Matrix metalloproteinases (MMPs) are involved in the remodeling processes of the extracellular matrix and the basement membrane. Most MMPs are composed of a regulatory, a catalytic, and a hemopexin subunit. In many tumors the expression of MMP-9 correlates with local tumor growth, invasion, and metastasis. To analyze the role of the hemopexin domain in these processes, the MMP-9 hemopexin domain (MMP-9-PEX) was expressed as a glutathione S-transferase fusion protein in Escherichia coli. After proteolytic cleavage, the isolated PEX domain was purified by size exclusion chromatography. In a zymography assay, MMP-9-PEX was able to inhibit MMP-9 activity. The association and dissociation rates for the interaction of MMP-9-PEX with gelatin were determined by plasmon resonance. From the measured rate constants, the dissociation constant was calculated to be $K_d = 2.4 \times 10^{-8}$ M, demonstrating a high affinity between MMP-9-PEX and gelatin. In Boyden chamber experiments the recombinant MMP-9-PEX was able to inhibit the invasion of melanoma cells secreting high amounts of MMP-9 in a dose-dependent manner. These data demonstrate for the first time that the hemopexin domain of MMP-9 has a high affinity binding site for gelatin, and the particular recombinant domain is able to block MMP-9 activity and tumor cell invasion. Because MMP-9 plays an important role in metastasis, this antagonistic effect may be utilized to design MMP inhibition-based cancer therapy.
hemopexin (PEX) domain. Because this construct lacks the known gelatin binding region of MMP-9, i.e. the fibronectin type II domain T2HU-2 within the catalytic domain, we assumed a second gelatin binding domain to be present within the MMP-9 hemopexin domain. We demonstrate a high affinity binding between MMP-9-PEX and gelatin. Furthermore, we show that MMP-9-PEX inhibits the migration of MMP-9-expressing melanoma cells.

EXPERIMENTAL PROCEDURES

Material and Methods—Culture reagents were from Sigma, Invitrogen, or Sarstedt (Berlin, Germany). All chemicals were purchased from Sigma (Germany), Amersham Biosciences, or ICN (Meckenheim, Germany). An affinity-purified goat polyclonal antibody raised against a peptide at the C terminus of murine MMP-9 (C-terminal domain, M-17) was purchased from Santa Cruz Biotechnology. A sheep polyclonal antibody against murine MMP-9 was a generous gift from G. Murphy (Cambridge, UK). A polyclonal rabbit GST antisemur, GST (Z-5) horseradish peroxidase, was from Santa Cruz Biotechnology. Purified MMP-2/MMP-9 gelatinases zymography standards (CC073) and purified murine MMP-9 (100 kDa, CC069) were from Chemicon International (Hofheim, Germany), and murine MMP-2 (444227) was from Calbiochem (La Jolla, CA, USA). The BD BioCoat Matrigel invasion chamber was purchased from BD Biosciences. Standard cloning procedures were performed as described (22). A control peptide (H-GIPETKKLK-OH) of >90% purity with a molecular weight of 1015.6 g/mol was produced by Jerini AG (Berlin, Germany).

Construction of Murine MMP-9-PEX—GST Fusion Proteins—GST fusion proteins were constructed using primers based on the published murine MMP-9 sequence (23). An antisense primer specific for the C-terminal nucleotides 1558–2187 was engineered to contain an internal XbaI site (5'–GGTGGTCCTAGACGTGGCACTGCGAGC–3'). A sense primer was engineered with an internal HinIII site (5'–ACTGCGAAGCCTTAGGGCCTACAGGA–3'). A full-length MMP-9 cDNA was a generous gift from S. Mauch (Leuven, Belgium) and serving as a PCR-template. Products were digested and ligated into pGEX-5X-1 plasmid (Amersham Biosciences).

Expression and Purification—The GST-MMP-9-PEX fusion protein was expressed in bacteria (BL21). Isopropyl-1-thio-β-D-galactopyranoside (IPTG)-induced log phase bacterial cultures containing these constructs were shock frozen and sonicated several times. After lysis, the insoluble parts were removed by centrifugation. GST fusion proteins were purified on Sepharose 4B-coupled glutathione beads (Amersham Biosciences). Beads were washed extensively with PBS, immobilized fusion proteins were digested with 5 mM factor Xa in 50 mM Tris/HCl pH 8, and the cleavage product was eluted with PBS according to the supplier's instructions. The amino acid sequence at the neo-N Xaa site (5’–GGTGGTCCTAGACGTGGCACTGCGAGC–3’) serves as a PCR-template. Products were digested and ligated into pGEX-5X-1 plasmid (Amersham Biosciences).

Western Blot—For antibody-based detection, buffer samples were separated by SDS-polyacrylamide gel electrophoresis utilizing separating gels of 10% polyacrylamide and stacking gels of 3% polyacrylamide. Lanes were loaded with 2 μg of total protein each. Following electrophoresis at 30 V, the proteins were transferred to nitrocellulose membrane. Membrane transfer was monitored by staining with Ponceau S. Blots were blocked with TBS-N (pH 7.6) containing 1% BSA, 20 mM Tris, 137 mM NaCl, and 0.1% Nonidet P40, and were washed and incubated with antibodies against the C terminus of MMP-9 or GST (dilution 1:10000). Signals were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences). Experiments were done in triplicate.

Circular Dichroism Spectroscopy—Circular dichroism (CD) measurements were obtained on an Aviv (Lakewood, NJ) 62DS CD spectrophotometer equipped with a temperature control unit calibrated with a 0.1% aqueous solution of α-10-camphersulfonic acid according to Chen and Yang (24). The spectral bandwidth was 1.5 nm. The time constant ranged between 1 and 4 s, and the cell path length was between 0.1 and 10 mm.

Calculation of Protein Concentrations—Protein concentrations were calculated from absorption spectra in the range of 240–320 nm using the method of Waxman et al. (25).

Mass Spectrometry—The purity and correct molecular mass of the protein were proven by ESI and MALDI-TOF MS. ~80 pmol of MMP-9-PEX was injected into an LC-MS-coupled electrospray single quadrupole mass spectrometer (Ettan ESI ToF, Amersham Biosciences). The protein was first desalted on a MicroTrap column (Michrom BioResources) before spraying into the ionization chamber. After determining the mass by MALDI-TOF (Ettan Maldi ToF Pro, Amersham Biosciences), ~3 pmol of MMP-9-PEX was crystallized in an excess of 3,5-dimethoxy-4-hydroxycinnamic acid. Deconvolution of mass spectra was performed using MagTran software (www.ionsource.com/links/programs.htm).

Zymography—MMP activity was assessed by gelatin zymography following the methods described previously (26, 27). Lanes were loaded with a definite amount of recombinant protein or serum-free supernantant as indicated in Figs. 3 and 5. Samples were preincubated with 4-aminophenyl mercuric acetate (APMA) at a final concentration of 2 mM for 2 h at 37 °C. Proteins were run on non-reducing SDS/polyacrylamide gel containing 1 mg/ml gelatin either with or without 0.41 μg of recombinant hemopexin domain (or albumin or peptide GIPET-KKLK as indicated in the Fig. 3 legend) per milliliter of gel. After electrophoresis in 25 mM Tris, 250 mM glycine, and 1% SDS, the gel was washed at room temperature with 2.5% Triton X-100, 5 mM CaCl2, 50 mM Tris/HCl, pH 7.5, and incubated again in the same buffer twice for 1 h. After rinsing the gel extensively with six changes of distilled water, it was treated overnight at 57 °C in 5 mM CaCl2, 50 mM Tris/HCl, pH 7.5, followed by Coomassie Blue staining (0.5% w/v) and destaining in methanol/acetic acid/water (10:10:80). Gelatin zymography depicts MMPs as negatively staining bands of gelatinolytic activity. Zymographic bands were scanned, and the optical density was determined using the Bio-Rad Gel-Doc system. The activity data of all samples analyzed by zymography were normalized by setting the activity of a defined sample to 1.0 as indicated in the Fig. 3 legend.

Surface Plasmon Resonance Studies—Gelatin was covalently immobilized to a carboxymethyl dextran matrix (Fisons, Loughborough, UK) at 10 μg/ml for 2 min in 10 mM sodium acetate buffer, pH 3.9, as recommended by the manufacturer. Binding experiments were performed at controlled temperature (15 °C) with 12 different concentrations of the purified hemopexin domain using the IASYSTEM (Fisons, Loughborough, UK) optical biosensor. Association was monitored for at least 2 min, the sample was replaced by 10 mM sodium acetate buffer, pH 3.9, and dissociation was monitored accordingly before the cuvette was regenerated with phosphate buffer/1 M sodium chloride, pH 7.4, and equilibrated again with 10 mM acetate buffer, pH 3.9. Association and dissociation affograms were analyzed by nonlinear regression with the FASTfit software (Fisons, Loughborough, UK), which uses the Marquardt-Levenburg algorithm for iterative data fitting.

Cells and Cell Treatment—Human malignant melanoma A375 cells were obtained from ATCC (CRL-1619), and rat mesangial cells were a generous gift from F. Mertens (RWTH, Aachen, Germany). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal-calf serum (FCS), 100 μg/ml streptomycin, and 60 μg/ml penicillin. Cells were grown at 37 °C in a water-saturated atmosphere in air/CO2 (9:1). Cells were assessed for the expression of MMP-2 and MMP-9 by zymography after culturing under serum-free conditions for 24 h.

Boyden Chamber Experiments—Transwell 0.64 cm2 filter inserts (8-μm pores) coated with a thin layer of Matrigel basement membrane matrix (125 μg/cm2) were rehydrated according to the customer's instructions either with or without recombinant MMP-9-PEX (1–500 μg/ml) and placed in wells of 24-well plates containing 0.5 ml of serum-free DMEM. Prior to seeding into the Transwell inserts, cells were released using trypsin/EDTA and sequentially rinsed with DMEM containing 10% FCS. Cells were resuspended and plated onto Matrigel-coated Transwell inserts in DMEM (5.0 × 104 cells/ml) and 250 μl were added to the upper chambers. DMEM containing 5% FCS was used as chemotactrant. Chambers were incubated for 22–24 h at 37 °C, 5% CO2 in a humidified tissue culture incubator. The cells on the upper surface of the filters were then removed using a cotton swab, and those remaining on the lower surface of the filters were fixed with 10% methanol and stained with hematoxylin and eosin.

The filters were rinsed with deionized water, dried, and examined using light microscopy. The number of cells in five random optical fields (400× magnification) from triplicate filters were averaged to obtain the number of migrating cells.
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RESULTS

Molecular Cloning, Purification, and Characterization of MMP-9-PEX—To obtain a soluble MMP-9 PEX domain we generated a GST fusion protein. The cDNA encoding the PEX domain (MMP-9 amino acids Glu-20 to Pro-729) was inserted into pGEX downstream of the sequence encoding GST and a recognition site for factor Xa (Fig. 1).

The GST fusion protein was expressed in *Escherichia coli*. Aliquots of bacterial lysates were subjected to SDS-PAGE. In Western blots the protein was detected with two different polyclonal antisera against the C terminus of murine MMP-9 (Fig. 2A, right, and not shown) or GST (Fig. 2A, left). A protein band representing the GST-MMP-9-PEX fusion protein was detectable with both antisera and corresponds to a molecular mass of about 52 kDa. Bacterial lysates were then applied to a glutathione-Sepharose column. After digestion with factor Xa, a about 52 kDa. Bacterial lysates were then applied to a glutathione-Sepharose column. After digestion with factor Xa, a

The recombinant protein was further purified by size exclusion chromatography (Fig. 2B). The three fractions containing detectable amounts of proteins (fractions 9, 20, and 34) were analyzed by SDS-PAGE and Western blotting using a sheep antiserum against GST, and a recognition site for factor Xa (Fig. 1).

We then identified and characterized MMP-9-PEX by mass spectrometry and circular dichroism, respectively. The purified protein was automatically desalted and analyzed on an Ettna LC-MS (Fig. 2D). The MS run presented in the lower panel of Fig. 2D corresponds to the main peak in the ion chromatogram (not shown). The determined experimental molecular mass for PEX is 25,034 Da (MALDI and ESI MS) and exhibits an offset of 42.58 Da compared with theoretical mass (24,991.42 Da, M). The MS run presented in the lower panel of Fig. 2D corresponds to the main peak in the ion chromatogram (not shown). The determined experimental molecular mass for PEX is 25,034 Da (MALDI and ESI MS) and exhibits an offset of 42.58 Da compared with theoretical mass (24,991.42 Da, M).

To specifically address the ability of MMP-9-PEX to directly bind gelatin, we performed surface plasmon resonance studies. Gelatin was immobilized to a carboxymethyl dextran matrix as described under “Experimental Procedures.” Binding experiments were performed at a controlled temperature with 12 different concentrations of purified hemopexin protein using the IASYSTEM optical biosensor. Association and dissociation affinograms (Fig. 4) were analyzed by nonlinear regression with FASTfit software (Marquardt-Levenburg algorithm for iterative data). From these data, an association rate of \( k_{on} = 3.83 \times 10^{9} \text{ M}^{-1} \text{s}^{-1} \) and a dissociation rate of \( k_{off} = 1.01 \times 10^{-2} \text{ s}^{-1} \) could be deduced. With these values, the dissociation constant of \( K_d = 2.64 \times 10^{-8} \text{ M} \) was calculated (Fig. 4, and Table I). Evidently the MMP-9-PEX domain is able to bind to gelatin.

Invasion of MMP-9-Expressing Cells in the Presence of MMP-9-PEX—These results prompted us to examine whether the recombinant MMP-9-PEX is able to interfere with the MMP-dependent invasion of mammalian tumor cells. Human malignant melanoma A375 cells expressing MMP-9 and MMP-2 and rat mesangial cells expressing MMP-2 as demonstrated by gelatin zymography (Fig. 5A) were cultured on Matrigel-coated membranes in Boyden chambers under serum-free conditions. FCS-containing medium was used as a chemottractant. Half of the chambers were preincubated with recombinant MMP-9-PEX domain in a concentration of 1, 10, 100, and 500 \( \mu \text{g/ml} \) media as indicated in Fig. 5B. A375 incubated with MMP-9-PEX showed decreased invasion and migration through the coated filters compared with control cells. Incubation with GIPETKKLM at 20 \( \mu \text{g/ml} \) as an additional control did not alter the migration pattern of A375 melanoma cells (Fig. 5B). Rat mesangial cells, expressing MMP-2 exclusively, however, showed no difference in migration in the presence of MMP-9-PEX (data not shown). Thus, with respect to gelatinases, recombinant MMP-9-PEX inhibited migration of predominantly MMP-9 expressing cells but not the migration of exclusively MMP-2 expressing cells.

DISCUSSION

Reported here is the gelatin binding property of the C-terminal hemopexin domain of murine gelatinase B, the first such report for any of the MMPs. In 1995, Li et al. (3) described the crystal structure of porcine synovial collagenase (MMP-1) consisting of a catalytic domain and a second domain of ~200 amino acids homologous to hemopexin, a heme-binding glycoprotein. The MMP-1 hemopexin domain contains four units of four-stranded antiparallel \( \beta \)-sheets stabilized on its 4-fold axis by a cation (3). The domain constitutes a four-bladed \( \beta \)-propeller structure and was assumed to control the specificity of MMPs, affecting both substrate and inhibitor binding (3). Two cysteines at either end are conserved in all hemopexin domains of the MMP family.

Since 1992 it has been known that the gelatin binding of MMP-9 is mediated by the second fibronectin-type II domain (T2HU-2), although the presence of another gelatin binding site could not be excluded. Although T2HU-2 mediates binding,
it is not the rate-limiting step in the hydrolysis of gelatin by the enzyme (29). Because Me₂SO inhibits gelatin binding but not its degradation (29), and the hemopexin structure depends on the joining of the two cystein residues susceptible to Me₂SO, this would suggest that Me₂SO inhibits gelatin binding by destroying the hemopexin structure. Therefore, we postulated a second gelatin binding site within the MMP-9 hemopexin domain.

To obtain high amounts of protein, the MMP-9 hemopexin domain was expressed as a GST fusion protein in *E. coli*. A short sequence of buffer amino acids at the N terminus of the protein (GIPETKKLM) was attributed to the cloning procedure and most probably does not influence the binding properties in a significant way. MMP-9 activity in gelatin zymography and the migration of MMP-9-producing tumor cells was not influenced by the peptide GIPETKKLM (Figs. 3 and 5). The precise mass of the hemopexin domain, including the buffer amino acids, was measured to be 25,034 Da by electrospray mass spectrometry (Fig. 2D), which is close to the estimated molecular mass. The difference of 42.58 Da compared with the theoretical mass of 24,991.42 Da (M + H⁺) might be explained by acetylation, a rather common phenomenon also observed for...
proteins from E. coli (30, 31). The difference between the experimental and theoretical mass of the protein is 0.55 Da, which is an acceptable error of the instrument. However there may also be differences due to oxidation reactions occurring somewhere in the protein. MMP-9-PEX contains three cysteines and two methionines, which may all be candidates to undergo oxidation. Immunoreactivity with two different polyclonal antibodies further verified the identity of the purified protein, and circular dichroism spectra indicated the correct folded protein. When the enhanced chemiluminesence (ECL) reaction was allowed to proceed, a few bands of higher molecular weight turned up on the Western blots, which might represent cross-linked multimeric forms of the recombinant protein.

Secondary structure analysis (28) of the far UV CD spectrum reflects the β-sheet character of the protein (α-helix = 21%, β-sheet = 35%, turn = 17%). Inspection of the x-ray structures of the hemopexin domains of MMP-2 and MMP-13 revealed β-sheets only and no helical secondary structure (32, 33). The overestimation of the calculated α-helical content of the MMP-9-PEX is due to the negative band around 228 nm, which might originate from aromatic side chain contributions (34).

For MMP-2 it could already be shown that the C-terminal domain exhibits strong binding properties for fibronectin and heparin and that binding depends on the structural Ca2+ but not Zn2+ ion in this domain (7). In these studies, however, the influence of the hemopexin domain on enzymatic activity was not tested. By gelatin zymography, an easily practicable in vitro assay to determine gelatinase activity, we demonstrated a reduction of MMP-9 activity as a consequence of the presence of recombinant MMP-9-PEX by 60–71%. A greater reduction in the case of smaller amounts of MMP-9 may be explained by reaching the nonlinear range of zymography and densitometry when loading high amounts like 1 ng of MMP-9 protein (Fig. 3, A and B).

To explain a reduction of MMP-9 activity in the presence of recombinant MMP-9-PEX, we carried out a comprehensive study to determine the kinetic parameters for the binding of MMP-9-PEX to gelatin. By plasmon resonance, we measured a dissociation constant of 2.64 × 10−8 M. The Kd values of TIMP-1 for the MMP-9 latent and active species are 35 and 23.9 nM for the high affinity site (35). That means that the binding affinity of gelatin to MMP-9-PEX is as high as the binding of MMP-9 to TIMP-1. These results indicated that the association of MMP-9-PEX and gelatin was rather rapid (k on ≈ 105 M−1 s−1), resulting in a very effective binding. Collectively, these findings support the importance of the C-terminal domain of MMP-9 for an efficient binding to the substrate gelatin. Others (36) have also characterized the binding parameters of pro-MMP-9 and demonstrated that the proenzyme binds with high affinity (Kd ∼ 20–30 mM) to the surface of a variety of cell types, and the Kd values of the α2(IV) chain of collagen IV for pro-MMP-9 were calculated to be ∼45 nM. However, because a pro-MMP-9-TIMP-1 complex and MMP-9 binds to α2(IV), the authors suggested that neither the C-terminal (binds to TIMP-1) nor the N-terminal domain of MMP-9 (which is processed during activation) is directly involved in α2(IV) binding, but the domain was not identified further. From our results, we concluded that the C-terminal domain of MMP-9, although known to bind TIMP-1 with high affinity (35, 37), is also able to bind to gelatin with a comparable high affinity.

We extended our characterization of MMP-9-PEX with respect to its impact on cell migration and found that migration of malignant human melanoma cells (A375) was reduced in the presence of recombinant MMP-9-PEX (Fig. 5). Because MMP-9-PEX was able to inhibit the migration of MMP-9-expressing
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**Table I**

|  | $k_{on}$ (association) | $k_{off}$ (dissociation) | $K_d$ (dissociation) |
|---|---|---|---|
| | $M^{-1} s^{-1}$ | $s^{-1}$ | $\mu M$ |
| | $3.83 \times 10^7 \pm 1.61 \times 10^6$ | $1.01 \times 10^{-2} \pm 4.58 \times 10^{-3}$ | $2.64 \times 10^{-8}$ |

**FIG. 5.** MMP-9-PEX inhibits invasion of A375 melanoma cells. A, zymography of the serum free supernatants of human malignant A375 melanoma cells, which secrete MMP-2 and MMP-9, and rat mesangial cells, which secrete MMP-2 only. An MMP-standard for human MMP-2 and MMP-9 is shown (left lane). B, invasion and migration of A375 melanoma cells were measured in a Boyden chamber in the presence of MMP-9-PEX at different concentrations as indicated (500 µg/ml equivalent to 20 µM). Cells were cultured with or without MMP-9-PEX for 24 h. For control, cells were incubated with GIPETK KLM (20 µg/ml equivalent to 20 µM) as indicated. Additional control cells were incubated with equal amounts of phosphate-buffered saline (0). Cells of the lower surface of the membranes were counted after staining with hematoxylin and eosin. γ axis, number of cells in [%]. Each bar represents the mean ± S.D. of three chambers. This is a representative result of three experiments. *, $p < 0.01$ compared with control.

A375 cells but not of mesangial cells known to express MMP-2 (but no detectable amounts of MMP-9), we speculate that MMP-9-PEX interferes specifically with the MMP-9-dependent migration. MMP-9 is not crucial for only migration and metastasis but also for hematopoiesis. Recently, it has been published (38) that recruitment of stem and progenitor cells from the bone marrow requires MMP-9-mediated release of serum Kit ligand (sKitL). The relative deficiency of serum Kit ligand at baseline or after myelosuppression in MMP-9−/− mice strongly suggests that MMP-9 plays a physiological role in releasing sKitL, setting up the stage for hematopoietic reconstitution. Thus, inhibition of MMP-9 (e.g. by MMP-9-PEX) may provide a novel mechanism for regulating hematopoiesis in sKitL-dependent myeloproliferative disorders.

We have presented MMP-9-PEX as an inhibitor of MMP-9 activity in vitro and in cell culture, which raises many questions and perspectives for further investigations. One important issue is the identification of the gelatin binding epitope within the MMP-9 hemopexin domain. In addition, the recombinant MMP-9-PEX offers the possibility for determining other substrate specificities. Another important question relates to the effects of MMP-9-PEX in vivo. Because the catalytically inactive MMP-9-PEX binds with high affinity to gelatin, a substrate of MMP-9, we consider MMP-9-PEX as a candidate to antagonize MMP-9-activity in the context of tumor cell invasion and metastasis. Recombinant MMP-9-PEX may be used as a new approach in the treatment of diseases with high MMP-9 activity such as melanomas or colorectal carcinomas.

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The Matrix Metalloproteinase 9 (MMP-9) Hemopexin Domain Is a Novel Gelatin Binding Domain and Acts as an Antagonist
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