β-Hematin Interaction with the Hemopexin Domain of Gelatinase B/MMP-9 Provokes Autocatalytic Processing of the Propeptide, Thereby Priming Activation by MMP-3[†]

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Received November 13, 2007; Revised Manuscript Received December 20, 2007

ABSTRACT: Gelatinase B or matrix metalloproteinase-9 is involved in inflammation and in autoimmune and vascular diseases. In contrast to the constitutive and homeostatic matrix metalloproteinase-2, matrix metalloproteinase-9 is an inducible enzyme. Furthermore, it needs tight regulation, and a major control mechanism of its enzymatic activity is the activation of the latent enzyme by proteolysis of the 87 residue propeptide. Activated matrix metalloproteinase-9 is detected in many vascular or hematological disease states, including in an experimental model for cerebral malaria with *Plasmodium berghei* ANKA. However, insight into its activation mechanism is incomplete. In view of the association with hemorrhagic and hemolytic diseases, it was studied whether and how hemoglobin and its derivatives might activate pro-matrix metalloproteinase-9. Incubation of matrix metalloproteinase-9 with hemin or β-hematin, the core constituent of hemozoin or malaria pigment, leads to differential autocatalysis of the propeptide, mediated by allosteric interaction with the hemopexin domain. The cleavage catalyzed by β-hematin coincides with the first cleavage by stromelysin-1/matrix metalloproteinase-3, and preincubation of matrix metalloproteinase-9 with β-hematin enhances the activation rate by matrix metalloproteinase-3 at least 6-fold. These findings suggest that reduction of hemorrhage and hemolysis might prevent matrix metalloproteinase-9-mediated inflammatory and vascular damages.

The matrix metalloproteinases (MMPs[1]) constitute a large family of soluble or membrane bound zinc-dependent proteases involved in the remodeling of the extracellular matrix and in the regulation of immune responses. They represent key players in physiological processes like growth, stem cell mobilization and wound repair, and are also involved in pathological conditions, such as tumor cell metastasis, inflammation and vascular and autoimmune diseases (*1*−*4*). The basic structural features of all MMPs are the presence of a propeptide and a Zn^{2+}-containing active site. Most MMPs possess a C-terminal hemopexin domain, important for binding of particular substrates, endogenous inhibitors and cell surface receptors (*5*). In addition, gelatinases have a fibronectin-like domain, involved in the binding to gelatin, and MMP-9 contains a unique O-glycosylated (OG) domain between the hemopexin domain and the active site (*1*, *4*, *6*). Human neutrophils store large amounts of this enzyme in their secretory granules, and degranulation, e.g., induced by chemotactic factors, results in rapid release of MMP-9 (*7*). Similar to most other MMPs, MMP-9 is secreted as a latent pro-enzyme that needs activation by the removal of the propeptide in order to catalyze substrate degradation. This key step of activation by proteolysis is an irreversible off−on switch, that needs efficient control by endogenous inhibitors, such as the tissue inhibitors of metalloproteinases (TIMPs) (*8*).

For a proMMP to become catalytically active, disruption of the coordination between the cysteine thiol group of the unique sequence motif PRCGXPD in the propeptide and the catalytic Zn^{2+} is required. This process is described as the cysteine-switch model (*9*) and can be catalyzed by several mechanisms (*10*). MMP-9 and other MMPs are artificially activated by chemical treatment with mercurial compounds, such as 4-aminophenylmercuric acetate (APMA) (*10*), with alkylating agents or with chaotropes like urea and detergents (*11*, *12*). Intriguingly, Bannikov et al. reported that binding of proMMP-9 to gelatin may lead to reversible activation through disengagement of the propeptide from the active site (*13*). Alternatively, *in vitro* studies have shown that reactive

[†] This study was supported by a special fund created for malaria research at the Rega Institute and the Geconcerteerde OnderzoeksActies (GOA/2007/15). P.E.V.d.S. is a postdoctoral fellow and N.G. a research assistant of the Fund for Scientific Research (F.W.O.-Vlaanderen).

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[1] Abbreviations: MMP, matrix metalloproteinase; OG, O-glycosylated; TIMP, tissue inhibitor of metalloproteinase; APMA, 4-aminophenylmercuric acetate; ROS, reactive oxygen species; HSA, human serum albumin; ΔHem, lacking the hemopexin domain; ΔOG, lacking the O-glycosylated domain; DTT, dithiothreitol; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperide-*N*-oxyl; EDTA; ethylene diamine tetraacetic acid; DMSO, dimethyl sulfoxide; sHz, synthetic hemozoin; SDS, sodium dodecyl sulfate; PVDF, polyvinylidene difluoride; cd, catalytic domain; ht_1-MMP-9 and ht_2-MMP-9, hemin-truncated forms of MMP-9; βt-MMP-9, β-hematin-truncated MMP-9.

10.1021/bi702260q CCC: $40.75
Published on Web 02/01/2008

oxygen species (ROS), such as hypochlorous acid (HOCl) (*14*) and peroxynitrite ($ONOO^-$) (*15*), can activate proMMPs by modifying the cysteine thiol groups in the propeptide, presumably followed by autocatalytic cleavage. Furthermore, S-nitrosylation of the cysteine residue in the prodomain and subsequent oxidation into a sulfinic ($-SO_2H$) or sulfonic ($-SO_3H$) acid derivative was shown to lead to permanent MMP-9 activation *in vivo* (*16*). These types of activation might be of importance under inflammatory and/or ischemic conditions. Finally, several proteases, such as the serine protease trypsin (*17*, *18*) and tissue kallikrein (*19*, *20*), mediate direct cleavage of the prodomain. In addition, different MMPs can activate each other, e.g., the two step cleavage of the propeptide of MMP-9 by MMP-3, also called stromelysin-1 (*21*−*23*).

In various pathologies, including cerebral ischemia and diabetic retinopathy, MMP-9 is upregulated and activated and contributes to degradation of the blood−brain barrier, the formation of edema and finally hemorrhage (*24*−*27*). A strong correlation between activated MMP-9 and hemoglobin levels, a marker for hemorrhage, was reported (*25*). Recently, Tajima et al. have studied intra-articular hemorrhage and they observed that hemoglobin in its turn can induce the expression and activation of MMP-9 and MMP-2 by synovial cells (*28*).

Under normal circumstances, the total hemoglobin and heme concentrations in the blood of an adult are ∼150 mg/mL and ∼8 mg/mL, respectively. However, during hemolysis and/or hemorrhage, significant amounts of hemoglobin are released in the circulation and/or surrounding tissues. The prosthetic group of hemoglobin, heme, is then released from hemoglobin and easily converted into hemin, the Fe^{3+} oxidation product of heme. Both heme and hemin are ubiquitous iron-containing compounds inherently dangerous by their oxidative properties in pathologic situations such as in hemolytic and other anemias, hemorrhage, trauma, malaria and other infections (*29*). Neutrophils can be recruited and activated by heme or hemin and subsequently produce interleukin-8 (*30*). This chemokine further triggers neutrophil chemotaxis and degranulation, resulting in the release of MMP-9 (*7*). Interestingly, heme or hemin also catalyzes the formation of ROS and triggers the oxidative burst in neutrophils (*30*). Moreover, Zamboni et al. described a potential link between serum iron and MMP-9 activation (*31*).

During malaria infection, hemoglobin is digested by the parasite inside the erythrocytes. To protect itself, the malaria parasite detoxifies free heme via crystallization into hemozoin or malaria pigment, a unique insoluble crystal composed of heme dimers and structurally identical to β-hematin (*32*, *33*). The destruction of erythrocytes by the parasite ends up in the release of hemozoin as well as hemoglobin and heme. It has been estimated that 400 μg/mL hemozoin is released in the circulation during each cycle of parasitic replication (∼36−48 h) at a parasitemia of 10%, which is commonly occurring in malaria patients (*33*). Recently, Prato et al. reported that phagocytosis of hemozoin by monocytes induces the production of MMP-9, which contributes to the disruption of endothelial basement membranes and the extravasation of blood cells (*34*). In addition, malaria pigment also has the capacity to attract and activate neutrophils (*35*), to catalyze the formation of ROS (*36*), and recently, hemozoin has been reported to be responsible for the suppression of erythropoiesis (*37*), one of the causes of malaria-associated anemia.

In the present study, a novel autocatalytic aminoterminal processing mechanism of proMMP-9 by hemin and β-hematin is reported and it is shown that the hemopexin domain functions in this truncation. Although several identified intermediate forms of MMP-9 are proteolytically inactive, the activation of the hemozoin-truncated MMP-9 by MMP-3 is significantly accelerated.

EXPERIMENTAL PROCEDURES

Proteins and Reagents. Recombinant human full-length proMMP-9 (92 kDa) was expressed in Sf9 insect cells and purified as previously described (*6*). The following recombinant variant proteins were generated by mutagenesis of full-length MMP-9 (*6*): MMP-9ΔHem, lacking the hemopexin domain (72 kDa); MMP-9ΔOG, lacking the OG domain (70 kDa) and MMP-9ΔOGHem, lacking both the hemopexin and the OG domain (48 kDa). MMP-9mutE, an inactive mutant (92 kDa) in which the catalytic Glu_{402} is mutated into Ala, was produced by *in vitro* mutagenesis in a similar way as the MMP-9mutEC mutant, in which both the catalytic Glu_{402} and Cys_{468} in the OG domain are mutated into Ala (*6*).

Human hemoglobin, hemin, human serum albumin (HSA), the redox reagent dithiothreitol (DTT, Cleland's reagent), the superoxide scavenger 4-hydroxy-2,2,6,6-tetramethylpiperide-*N*-oxyl (TEMPOL) and the metalloproteinase inhibitors ethylene diamine tetraacetic acid (EDTA) and *o*-phenanthroline were purchased from Sigma-Aldrich (St. Louis, MO). Hemin was dissolved at 20 mg/mL in dimethyl sulfoxide (DMSO). The protease inhibitor cocktail (Complete, EDTA-free) was obtained from Roche Diagnostics (Mannheim, Germany). Recombinant human TIMP-1 was purchased from R&D systems.

For the preparation of synthetic hemozoin (sHz) or β-hematin, a modification of the method described by Jaramillo et al. (*38*) was applied. Briefly, 175 mg of hemin chloride was solubilized in 17.5 mL of 0.1 N NaOH and neutralized with 1.75 mL of 1 N HCl. Next, 32.72 mL of 1 M sodium acetate, pH 4.8, was added and the mixture was incubated for 3 h at 60 °C. After incubation, 0.58 mL of 10% sodium dodecyl sulfate (SDS) was added followed by centrifugation for 15 min at 20800*g*. The pellet was sonicated in 100 mM sodium bicarbonate, pH 9.0, 0.5% SDS and again centrifuged. Next, the pellet was washed 4 times in 2% SDS and 2 times in milliQ water to wash out SDS. The β-hematin was dried at 37 °C overnight and crushed with a pestle and mortar to decrease the particle size. Finally, it was suspended in PBS and homogenized in a potter tube. The size of the crystals was ≤1 μm as determined by microscopical analysis.

Incubation of ProMMP-9 with Hemoglobin, Hemin or β-Hematin. The different recombinant proMMP-9 variants (final concentration of 1 μM) were incubated and shaken at 37 °C for the indicated time intervals with the indicated concentration of hemoglobin, hemin (dissolved at 20 mg/mL in DMSO as a stock solution) or β-hematin in assay buffer (100 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $CaCl_2$ and 0.01% Tween 20). To evaluate the effect of DMSO during the incubation, proMMP-9 was incubated with β-hematin in the presence of DMSO.

In addition, hemin from the stock solution was diluted in assay buffer in the presence or absence of HSA (1%) and preincubated at room temperature for 15 min. After centrifugation (15 min, 20000*g*) of the hemin solution, the supernatant and the pellet were separated and incubated with proMMP-9 at 37 °C for 48 h. The incubations of recombinant human proMMP-9 with β-hematin were also performed in the presence of HSA (1%), DTT (100 mM; 3 mM and 0.5 mM), the superoxide scavenger TEMPOL (4 mM), the protease inhibitor cocktail (Complete, EDTA-free), EDTA (100 mM and 10 mM) or *o*-phenanthroline (10 mM and 1 mM) as indicated. After incubation, 0.3 ng of MMP-9 of each sample was analyzed by gelatin zymography (7.5% or 10.5% acrylamide gels) as described previously (*39*). As a molecular weight standard, a mixture of 0.1 ng of recombinant human full-length proMMP-9 (92 and 200 kDa) and 0.1 ng of two different recombinant MMP-9 mutants, MMP-9ΔHem (72 and 144 kDa) and MMP-9ΔOGHem (48 kDa), all produced in Sf9 insect cells (*6*), was used. In addition, some samples were also analyzed with the use of SDS−PAGE followed by Coomassie Blue staining or silver staining.

Western Blot Analysis. For the Western blot analysis, two primary antibodies were used: the monoclonal antibody REGA-3G12, which binds part of the catalytic domain (*40*), and a polyclonal antibody, only recognizing the propeptide of MMP-9. For the production of the polyclonal antibody, a peptide corresponding to the propeptide of human MMP-9 (residues 1−87) was synthesized using Fmoc chemistry on an automated peptide synthesizer (433A, Applied Biosystems, Foster City, CA). After deprotection and purification, this peptide was used for the immunization (100 μg, in complete Freund's adjuvant) and for boosting (7× with a 4 week time interval schedule, 100 μg in incomplete Freund's adjuvant) of a female rabbit. Serum was taken 10 days after the final boost and was initially purified on a protein A Sepharose column (G.E. Healthcare) and thereafter affinity-purified on a column containing the immobilized recombinant full-length proMMP-9. The polyclonal antibody recognizes synthetic propeptide and intact proMMP-9, but not MMP-3-activated MMP-9. Antigen detection on Western blots was revealed as described (*40*).

Peptide Sequencing/Identification of Cleaved Fragments. Recombinant human proMMP-9 (10 μg, final concentration of 1 μM) incubated at 37 °C with hemin or β-hematin (750 μg/mL) for 48 and 24 h, respectively, was separated by reducing SDS−PAGE (7.5%) and transferred onto a polyvinylidene difluoride (PVDF) membrane by semi-dry electroblotting. After Coomassie Brilliant Blue (0.1%) staining, the bands of truncated MMP-9 were excised and subjected to protein sequencing by Edman degradation (Procise 491cLC, Applied Biosystems).

Determination of MMP-9 Activation Velocity and Activity. After a preincubation with or without hemin or β-hematin, the supernatant, containing 1 μM recombinant human MMP-9, was subjected to activation by the catalytic domain of MMP-3 at a molar ratio of 1:100 (MMP-3:gelatinase B) in assay buffer. Samples were taken at different time points and analyzed by zymography. Zymolytic bands were quantified by scanning densitometry. The activity was determined according to Knight et al. (*41*) by using a fluorogenic peptide substrate (7-methoxycoumarin-4yl)Acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-Ala-Arg-NH_2 (R&D Systems, U.K.). The substrate (2 μM) was incubated and stirred with MMP-9 (0.5 nM) in a fluorimeter at 37 °C.

RESULTS

Hemin and β-Hematin Induce Truncation of MMP-9 in Vitro. Various pathologic conditions are associated with hemorrhage or hemolysis, leading to the release of considerable amounts of hemoglobin and heme into the extracellular milieu. The direct effect of diverse concentrations of hemoglobin or hemin on recombinant human proMMP-9 was assessed *in vitro* by incubating the enzyme at 37 °C. At different time points after incubation, samples of the suspensions were collected and analyzed by gelatin-zymography (Figure 1).

In contrast to hemoglobin (panel A), incubation of proMMP-9 with hemin decreased the relative molecular weight of the enzyme in a time dependent manner, suggesting that processing occurred (panel B). Already after 7 h incubation of proMMP-9 with hemin concentrations of 150 μg/mL and 750 μg/mL (corresponding to less than 2 and 10% of the total heme concentration in normal blood), the zymography showed two zymolytic bands, both being hemin-truncated forms of MMP-9 (ht_1- and ht_2-MMP-9, see enlargement in Figure 1). Larger amounts (2 and 0.75 mg/mL) of hemin resulted in the degradation of MMP-9 after 24 to 48 h incubation. In addition, the amounts of the MMP-9 oligomers were reduced during the incubation in the presence of hemin, but not of hemoglobin, indicating that an interaction between hemin and the MMP-9 oligomers might result in dissociation/degradation. To investigate whether monomeric hemin, and not aggregates, is also capable to induce truncation of proMMP-9, hemin was partially solubilized with HSA (1%), the most prominent plasma protein with heme-binding capacity. After centrifugation, proMMP-9 was added to the supernatant and to the pellet. Following a 48 h incubation period, the same hemin-truncated forms were observed in the supernatant by zymography, whereas there was no processing in the absence of HSA (Figure 1, panel C). Truncation also occurred in the pellets, independent of the presence or absence of the serum protein HSA. In addition, HSA apparently prevented the oligomers from degradation by hemin.

During malaria infection, hemoglobin inside red blood cells is digested by the parasites resulting in the production of a chemically inert crystalline substance called hemozoin. This malaria pigment is released in the circulation as the red blood cells burst. In its pure form, hemozoin is identical to β-hematin, the synthetic form of hemozoin. Hence, proMMP-9 was incubated with β-hematin and this resulted in truncation of the enzyme as shown in Figure 2.

After 48 h in the presence of 150 μg/mL β-hematin (corresponding to the hemozoin amounts released at each replication cycle at a parasitemia of 2.5%), the zymography showed only one β-hematin-truncated MMP-9 form, denoted as βt-MMP-9. As with hemin, the use of higher amounts of β-hematin resulted in degradation of MMP-9 and the oligomers disappeared during the incubation. Panel D in Figure 2 shows the concentration-dependent formation of the β-hematin-truncated MMP-9 form after overnight incubation,

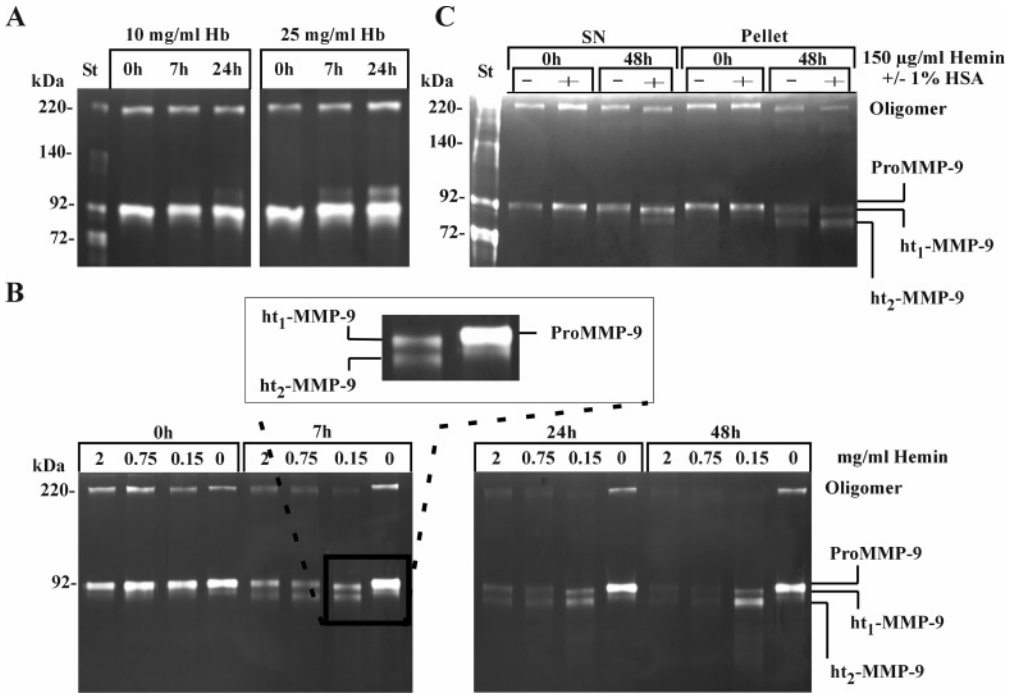

FIGURE 1: Hemin-mediated truncation of proMMP-9. Gelatin zymography analysis of samples of recombinant human proMMP-9 incubated with the indicated concentrations of hemoglobin (Hb) (panel A) or hemin (panels B and C). In panel C, hemin was dissolved in assay buffer in the presence (+) or absence (−) of 1% human serum albumin (HSA). After centrifugation, proMMP-9 was added to the supernatant (SN) or the pellet and incubated for 0 to 48 h as indicated. Representatives of at least 2 independent experiments are shown. ht_1-MMP-9 and ht_2-MMP-9 indicate hemin-truncated forms of MMP-9. St is a molecular weight standard preparation of domain deletion variants of MMP-9 (indicated in kDa).

expressed as scanning densitometry units of zymolysis bands. At a β-hematin concentration of 300 μg/mL, proMMP-9 was almost completely truncated. Remarkably, partial truncation of proMMP-9 even occurred at a concentration as low as 20 μg/mL of β-hematin (inset in panel D). Here also, incubation with larger amounts of β-hematin (higher than 600 μg/mL) resulted in the degradation of MMP-9. The incubations with β-hematin were also repeated in the presence of HSA (1%) (Figure 2, right panels) and truncation of proMMP-9 was corroborated. HSA stabilized MMP-9 monomers in the presence of high concentrations of β-hematin. As in the cases with hemin, HSA at the lowest β-hematin concentration (150 μg/mL) prevented the oligomers from degradation/dissociation, without further influence on truncation.

The Truncated Forms of MMP-9 Are Proteolytically Not Active. According to the preceding data, proMMP-9 is processed into truncated forms in the presence of hemin or β-hematin. To analyze whether these forms were proteolytically active in solution, the activity of the incubated samples was measured using a fluorogenic substrate-conversion assay. Surprisingly, although an increase in the electrophoretic mobility of MMP-9 is usually interpreted as activation, none of the observed processed forms of MMP-9 had any proteolytic activity in solution. Therefore, it was investigated whether the decrease in molecular size was caused by aminoterminal truncation.

Toward this end, proMMP-9 processed by hemin or β-hematin was separated by SDS−PAGE and, after blotting, the Coomassie Blue stained protein bands were excised and identified by automated Edman degradation (Table 1). Incubation of proMMP-9 with hemin resulted in cleavage between Asp_{15} and Leu_{16} (ht_1-MMP-9) and between Pro_{62} and Glu_{63} (ht_2-MMP-9), which is in agreement with the mobility of the two truncated forms as observed on zymography (Figure 1). Both processed MMP-9 forms are subsequently denominated as L_{16}MMP-9 and E_{63}MMP-9. In the presence of β-hematin, truncation occurred between Glu_{40} and Met_{41} and between Leu_{52} and Leu_{53} (both corresponding to βt-MMP-9). These two forms were not resolved from each other on zymography, were formed in approximately equal abundance (as estimated by Edman degradation) and will be henceforth indicated as M_{41}/L_{53}MMP-9. Interestingly, the cleavage site between Glu_{40} and Met_{41} formed after incubation with β-hematin is identical to the first cleavage site by MMP-3. This 86 kDa intermediate becomes susceptible to the second cleavage by MMP-3 at the Arg_{87}−Phe_{88} bond, resulting in the conversion into an active 82 kDa form (*21*).

The MMP-9 Hemopexin Domain and Proteolytic Activity Are Crucial for Hemin or β-Hematin To Induce Truncation of ProMMP-9. Various oxidative protein modifying reactions are mediated by heme or hemozoin (*42*). In addition, ROS can modify the cysteine thiol groups and activate proMMPs (*14*). To assess whether the truncation of proMMP-9 is caused by the formation of ROS, catalyzed by hemin or β-hematin, the incubations were repeated in the presence of different concentrations of DTT or TEMPOL. The truncation process, induced by β-hematin, was not inhibited by either DTT or TEMPOL (Figure 3), indicating that the mechanism responsible for the truncation of MMP-9 was not oxidative. The same conclusion could be drawn from the incubations of proMMP-9 with hemin (data not shown). Based on these results, it was concluded that the processing of proMMP-9 after incubation with hemin or β-hematin was not caused by ROS.

To analyze whether particular domains of MMP-9 are important for the truncation process, incubation experiments

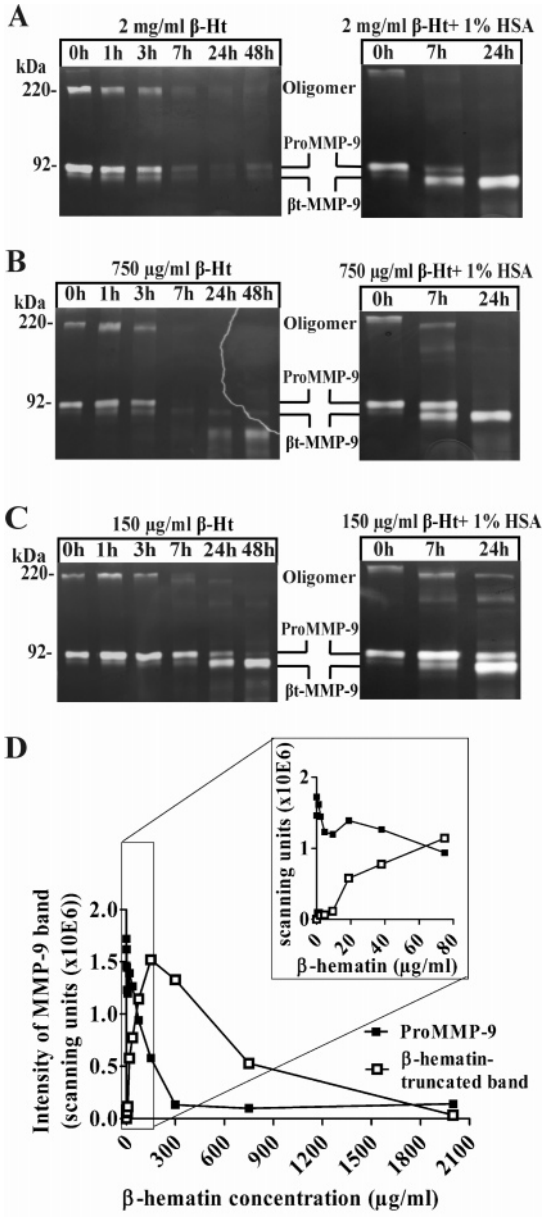

FIGURE 2: β-Hematin-induced processing of proMMP-9. Gelatin zymography analysis of samples of recombinant human proMMP-9 incubated with 2 mg/mL (panel A), 750 µg/mL (panel B) and 150 µg/mL (panel C) β-hematin (β-Ht) in the presence (right panels) or absence (left panels) of 1% human serum albumin (HSA). Samples were collected at the indicated time points after incubation. The molecular weights (in kDa) and the β-hematin-truncated form of MMP-9 (βt-MMP-9) are indicated on a representative zymography of at least 2 independent experiments. Panel D: ProMMP-9 was incubated overnight with β-hematin, and the concentration-dependent formation of the truncated form of MMP-9 was analyzed by zymography and quantified by scanning densitometry. The amount of proMMP-9 and the β-hematin-truncated form of MMP-9 is expressed as scanning units, deduced from zymolysis of each lane. The inset provides a more detailed view at β-hematin concentrations below 80 µg/mL. These results are the average of at least two independent experiments with similar results.

were performed using different recombinant variants of the enzyme lacking the OG domain (70 kDa), lacking the hemopexin domain (72 kDa), or lacking both the OG and the hemopexin domain (48 kDa) (Figure 4, panel A). These recombinant variants can be activated by MMP-3 at similar rates and display similar catalytic activities as compared to full-length MMP-9 (*6*). The results in Figure 4 indicate that,

Table 1: Aminoterminal Sequence of Intact and Hemin- or β-Hematin-Truncated ProMMP-9

MMP-9	aminoterminal sequence
ProMMP-9	D_{-5}PSSRA_1PRQRQSTLVLFPG DLRTNL...[a]
hemin-truncated MMP-9	
ht_1-MMP-9[b]	L_{16}RTNLTDRQLAEEYL...[c]
ht_2-MMP-9[b]	E_{63}TGELDSA...[c]
β-hematin-truncated MMP-9 (βt-MMP-9[b])	M_{41}RGESKSLGPALLLQKQ...[c] L_{53}LLQKQLSLPETGELDSA...[c]
MMP-3-incubated MMP-9	
intermediate form	M_{41}RGES...[d]
activated form	F_{88}QTFE...[d]

[a] As determined by mass spectrometry of a tryptic digest of recombinant MMP-9, including 5 expression vector-derived amino acids (D_{-5}PSSR). [b] ht_1-MMP-9 and ht_2-MMP-9, hemin-truncated forms of MMP-9; βt-MMP-9, β-hematin-truncated MMP-9. [c] As determined by Edman degradation. [d] For comparison, first and second cleavage site by MMP-3 as determined by Ogata et al. (*21*) and Sang et al. (*22*).

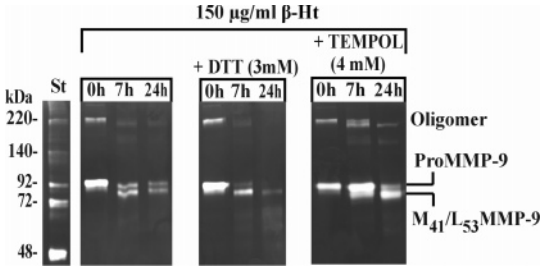

FIGURE 3: ProMMP-9 incubated with β-hematin in the presence of antioxidants. Gelatin zymography analysis of samples of recombinant human proMMP-9 incubated with 150 µg/mL β-hematin (β-Ht) in combination with the antioxidants dithiothreitol (DTT, 3 mM) and the superoxide scavenger 4-hydroxy-2,2,6,6-tetramethylpiperide-*N*-oxyl (TEMPOL, 4 mM). Samples were taken at the indicated time points after incubation. The molecular weights of a standard gelatinase preparation (St) are indicated in kDa. These zymographies are representative of at least 2 independent experiments.

in contrast to the full-length MMP-9, no hemin- or β-hematin-induced truncation was observed when the hemopexin domain is missing (panels B, C and D). In the absence of the OG domain, the processing of MMP-9 by hemin (panel B) or β-hematin (panels E and F) still occurred. However, this processing of MMP-9ΔOG in the presence of 300 µg/mL β-hematin was less efficient when compared to full-length MMP-9 (inset in panel F). Similar as with the full-length MMP-9, degradation of the MMP-9ΔOG occurs in the presence of high concentrations of β-hematin (panel F). These data, in addition to the homology of the hemopexin domain with the heme-binding protein hemopexin, strongly suggest that hemin or β-hematin interact with the hemopexin domain of MMP-9 and that the OG domain somehow facilitates the truncation process, possibly by providing molecular flexibility (*43*).

An interaction between hemin or β-hematin and the hemopexin domain is not sufficient to explain the partial truncations of the propeptide of MMP-9. To investigate if these truncations, which are actually peptide bond cleavages, occurred autocatalytically, the incubations were repeated using an inactive mutant of MMP-9, MMP-9MutE. In this mutant, the catalytic Glu_{402} in the active site, which is essential for the proteolytic activity, is mutated into Ala. The absence of truncation of the inactive MMP-9 mutant by

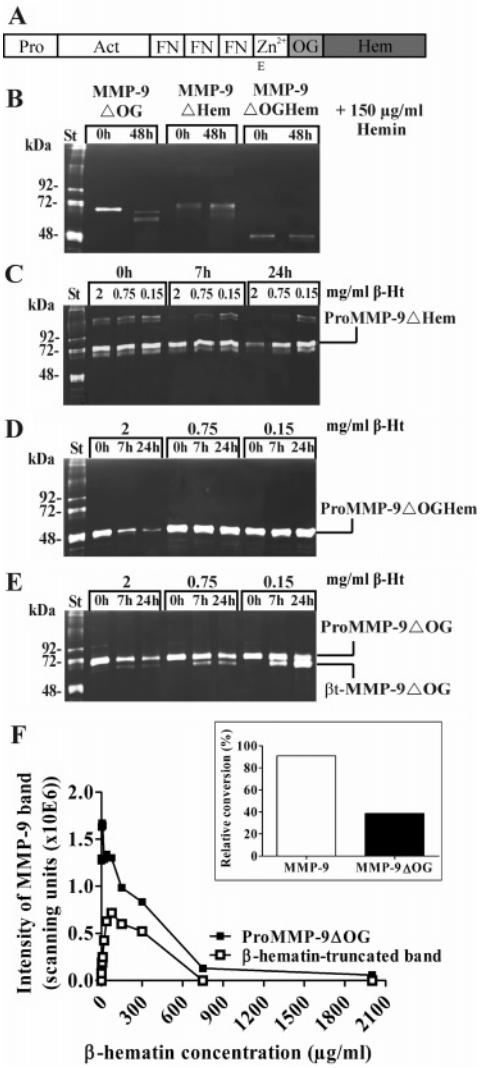

FIGURE 4: Influence of the hemopexin and OG domains of MMP-9 on its truncation by hemin or β-hematin. Gelatin zymography analysis of samples of recombinant domain deletion mutants of proMMP-9, lacking the hemopexin and/or OG domains, incubated with the indicated concentrations of hemin or β-hematin (β-Ht). Samples were taken at indicated time intervals after incubation. Panel A: Schematic domain structure of full-length MMP-9 with the O-glycosylated (OG) and hemopexin (Hem) domain indicated in gray scales. The catalytic Glu_{402} (E) in the Zn^{2+}-binding domain is shown underneath. Panel B: Zymography of samples of the mutants (MMP-9ΔOG, MMP-9ΔHem and MMP-9ΔOGHem) incubated for 48 h with 150 μg/mL hemin. Samples are ordered as specified on the figure. The molecular weights of a standard gelatinase preparation (St) are indicated in kDa. Panels C, D and E: Zymography analysis of samples of MMP-9ΔHem, MMP-9ΔOGHem and MMP-9ΔOG, respectively, incubated with the indicated concentrations of β-hematin. The zymographic patterns after addition of hemin or β-hematin without incubation (0 h) were identical to those of the recombinant variants without addition of heme derivatives. In particular, the double band of the MMP-9ΔHem variant was also observed in the original preparation (data not shown) and in the molecular weight standard. The figure shows representative zymographies of at least 2 independent experiments. Panel F: Intensity of proMMP-9ΔOG and the β-hematin-truncated form of MMP-9ΔOG, expressed as scanning units of zymolysis bands, after overnight incubation with different concentrations of β-hematin. The inset shows the relative conversion of the full-length proMMP-9 compared to proMMP-9ΔOG into its β-hematin-truncated form at a β-hematin concentration of 300 μg/mL. These results are the average of at least two independent experiments with similar results. Pro, prodomain; Act, active site domain; FN, fibronectin repeats; Zn^{2+}, Zn^{2+}-binding domain.

hemin or β-hematin suggests that not only the hemopexin domain but also an intact active site is necessary to process proMMP-9 into intermediate forms, indicating autocatalysis (Figure 5, panel A). In addition, the incubations of pro-MMP-9 with β-hematin were also performed in combination with several (metallo)-protease inhibitors (Figure 5, panel B). Addition of a protease inhibitor cocktail, which inhibits serine and cysteine-dependent proteases, did not inhibit the processing of proMMP-9. In contrast, *o*-phenanthroline or EDTA, both metalloprotease inhibitors, prevented the truncation, suggesting that MMP activity is involved. In the presence of the endogenous MMP-9 inhibitor TIMP-1, the truncation of MMP-9 was inhibited.

β-Hematin, and Not Hemin, Accelerates the Activation of MMP-9. The results thus far indicate that interaction of hemin or β-hematin with the hemopexin domain of MMP-9 and catalysis by an intact active site are essential for the processing of proMMP-9 into different truncated forms. Although the propeptide of these forms is shortened, the enzyme remains inactive. Truncation of the propeptide by hemin or β-hematin provokes the question whether this may affect the activation process of the enzyme. Hence, pro-MMP-9 was preincubated with or without hemin or β-hematin and, subsequently, the catalytic domain of MMP-3 (MMP-3cd), an efficient activator of proMMP-9, was added. In addition, hemin or β-hematin was also incubated simultaneously with proMMP-9 and MMP-3cd. As previously described (*21*) and as visualized on zymographies in panels A of Figures 6 and 7, the addition of MMP-3cd to proMMP-9 resulted in the two step cleavage of the enzyme. Starting from 10 min after MMP-3cd addition, a second gelatinolytic band, corresponding to the 86 kDa intermediate M_{41}MMP-9 form, appeared. The further conversion of this intermediate form into the activated 82 kDa F_{88}MMP-9 was completed after 4 h. A similar pattern was observed when, after a preincubation with hemin, the two truncated MMP-9 forms, L_{16}MMP-9 and E_{63}MMP-9, were subjected to the activation process. The processed L_{16}MMP-9 form was converted into the M_{41}MMP-9 intermediate 30 min after MMP-3cd addition, and half an hour later, the activated 82 kDa enzyme species appeared (Figure 6, panels A and C). Total activation was achieved 4 h after MMP-3cd addition, analogous to the control sample. When proMMP-9, hemin and the MMP-3cd were incubated simultaneously (Figure 6, panels B and C), no major difference in activation kinetics was observed compared to the (pre)incubation with and without hemin, although a small amount of partially truncated MMP-9 remained visible after 4 h of simultaneous incubation (Figure 6, panels A and C).

In contrast, after proMMP-9 was preincubated with β-hematin, the activation course was significantly accelerated (Figure 7). During overnight incubation with β-hematin, two truncated MMP-9 forms were generated, M_{41}MMP-9 and L_{53}-MMP-9. These forms were completely converted into the 82 kDa activated MMP-9 form only 30 min after MMP-3cd treatment (Figure 7, panels A and C), whereas this took 4 h without β-hematin. The course of MMP-9 activation was also assessed through activation of proMMP-9 in the presence of β-hematin. Panel B of Figure 7 reveals that, 1 h and 30 min after the addition of MMP-3, the activation rate was increased compared to the control incubation, suggesting that not only a preincubation with β-hematin but also the presence

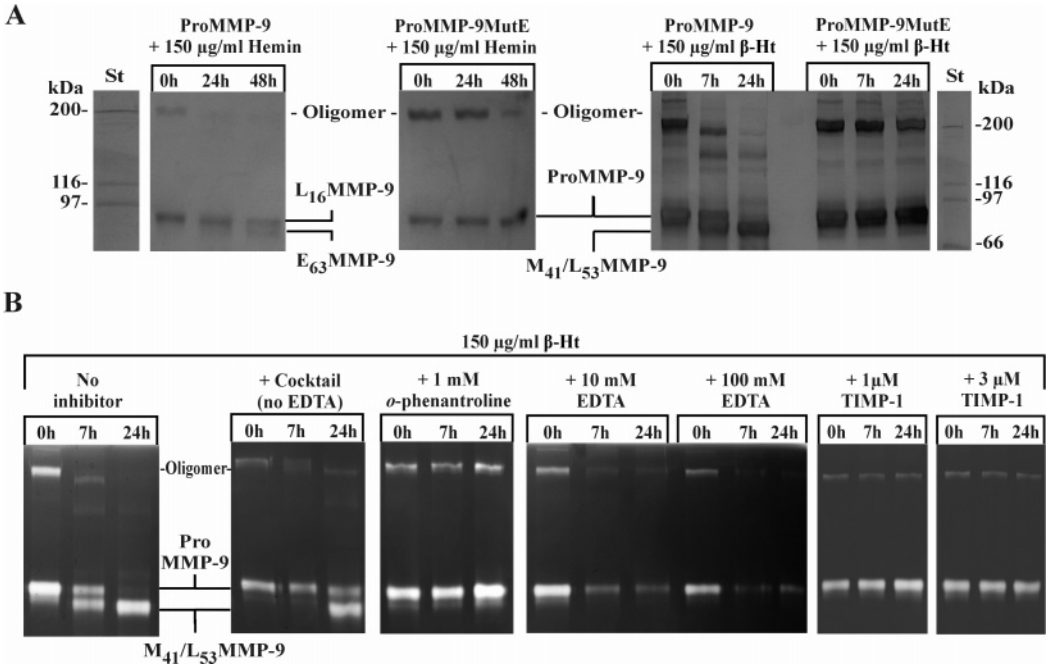

FIGURE 5: The intact active site of proMMP-9 is essential for the hemin- or β-hematin-induced truncations. SDS−PAGE and gelatin zymography analysis of samples of recombinant human proMMP-9 incubated with 150 µg/mL of hemin or β-hematin. Samples were taken at the indicated time intervals after incubation. Panel A: Hemin (Coomassie Blue stain) or β-hematin (β-Ht, silver stain) was incubated with the active and inactive mutant of MMP-9 and separated by SDS−PAGE. Panel B: Incubation of proMMP-9 with β-hematin without inhibitor, with a protease-inhibitor cocktail against serine and thiol proteases, with 1 mM *o*-phenanthroline, with 10 mM and 100 mM EDTA or with 1 and 3 µM TIMP-1. These SDS−PAGEs and zymographies are the representatives of at least 2 independent experiments. St, standard molecular weight marker.

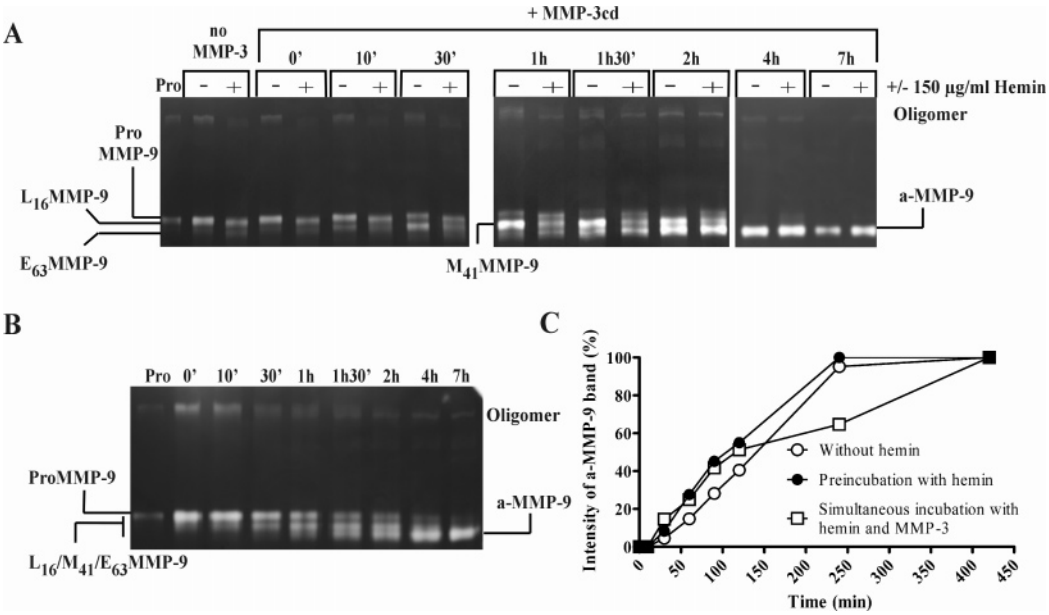

FIGURE 6: Influence of hemin on the activation of proMMP-9 by MMP-3. Gelatin zymography analysis of samples of recombinant human proMMP-9 preincubated at 37 °C for 48 h with (+) or without (−) 150 µg/mL of hemin and subsequently subjected to activation by MMP-3cd. Samples were taken at different time points after MMP-3cd treatment. Panel A: After preincubation of proMMP-9 with or without hemin, MMP-3cd was added to the incubation mixture and further incubated for the indicated time intervals. Panel B: Simultaneous incubation of proMMP-9, hemin and MMP-3cd. For the control without hemin, see panel A. Panel C: The zymolytic bands shown in panels A and B were quantified by scanning densitometry and the relative density of the activated MMP-9 (a-MMP-9) band, corresponding to F_{88}MMP-9, is indicated as percentage of total zymolysis of each lane. The zymographies are the representatives of at least 2 independent experiments and are averaged in panel C.

of the malaria pigment during the activation process influences the activation kinetics.

In conjunction with the results in panels A, B and C of Figures 6 and 7, Western blot analysis was performed on proMMP-9 and on the truncated MMP-9 forms before and after MMP-3 treatment (Figure 8, panels A and B). Two antibodies were used for detection: the monoclonal antibody REGA 3G12 which binds part of the catalytic domain and recognizes the denatured pro- and activated form of MMP-9 (*40*), and a specific polyclonal antibody which only detects the propeptide of the enzyme. As a corroboration of the sequence analysis, the truncated MMP-9 forms, generated

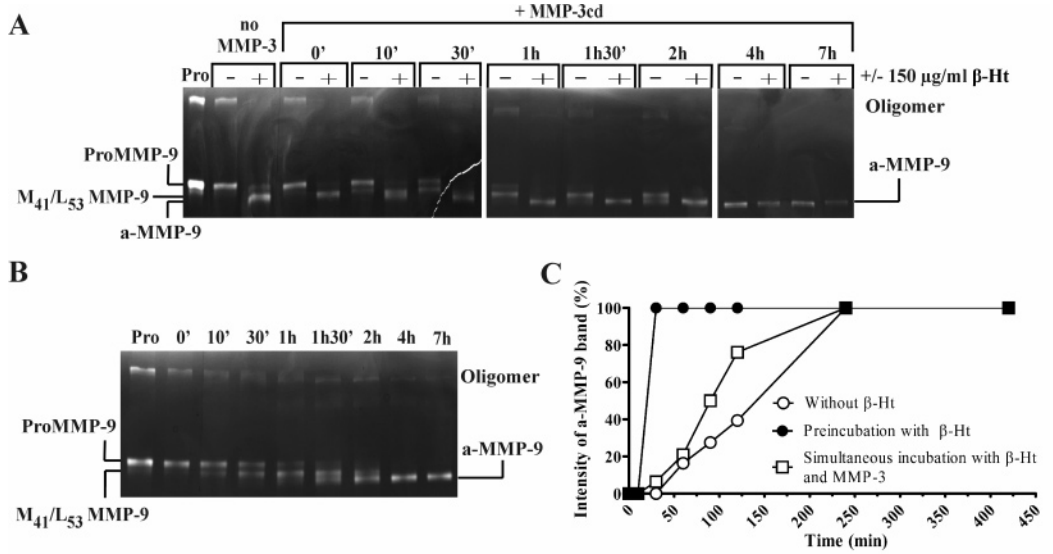

FIGURE 7: Influence of β-hematin on the activation of proMMP-9 by MMP-3. Gelatin zymography analysis of samples of recombinant human proMMP-9 preincubated at 37 °C for 24 h with (+) or without (−) 150 μg/mL of β-hematin (β-Ht) and subjected to activation by MMP-3cd. Samples were taken at different time points after MMP-3cd treatment. Panel A: After preincubation of proMMP-9 with or without β-hematin, MMP-3cd was added to the incubation mixture and further incubated for the indicated time intervals. Panel B: Simultaneous incubation of proMMP-9, β-hematin and MMP-3cd. For the control without β-hematin, see panel A. Panel C: The zymolytic bands shown in panels A and B were quantified by scanning densitometry and the relative density of the activated MMP-9 band (a-MMP-9), corresponding to F_{88}MMP-9, is indicated as percentage of total zymolysis of each lane. These zymographies are the representatives of at least 2 independent experiments and are averaged in panel C.

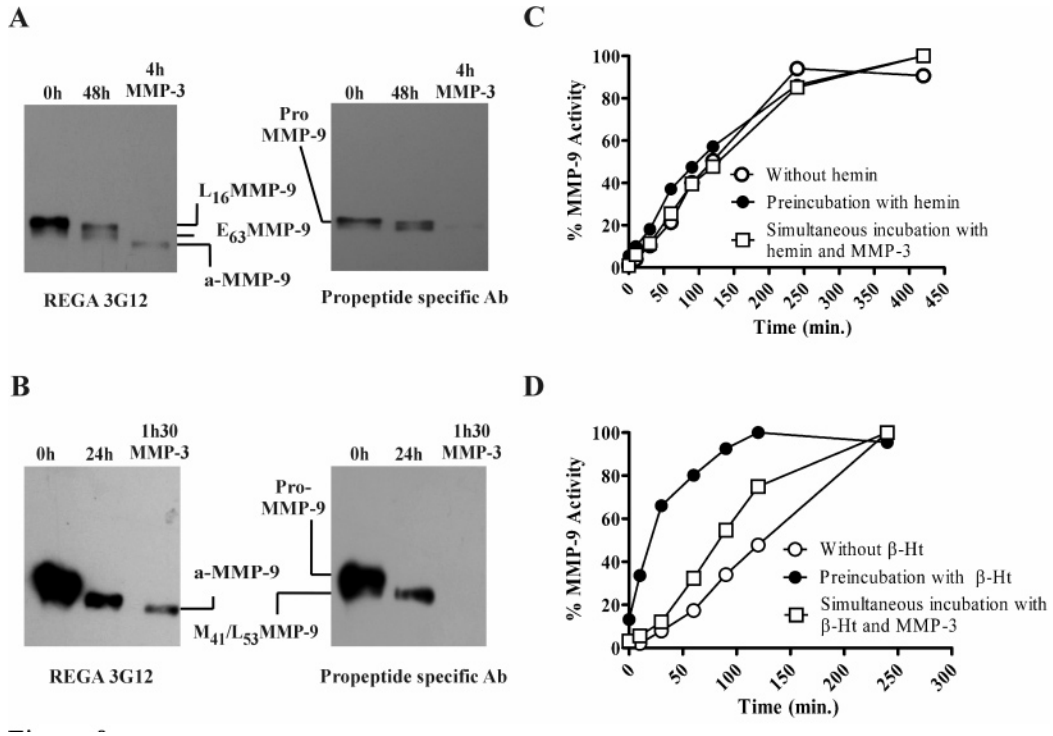

FIGURE 8: Propeptide detection and activation velocity measurement. Panels A and B: Western blot analysis of proMMP-9 before and after incubation with hemin (A) or β-hematin (B) and also after the subsequent incubation with MMP-3cd. The monoclonal antibody REGA 3G12, which recognizes both pro- and activated MMP-9 (a-MMP-9), and a polyclonal antibody, only recognizing the propeptide, were used. Panels C and D: At several time intervals after addition of MMP-3cd to the hemin (C) or β-hematin (D) truncated forms or after a simultaneous incubation of proMMP-9 and MMP-3cd with hemin (C) or β-hematin (D), the enzymatic activity was measured, using a fluorogenic peptide substrate in solution. The data shown here are the averaged representatives of at least 2 independent experiments.

during preincubation with hemin (panel A) or β-hematin (panel B), were still found to possess part of the prodomain attached, as binding of both antibodies was detected. After MMP-3cd treatment, only the monoclonal antibody REGA 3G12 recognized the enzyme. Absence of reactivity with the propeptide-specific polyclonal antiserum confirmed the complete removal of the propeptide. To further verify the results of Figures 6 and 7, the activity of MMP-9 after MMP-3cd treatment was measured in solution with a fluorogenic substrate (Figure 8, panels C and D). In support of the data in Figure 6, 50% of the maximal MMP-9 activity was reached 2 h after MMP-3cd addition and this activation rate

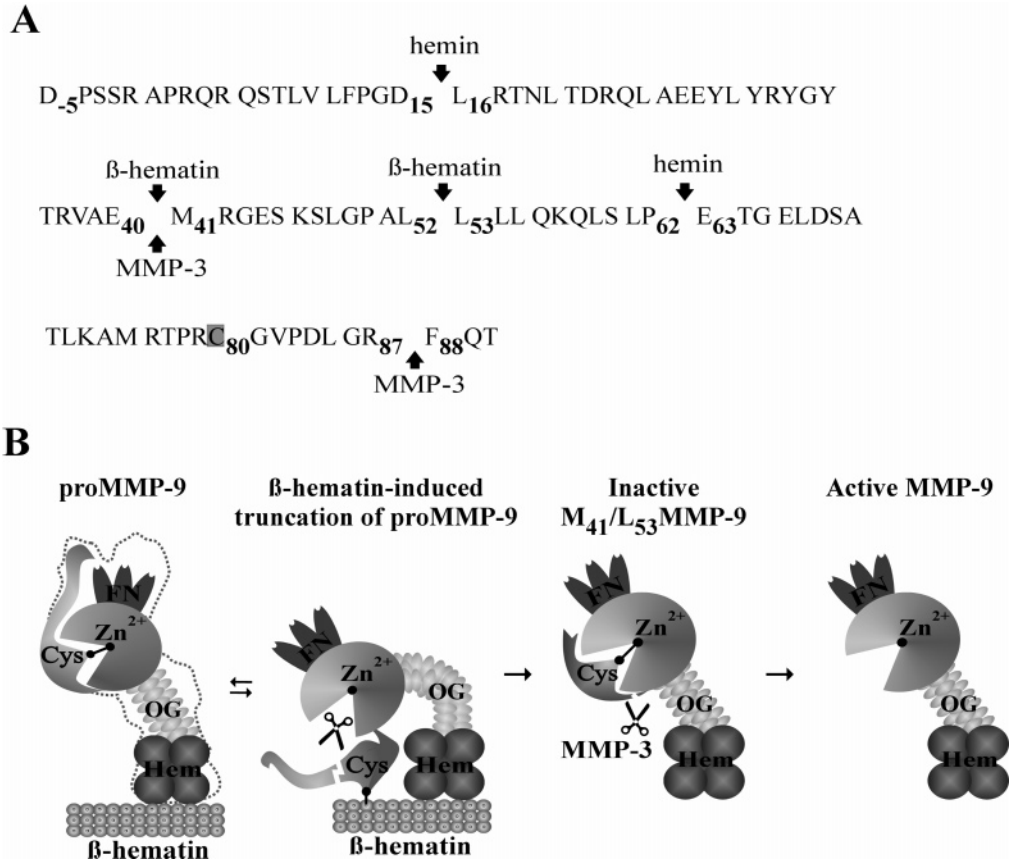

FIGURE 9: Mechanism of the β-hematin-induced priming of MMP-9-activation. Panel A: Experimental amino acid sequence of the propeptide of the used recombinant human proMMP-9 with indication of the cleavage sites after hemin (L_{16}MMP-9 and E_{63}MMP-9) or β-hematin (M_{41}MMP-9 and L_{53}MMP-9) preincubation. In addition, the first and second cleavage sites by MMP-3 are shown. Panel B: Model of the stepwise β-hematin-induced truncation of proMMP-9. The schematic representation of proMMP-9 is based on structural data of full-length MMP-9 (*43*), the contour of which is shown as a dotted line. In proMMP-9, the propeptide aminoterminus is situated nearby one of the three fibronectin (FN) repeats and contains a cysteine sulfhydryl group (Cys) that is coordinated to the active-site zinc ion (Zn^{2+}). With its hemopexin (Hem) domain, proMMP-9 interacts with β-hematin, leading to a conformational rearrangement of the enzyme, presumably involving the flexible nature of the OG domain (*43*). Subsequently, the Cys of the propeptide hypothetically interacts with the Fe^{3+} of β-hematin, thereby reversibly destabilizing the latency state and enabling autocatalysis as indicated in panel A. By circumventing the first step in the MMP-3 activation reaction, this truncated propeptide can be faster processed by MMP-3 into active MMP-9, compared to the intact proMMP-9. FN, fibronectin repeats; OG, O-glycosylated domain.

was not altered by incubating the enzyme with hemin before or during the activation process (panel C). In contrast, the β-hematin-truncated M_{41}/L_{53}MMP-9 forms were activated approximately 6 times faster. Furthermore, simultaneous addition of β-hematin also resulted in accelerated activation of proMMP-9 by MMP-3cd (panel D).

DISCUSSION

This study was incited by various biological observations, including the detection of activated MMP-9 in relation with hemorrhage (*25*), chronic venous disease (*31*), cerebral ischemia and reperfusion (*26*), stroke (*24*, *27*) and the regulated expression of MMP-9 in cerebral malaria (*44*). Since all these diseases have hemolysis in common, the question whether and which hemoglobin-derived molecules might assist in the activation of proMMP-9 was addressed.

Initially, incubations of proMMP-9 with preparations of human hemoglobin were performed, but no truncation was observed. Hence, hemoglobin derivatives were studied. Incubation of proMMP-9 with hemin or β-hematin (the core constituent of hemozoin) indicated processing of the enzyme, as analyzed by zymography. Although these processed MMP-9 forms were found to be truncated at the aminoterminus, they still contained the propeptide cysteine for latency and thus were proteolytically inactive in solution. This indicates that a decrease in molecular weight on zymography does not always correspond to complete removal of the propeptide, yielding activity of the pro-enzyme, and therefore, careful interpretation of electrophoretic mobility shifts of MMPs is required. To evaluate the functional effect of propeptide processing by hemin or β-hematin, the MMP-3-mediated activation was compared between the truncated MMP-9 forms and full-length proMMP-9. The data prove that the activation process of the β-hematin-truncated forms, M_{41}MMP-9 and L_{53}MMP-9, was significantly accelerated compared to the activation of intact proMMP-9 or proMMP-9 truncated by hemin. Furthermore, the activation rate was also increased when intact proMMP-9 was treated with MMP-3 in the continued presence of β-hematin. One of the two β-hematin-truncated forms, namely M_{41}MMP-9, is identical to the initial 86 kDa intermediate produced in the stepwise activation of proMMP-9 by MMP-3 (*21*, *22*). By circumventing the first step of this activation reaction, the β-hematin processed form of MMP-9 shows a 6 times increased activation rate.

To decipher the mechanism of this truncation process, chemical inhibitors and previously developed MMP domain deletion mutants were used. A direct oxidative process by the heme derivatives was excluded by simple biochemical tests. Hemopexin domains are well conserved liganding modules in the MMP family (*5*) and were previously shown to determine intermolecular interactions between MMP-9 and TIMP-1 and cargo receptors (*6*). Interestingly, hemopexin is a high-affinity heme-binding plasma protein, and is the physiological transporter of heme and the first line of defense against intravascular heme-mediated oxidative damage (*45*). Furthermore, both hemopexin itself and the hemopexin domain of MMP-9 hold a binding site for the low-density lipoprotein receptor-related protein (LRP-1/CD91), an endocytotic receptor which is predominant on hepatocytes and on macrophages (*6*). Therefore, the involvement of the hemopexin domain was examined. In the present study, it is established that hemin and β-hematin interact with the hemopexin domain of MMP-9, presumably leading to a conformational change of the enzyme (Figure 9). This structural rearrangement is assisted by the OG domain, which has been shown to possess a high degree of flexibility (*43*). Subsequently, the propeptide of MMP-9 might dynamically interact with hemin or β-hematin, leading to a transiently accessible active site with an interruption of the bond between the cysteine of the propeptide and the catalytic Zn^{2+}. Whereas binding of proMMP-9 to gelatin is suggested to lead to reversible activation (*13*), interaction with hemin or β-hematin results in irreversible truncation of the NH_2-terminal propeptide. For the latter to occur, also the catalytic domain is required. This finding was demonstrated with a novel mutant of proMMP-9 in which only the catalytic Glu was replaced by an Ala. With this mutant, it was proven that autocatalysis is involved in the processing of proMMP-9 by hemin and β-hematin. The reproducibility of the experiments, the dose dependencies, the kinetic data as well as all corroborations by Western blot, protein staining and activity tests validate the proposed scheme in Figure 9. Similar to recent findings on MMP-9 activation by tissue kallikrein (*20*) and the transient activation of gelatinase B by gelatin (*13*), this novel type of activation priming is induced by allosteric interaction, since the hemopexin domain is essential for the β-hematin-triggered truncation of MMP-9.

Recently, it was shown that the *Plasmodium* DNA on hemozoin stimulates, via Toll-like receptor-9, the expression of TNF-α (*46*), which may further induce the expression of MMP-9. Correspondingly, Prato et al. reported that hemozoin-feeding to adherent monocytes induces the expression of MMP-9 in a TNF-α dependent manner (*34*). *In vivo*, this increased MMP-9 expression might contribute to the disruption of the glia limitans in the central nervous tissue and the extravasation of blood cells (*47*). Interestingly, in human cerebral malaria, clusters of hemozoin particles have been detected in the brain microvasculature (*48*, *49*). In mice, infection with *Plasmodium berghei* ANKA results in cerebral malaria characterized by MMP-9 activation in the brain (*44*). These infected mice developed typical microvascular damage with vascular leak and hemorrhage in the brain, in line with proposed functions of this proteinase (*50*). It is therefore tempting to speculate that interaction of MMP-9 with β-hematin could affect the activation process of MMP-9 and thereby accelerate the blood−brain barrier breakdown during cerebral malaria. Whether this interaction also occurs *in vivo* remains to be proven. However, since the used concentrations of β-hematin are achieved *in vivo*, it is probable that the data presented in this study are biologically relevant, in particular in malaria but also in conditions associated with hemorrhage and hemolysis.

Besides activation of Toll-like receptor-9 by β-hematin and subsequent induction of MMP-9 and aside the formation of lipoperoxides, the present study documents a third mechanism by which hemozoin may induce immunopathological changes. Here, it is shown that β-hematin primes MMP-9 activation with the help of the hemopexin domain by an autocatalytic process. This priming results in more efficient activation by MMP-3 *in vitro* and might be relevant in many disease states accompanied by hemolysis, such as malaria infections.

ACKNOWLEDGMENT

The authors are grateful to Prof. Pauline M. Rudd (University College Dublin, Ireland) for critical reading of the manuscript and to Bénédicte Cauwe (Laboratory of Immunobiology, Rega Institute) for helpful discussions concerning this study.

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BI702260Q