by the Geconcerteerde OnderzoeksActies (GOA 2013/015) and C1 funding of KU Leuven (16/17/010), the Charcot Foundation of Belgium and the Research Foundation of Flanders.
(FWO-Vlaanderen), the Rega Foundation and the Deutsche Forschungsgemeinschaft SFB 877 (project A9).

**Conflict of interest**

The authors declare that they have no conflicts of interest with the contents of this article.

**Author contributions**

LB and GO designed research. LB, EU-B, EM, JV, VR and PP performed research. DC, MG-A, PB, WS, CB-P provided recombinant proteins. JJ, EM and PW provided CDG patient samples and LB and GO wrote the paper. All authors critically improved the manuscript and agreed with the final version.

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