Mutations in the Catalytic Domain of Human Matrix Metalloproteinase-1 (MMP-1) That Allow for Regulated Activity through the Use of Ca^{2+}

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Background: MMP-1 is an enzyme that requires metal binding for activity.

Results: Two mutations in residues involved in metal binding render the enzyme regulatable by calcium.

Conclusion: The mutant MMP-1 protein is unstable and loses activity in low calcium.

Significance: The conditional activity of MMP-1 as a function of calcium may be an effective treatment for fibrotic diseases.

Conditionally active proteins regulated by a physiological parameter represent a potential new class of protein therapeutics. By systematically creating point mutations in the catalytic and linker domains of human MMP-1, we generated a protein library amenable to physiological parameter-based screening. Mutants screened for temperature-sensitive activity had mutations clustered at or near amino acids critical for metal binding. One mutant, GVSK (Gly159 to Val, Ser208 to Lys), contains mutations in regions of the catalytic domain involved in calcium and zinc binding. The in vitro activity of GVSK at 37 °C in high Ca^{2+} (10 mM) was comparable with MMP-1 (wild type), but in low Ca^{2+} (1 mM), there was an over 10-fold loss in activity despite having similar kinetic parameters. Activity decreased over 50% within 15 min and correlated with the degradation of the activated protein, suggesting that GVSK was unstable in low Ca^{2+}. Varying the concentration of Zn^{2+} had no effect on GVSK activity in vitro. As compared with MMP-1, GVSK degraded soluble collagen I at the high but not the low Ca^{2+} concentration. In vivo, MMP-1 and GVSK degraded collagen I when perfused in Zucker rat ventral skin and formed higher molecular weight complexes with 2-macroglobulin, an inhibitor of MMPs. In vitro and in vivo complex formation and subsequent enzyme inactivation occurred faster with GVSK, especially at the low Ca^{2+} concentration. These data suggest that the activity of the human MMP-1 mutant GVSK can be regulated by Ca^{2+} both in vitro and in vivo and may represent a novel approach to engineering matrix-remodeling enzymes for therapeutic applications.

The matrix metalloproteinases (MMPs)3 are characterized by their dependence on zinc and calcium for activity. The MMPs along with the prolylhydroxylases, ADAMs (a disintegrin and metalloproteinase), serralysins, and astacins, comprise the metzincin subfamily of metalloproteases (1). Although MMPs are capable of effecting the turnover and degradation of proteins that compose the extracellular matrix, there are a large number of non-extracellular matrix proteins including cell adhesion molecules, growth factors, cytokines, chemokines, tyrosine kinase receptors, and MMPs themselves that are processed by MMPs (2). As expected by the plethora of substrates, MMP activity is an important component of many diverse physiological processes including wound healing, bone remodeling, organogenesis, and inflammation. However, MMP activity is also associated with various pathological conditions such as cancer, rheumatoid arthritis, vascular disease, and fibrosis (1, 3, 4).

The majority of MMP proteins are composed of a prodomain, catalytic domain, and hemopexin domain. The prodomain contains the consensus sequence PRCXXP, which serves an autoinhibitory function by utilizing the cysteine residue to coordinate to the Zn^{2+} molecule located in the active site of the catalytic domain (5). The catalytic domain contains three conserved histidine residues in the consensus sequence HEXGHXXGXXH that ligate the active site Zn^{2+} (6). This interaction maintains the enzyme in an inactive state until removal of the prodomain either by proteolysis or by destabilization renders the protein active (7). The hemopexin domain is located C-terminal of the conserved methionine turn (6) and facilitates substrate recognition. Other protein-protein interactions mediated by the hemopexin domain are important for enzyme activation, localization, internalization, and degradation (8).

Interstitial collagenase, also known as MMP-1, was originally identified in 1962 based on its activity during tadpole tail morphogenesis (9). Native collagen types I, II, and III are proteolyzed by MMP-1 at the peptide bond between Gly^{775}-Ile^{776} or Gly^{775}-Lys^{776}, resulting in the generation of the distinctive three-quarter and one-quarter fragments (10). These fragments denature into gelatin at physiological temperatures and are further degraded by other MMPs and proteases. In addition to the active site zinc, a second zinc molecule is bound in the catalytic domain and serves a structural function. In addition to zinc, three calcium molecules bind to the catalytic domain and...
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one binds to the hemopexin domain (Ref. 11 and references therein). The calcium molecules have also been shown to play an important role in domain stabilization and in the regulation of catalytic activity (12–14). As expected, the amino acids important for the binding of the zinc and calcium molecules within the catalytic domain are highly conserved among MMP family members (11).

The process of protein evolution has the potential to enhance or modify the pre-existing properties of an enzyme to tailor them toward a specific need (15, 16). For example, a protein may be evolved to have activity only in a particular physiological microenvironment. These unique microenvironments can be defined by differences in pH, temperature, ion concentration, oxidation state, and other physiological parameters. Recent advances in high throughput protein mutagenesis make possible the construction of comprehensive libraries of protein variants that can be screened for conditional activity under defined physiological conditions. We have generated such a library of human MMP-1 protein variants by systematically mutating the catalytic and linker domains of the protein. Here we report on the creation of a human MMP-1 protein whose activity can be modulated by the concentration of Ca2+. The ability to control the in vivo activity of MMP-1 may provide a unique approach to creating matrix-remodeling enzymes for therapeutic applications such as fibrotic diseases.

EXPERIMENTAL PROCEDURES

Generation and Screening of MMP-1 Mutants—A comprehensive positional evolution (CPE™, BioAtla) library of single point mutants in the catalytic domain and linker region of MMP-1 was created. The library contained 15 random amino acid variants at each of the 178 amino acids mutated (from Phe to Ala). Potential single amino acid mutants were screened (as described below) against fluorogenic peptide substrate IX (Mca-K-P-L-G-L-Dpa-A-R-NH2, (R&D Systems (17)) for decreased catalytic activity at 37 °C relative to 25 °C and for sensitivity but did express protein at high levels. Therefore the two mutations were combined to make a double mutant, GVSK, which expressed protein at levels sufficient to pursue further studies.

MMP-1 Purification and Activation—Wild type and mutant MMP-1 cDNAs were subcloned into pET26b vectors (EMD Biosciences) for protein expression. Transformed BL21(DE3) bacteria were induced with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside and incubated overnight at 25 °C. The next morning, the bacteria were centrifuged, and the pellet was resuspended in OS (osmotic shock) buffer containing 0.5 mg/ml lysozyme and 25 units/ml Benzonase (Sigma) and incubated for 30 min at room temperature. An equal volume of cold water was added and incubated on ice for an additional 30 min. The clarified supernatant was obtained by centrifugation (3000 × g, 20 min at 4 °C) and incubated with a 20% volume (v/v) of Q-Sepharose (GE Healthcare) for 1 h at 4 °C. The mixture was filtered through a 0.22-μm filter (Millipore), combined with a filtered Q-Sepharose wash and loaded onto a 5-ml SP Sepharose fast flow column (GE Healthcare). The protein was eluted with a 0–70% gradient (Buffer A: 25 mM Tris, pH 7.5, 75 mM NaCl, 10 mM CaCl2; Buffer B: 25 mM Tris, pH 7.5, 1 mM NaCl, 10 mM CaCl2). Peak fractions were pooled, diluted 8-fold in Buffer A, and reloaded onto the column. After the second elution, peak fractions were pooled, concentrated, and further purified by size-exclusion chromatography using a Sephadex 200 10 × 300-mm column (GE Healthcare) in Buffer A. Peak fractions were again pooled and concentrated, and the concentration of the purified protein was determined. Purified full-length MMP-1 was activated by proteolytic cleavage using immobilized trypsin. 200 μl of trypsin immobilized on beaded agarose (Pierce) was washed with 1 ml of TCN buffer (50 mM Tris, pH 7.5, 10 mM CaCl2, 150 mM NaCl) three times. The trypsin beads were then mixed with 1 ml of purified MMP-1 (~3 mg/ml) and incubated for 2 h at room temperature with rotation. Beads were separated from the activated protein by using a Zeba desalt spin column (Thermo Scientific). The concentration of the activated protein was determined, and the activity was assessed by 4–20% SDS-PAGE analysis and the in vitro MMP-1 peptide activity assay.

In Vitro MMP-1 Activity Assay—Purified MMP-1, GVSK, or perfusate samples were incubated with fluorogenic peptide substrate IX and assayed for activity. Purified MMP-1 or MMP-1 from a perfusate sample was diluted in TCN buffer (1 or 10 mM CaCl2) to a concentration of 1 μg/ml. A series of seven, 3-fold serial dilutions was then made from this starting concentration. 100 μl of each sample was then added to a 96-well FLUOTRAC 200 black plate (Greiner Bio-One) containing 5 μl of the fluorogenic substrate (final concentration of 200 μM). Plates were read using the SpectraMax M3 fluorescent plate reader (Molecular Devices) at 405 nm. Each sample was measured in duplicate, and the activity was calculated by averaging the readings for each sample within the linear range of the assay. Alternatively, activated MMP-1 or GVSK was diluted to a final concentration of 1 μg/ml in TCN buffer (1 or 10 mM CaCl2) containing 10% Zucker rat serum in a final vol-

4 Throughout this study, the designation GVSK is used to indicate the double mutant G159V/S208K.
ume of 120 μl. The samples were incubated for various times at 25 °C. After the incubation, 95 μl of each sample was added to a 96-well FLUOTRAC 200 black plate containing 5 μl of the fluorogenic substrate (final concentration of 100 μM). The plates were read, and the activity was calculated as described above. Each sample was assayed in triplicate.

Kinetic Parameter Analysis—Purified MMP-1 and GVS were incubated with a series of 2-fold dilutions (0.95–62 μM final concentration) of fluorogenic peptide substrate IX in TCNB buffer (TCN buffer with 0.05% Brij-35 for protein stabilization at low protein concentrations) with either 1 mM or 10 mM CaCl₂. 100 μl reactions were incubated at 37 °C in a 96-well FLUOTRAC 200 black plate. Plates were read using the SpectraMax M3 fluorescent plate reader (Molecular Devices) at 405 nm. The conversion factor was obtained by incubation of substrate IX with 0.25 μM MMP-1 for 24 h at 37 °C. Hydrolysis rates were obtained from fluorescence versus time plots using data points from the linear portion of the curve. The slope from these plots was divided by the fluorescence change corresponding to complete hydrolysis and then multiplied by the substrate concentration to obtain hydrolysis rates in μM s⁻¹. Kinetic parameters were obtained by Lineweaver-Burk, Eadie-Hofstee, and Hanes-Woolf analysis. Each experiment was performed three times, and each sample was assayed in duplicate.

In Vitro Soluble Collagen I Digestion—MMP-1 and GVS at a concentration of 0.1 mg/ml were preincubated for 2 h at either 25 °C or 37 °C in TCN buffer containing either 1 mM or 10 mM CaCl₂. Fluorescein-labeled collagen isolated from calf skin (Elastin Products) was diluted 10-fold in the two TCN buffers to a final concentration of 0.35 mg/ml. After the preincubation, 45 μl of the diluted collagen was mixed with 5 μl of enzyme, and the mixtures were incubated for 2 h at 25 °C. The digestion reactions were stopped with the addition of gel sample buffer and were heated for 5 min at 99 °C prior to 4–20% SDS-PAGE. Gels were analyzed by Coomassie Blue staining.

In Vivo MMP-1 Activity—The in vivo activity of MMP-1 and GVS were assayed by perfusing the enzyme in the ventral skin of Zucker rats. All animals were treated in accordance with protocols approved by the Institutional Animal Care and Use Committee at Halozyme Therapeutics, Inc. The ventral surface of ~6–12-month-old male Zucker rats (Harlan Laboratories) was shaved, and then 1 ml of TCN buffer (1 or 10 mM CaCl₂) containing 1 μg/ml rHuPH20 (recombinant human PH20 hyaluronidase, Halozyme Therapeutics) and 2.5 μg/ml epinephrine (Sigma) was perfused (PHD 2000 infuse/withdraw, Harvard Apparatus) at a rate of 0.12 ml/min at up to five different sites. After 10 min, 1 ml of MMP-1 or GVS at a concentration of 50 μg/ml in TCN buffer (1 or 10 mM CaCl₂) was perfused at a rate of 0.12 mg/ml. At various time points after the completion of the second perfusion, 1-cm full thickness biopsies of the treated sites were taken and fixed in Bouin’s solution (Sigma) for histological analysis. Perfusate that collected in the area vacated by the biopsied tissue was transferred with a pipette to Eppendorf tubes and kept at ~20 °C until further analysis.

Western Blotting and Immunoprecipitation—~4 μg of each perfusate sample (~5–20 μl) was mixed with gel sample buffer and heated for 5 min at 99 °C prior to electrophoresis. Samples were analyzed by either 8% or 4–20% SDS-PAGE (Invitrogen). After electrophoresis, the gels were blotted onto nitrocellulose membranes (Invitrogen). The blots were blocked for 30 min with 1% milk in PBST (0.05% Tween 20 (Sigma) in phosphate-buffered saline (EMD Chemicals)). After three PBST washes, the blots were incubated for 1 h at room temperature with 1:1000 dilutions of either a rabbit polyclonal anti-collagen I antibody (Abcam) or a goat anti-human MMP-1 antibody (R&D Systems). After washing with PBST, the blots were incubated for 1 h at room temperature with either an anti-goat or an anti-rabbit HRP-conjugated secondary antibody at a 1:2000 dilution (Calbiochem). After washing with PBST, the blots were developed using the TMB insoluble reagent (Calbiochem). To determine the immunoprecipitation experiments, ~12 μg of total protein from the rat perfusates (~15–30 μl) was mixed with 5 μl of an anti-rabbit α2-macroglobulin rabbit antibody (Alpha Diagnostic International) and brought to a final volume of 200 μl with PBST. After overnight incubation at 4 °C, 40 μl of protein A/G beads (Pall Scientific) was added and incubated with rotation for 3 h at room temperature. Each sample was then washed four times with 1 ml of PBST, and 30 μl of gel sample buffer was added. The samples were heated for 5 min at 99 °C, electrophoresed on a 4–20% gel, and then transferred to a nitrocellulose membrane. The blot was probed with the anti-human MMP-1 antibody as described above.

MMP-1 Solid Phase Capture ELISA—The concentration of MMP-1 and GVS from the rat perfusates was determined by ELISA. 96-well 4HBX plates (Immulon) were coated with 100 μg/ml of an anti-human MMP-1 antibody (R&D Systems) at 1 μg/ml in 100 mM sodium phosphate buffer, pH 7.2, overnight at 4 °C. The next day, the plates were washed five times with 300 μl/well of PBST and blocked with 200 μl/well of PBST (0.05% Tween 20, Sigma) for 1 h at room temperature. Purified human MMP-1 (R&D Systems) standards were prepared by diluting into PBST at an initial concentration of 200 μg/ml. A series of six 3-fold dilutions was prepared from this starting concentration. Per fusate samples were initially diluted 1:100, and six 3-fold dilutions were prepared from this initial dilution. 100 μl/well of each dilution was incubated for 2 h at room temperature. The plates were washed five times with 300 μl/well of PBST and incubated with 100 μl/well of a biotin-conjugated anti-human MMP-1 antibody (R&D Systems) at 0.25 μg/ml in PBST for 2 h at room temperature. After another wash step, the plates were incubated with 100 μl/well of streptavidin-HRP (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. After the final wash, 100 μl/well of SureBlue TMB microwell peroxidase substrate (Kirkegaard & Perry Laboratories, Inc.) was added to develop the reaction. After ~5 min, 100 μl/well of TMB stop solution (Kirkegaard & Perry Laboratories, Inc.) was added to stop the reaction, and the plates were read using the SpectraMax M3 fluorescent plate reader (Molecular Devices) at 450 nm. Each perfusate sample was assayed in duplicate, and the concentration was calculated by averaging the readings for each sample within the linear range of the assay. Each experiment was performed in triplicate.

Keloidal Collagen Digestion—8 μm frozen sections were prepared from a human keloid biopsy. Sections on slides were covered with 150 μl of either 10 mM TCN buffer or 1.6 mg/ml

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GVSK in 10 mM TCN buffer for 2 h at 37 °C. Sections were then fixed for 5 min with 10% neutral buffered formalin (VWR International) at room temperature. The slides were subsequently stained with hematoxylin and eosin (H&E) and analyzed by light microscopy. Micrographs were taken with a Nikon Eclipse TE2000-U microscope using Spot software version 4.6 (Spot Imaging Solutions).

RESULTS

To create conditionally active mutants of MMP-1, a comprehensive library of single point mutants in the catalytic and linker domain was generated. 15 amino acid variants of 178 amino acids beginning at Phe81 and ending with Ala236 were made. These mutations corresponded to the N-terminal amino acid in the activated protein after cleavage of the prodomain to the final amino acid of the linker domain (18). As a first screen for conditional activity, over 2600 mutated proteins were assayed for decreased catalytic activity at 37 °C relative to 25 °C. 26 single amino acid mutants meeting this requirement were obtained. Although a small number of mutations were at or near residues involved in Zn2⁺ binding (catalytic or structural), the majority of these point mutations were located in amino acids that were either directly involved or adjacent to amino acids that participate in the coordination of two of the three calcium molecules (Calcium 1 and Calcium 3) of the catalytic domain (11, 18). These results led us to examine the effects that the concentration of Ca2⁺ and Zn2⁺ would have on the in vitro and in vivo activity of these mutants. A representative mutant, GVSK, which has a glycine to valine mutation at amino acid 159, a residue directly involved in Ca2⁺ coordination, and a serine to lysine mutation at amino acid 208, a residue adjacent to an amino acid involved in the binding of the catalytic Zn2⁺ molecule (18), was chosen for further characterization.

To generate activated wild type MMP-1 (henceforth referred to as MMP-1) and GVSK protein, the full-length purified proteins were incubated with immobilized trypsin. The trypsin activation of proMMP-1 resulted in the removal of the prodomain and a change in apparent molecular mass from 57 kDa to ~43 kDa (Fig. 1A). In addition to the activated protein, there were two other proteins (near the 22 kDa marker) which, based on N-terminal sequencing, were determined to be the catalytic and hemopexin domains (data not shown). These were probably generated by autocleavage in the linker region (19). Once activated, MMP-1 was assayed against a fluorogenic peptide substrate to determine its activity. Activated MMP-1 exhibited extensive activity against the peptide substrate and was ~78 times more active than the full-length, unactivated protein (Fig. 1B), indicating that the change in the molecular weight of the protein correlated with increased activity. The activation of the GVSK mutant was essentially identical to the wild type protein (data not shown).

The in vitro catalytic activity of activated MMP-1 and GVSK against a fluorescent peptide substrate was first assayed as a function of Ca2⁺ concentration to determine whether the mutations in GVSK had an effect on activity. The proteins were incubated at 37 °C for 2 h at 1, 2, 5, and 10 mM Ca2⁺. At 10 mM Ca2⁺, a concentration sufficient for maximal activity (12), the activity of MMP-1 was higher than the activity of GVSK, indicating that the mutations had decreased the specific activity of GVSK (Fig. 2). With decreasing concentrations of Ca2⁺, the in vitro catalytic activity of MMP-1 was essentially unaffected. In contrast, at 2 mM Ca2⁺, the activity of GVSK had decreased 3-fold as compared with its activity at 10 mM. As the concentration was further decreased to 1 mM, the activity had decreased ~10-fold (Fig. 2). However, when incubated at 25 °C, GVSK lost less than half of its activity, suggesting that temperature and Ca2⁺ concentration both had an effect on activity (data not shown).

To ascertain whether the calcium sensitivity of GVSK was attributable to one of the two mutations, the activated single mutants GV and SK were incubated at 37 °C for 2 h with 1 and 10 mM Ca2⁺ and assayed for activity against the fluorescent peptide substrate. Analogous to MMP-1, the activity of SK was similar at both 1 mM and 10 mM Ca2⁺ (data not shown). In contrast, the activity of GV was decreased ~4-fold in 1 mM Ca2⁺ as compared with its activity in 10 mM Ca2⁺ (data not shown). These data suggest that the calcium sensitivity of GVSK is due to the Gly159 to Val mutation. These results were...
not surprising as residue 159 is directly involved in Ca\(^{2+}\)
coordination.

The effect of Zn\(^{2+}\) concentration on the in vitro catalytic activity of activated GVSK and MMP-1 was examined next. The proteins were incubated at 37 °C for 2 h with either 1 μM or 10 μM Zn\(^{2+}\) in TCN buffer (1 and 10 mM Ca\(^{2+}\)). No difference in the in vitro activity of MMP-1 or GVSK was observed as a function of the Zn\(^{2+}\) concentration in either the 1 mM or 10 mM Ca\(^{2+}\) TCN buffer (data not shown). As a result of these findings, the effect of the Zn\(^{2+}\) concentration on GVSK activity was not pursued further.

To determine whether the decreased activity of GVSK in 1 mM Ca\(^{2+}\) was due to changes in the kinetic parameters of the enzyme, the \(K_m\) and \(k_{cat}\) were determined for both GVSK and MMP-1 at the low and high calcium concentrations. The \(K_m\) and \(k_{cat}\) of MMP-1 in either 1 mM or 10 mM Ca\(^{2+}\) were essentially identical (Table 1) and were very similar to those that have been previously reported (17). The \(k_{cat}/K_m\) of GVSK in 10 mM Ca\(^{2+}\) was similar to those for MMP-1 despite slightly lower \(K_m\) and \(k_{cat}\) values. The \(k_{cat}/K_m\) of GVSK in 1 mM Ca\(^{2+}\) was ~2-fold less than the other three samples (Table 1). Thus, it does not appear that the activity difference between the two observed as a function of calcium concentration (Fig. 2) was due to different kinetic parameters.

The calcium-dependent activity of activated GVSK as compared with MMP-1 was further characterized by assaying the activity as a function of time and calcium concentration. The activity of MMP-1 at 37 °C at either the low (1 mM) or high calcium concentration (10 mM) increased slightly at the early time points and by the end of the experiment was similar to the starting activity (Fig. 3A). GVSK at high calcium showed the same temporal pattern of activity as MMP-1 with the only difference being that the specific activity was slightly lower at the later time points (60 min onwards, Fig. 3A). However, there was no loss of activity during the course of the experiment at 10 mM Ca\(^{2+}\). In contrast, GVSK in low calcium had a lower starting specific activity, and by 15 min, the activity had decreased 2-fold. The loss of activity continued over time, and by 3 h, there was almost no detectable activity (Fig. 3A).

As previously mentioned, calcium has been shown to play an important role in domain stabilization and tertiary structure formation (12–14). In fact, under certain conditions, including low calcium concentrations, MMP-1 and MMP-3 have been shown to undergo autolysis (13, 19). To determine whether the loss of activity in GVSK was due to protein degradation, activated GVSK and MMP-1 were incubated in either low or high calcium buffer at 25 °C and 37 °C. At various time points, aliquots were taken and analyzed by SDS-PAGE. In 10 mM Ca\(^{2+}\), whether incubated at 25 °C or 37 °C, both MMP-1 and GVSK showed no evidence of degradation as the amount of the activated protein at 2 h was similar to the starting amounts (Fig. 3B). MMP-1 was also stable in 1 mM Ca\(^{2+}\) at both temperatures (bottom two gels). GVSK was also stable in 1 mM Ca\(^{2+}\) when incubated at 25 °C. However, at 37 °C in 1 mM Ca\(^{2+}\), the amount of activated GVSK was significantly decreased after 30 min and was almost gone after 2 h (Fig. 3B). Based on these observations and the kinetic data, GVSK activity loss correlated with the degradation of the protein.

The ability of activated MMP-1 and GVSK to digest soluble collagen I, a physiological substrate, was examined next. Prior to incubation with collagen I, MMP-1 and GVSK were preincubated for 2 h at either 25 °C or 37 °C in either 1 mM or 10 mM Ca\(^{2+}\). The soluble collagen fraction contained three sets of bands: a high molecular mass band above the 250 kDa marker, a doublet below the 250 kDa marker, and a doublet of ~120 kDa (Fig. 4, Col I lane). Treatment of soluble collagen with MMP-1 in 10 mM Ca\(^{2+}\) that was preincubated at 25 °C resulted in the decrease in the molecular masses of all three of the sets of bands (Fig. 4). The digestion of the sets of bands was almost complete and, in addition, there other bands appeared near the 50- and

### TABLE 1

| Sample | [Ca\(^{2+}\)] | \(k_{cat}\) | \(K_m\) | \(k_{cat}/K_m\) |
|--------|---------------|-------------|----------|----------------|
| MMP-1  | 10 \(\mu M\)  | 4.54 ± 1.49 | 21.13 ± 7.00 | 216,000 ± 41,500 |
| MMP-1  | 1 \(\mu M\)   | 5.34 ± 2.07 | 20.57 ± 5.35 | 254,000 ± 52,400 |
| GVSK   | 10 \(\mu M\)  | 2.97 ± 1.00 | 11.0 ± 2.56 | 265,800 ± 44,600 |
| GVSK   | 1 \(\mu M\)   | 2.26 ± 0.64 | 16.53 ± 3.47 | 135,300 ± 15,600 |

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To determine the fate of the perfused protein, Zucker rat skin was treated with either activated MMP-1 (1 mM Ca\(^2+\)) or activated GVSK (10 mM Ca\(^2+\)), and perfusate was isolated at various times after perfusion. As early as 5 min after perfusion, there were multiple higher molecular weight bands in addition to the activated protein when assayed by Western blot using an antibody that recognizes predominantly the monomeric form of the protein (data not shown). Therefore, the amount of protein detected in the perfusates is primarily composed of the uncomplexed form of the enzyme. The concentration of MMP-1 in 1 mM Ca\(^2+\) measured in the perfusate was ~60% of the initial concentration after 30 min (Fig. 5C), and by 90 min, it was ~25% of the starting concentration. The results for MMP-1 in 10 mM Ca\(^2+\) were essentially identical (data not shown). The concentration of GVSK in 10 mM Ca\(^2+\) was ~40% of the initial concentration after 30 min and was significantly less at 90 min as compared with MMP-1 (~10% as compared with ~26%). In striking contrast, when GVSK in 1 mM Ca\(^2+\) buffer was perfused, by 5 min after perfusion, the concentration of the monomeric protein had already decreased by ~60%, and by 30 min after perfusion, it was only ~15% of the original concentration (Fig. 5C). These results indicated that GVSK was clearly more susceptible to complex formation, especially at the lower calcium concentration.

It has been long been established that MMP-1 can be inhibited by α2-macroglobulin through the formation of higher molecular weight complexes (20–22). Although the level of α2-macroglobulin is high in plasma (~2.2 mg/ml or 3 μM), it can also approach these levels in the interstitial fluid (23). These observations suggested that the higher molecular weight MMP-1 reactive bands as detected by Western blot using an antibody (Fig. 6B). When mixed with Zucker rat serum, there were multiple higher molecular weight bands that were reactive with the MMP-1 antibody (Fig. 6A, lanes 4 and 5). These same bands were present in the perfusate samples treated with either MMP-1 or GVSK, indicating that the protein that MMP-1 is interacting with when perfused in the skin may be serum-derived.

To confirm that the higher molecular weight complexes formed in the skin contained α2-macroglobulin, an immunoprecipitation of the perfusates was performed. The perfusate sample derived from buffer alone, when immunoprecipitated with an anti-α2-macroglobulin antibody and subsequently blotted with an anti-MMP-1 antibody, revealed a nonspecific band at ~60 kDa but nothing in the higher molecular mass range (Fig. 6B, fourth lane from left). In contrast, the same higher molecular mass bands observed in either MMP-1-treated or GVSK-treated skin perfusates (Fig. 5B) were present in the 5- and 90-min perfusate samples treated with MMP-1, indicating that MMP-1 is complexing with α2-macroglobulin in the skin in vivo.
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A

GVS K | MMP-1 | 50 µg/ml protein

MW 1 1 10 10 [Ca²⁺] mM

kDa
250
148
98
64
50
36

B

MMP-1 | GVS K

MW pre-inj 5' 90' 15' 5' 90' 15' 50 µg/ml protein

kDa
250
148
98
64
50
36

C

% of monomeric protein
110
100
90
80
70
60
50
40
30
20
10

1 mM Ca²⁺ MMP-1
10 mM Ca²⁺ GVS K
1 mM Ca²⁺ GVS K
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The effect that MMP-1 complex formation with α2-macroglobulin had on the activity of the protein was determined by incubating activated MMP-1 and GVSK with 10% Zucker rat serum in either 1 mM or 10 mM Ca\(^{2+}\) buffer for various times and assaying for activity. As GVSK in 1 mM Ca\(^{2+}\) is unstable at 37 °C, the assay was performed at 25 °C to correlate changes in activity as a function of complex formation and not protein degradation. When MMP-1 in 1 mM Ca\(^{2+}\) buffer was incubated with serum, the activity did not change over the course of the experiment (Fig. 6C). On the other hand, GVSK in 10 mM Ca\(^{2+}\) exhibited a gradual loss in enzymatic activity, and by the end of 2 h, ~74% of the original enzymatic activity remained (Fig. 6C). Strikingly, when GVSK in 1 mM Ca\(^{2+}\) buffer was incubated with serum, the loss in activity was rapid. A third of the activity was lost after 15 min, and less than 50% remained after 30 min. By the end of the 2-h experiment, GVSK in 1 mM Ca\(^{2+}\) retained only ~17% of the initial enzymatic activity. The decreased calcium concentration clearly rendered GVSK more susceptible to enzymatic inactivation by serum.

A goal of the generation of conditionally active mutants of MMP-1 has been to assess their potential use as therapeutic agents in the treatment of fibrotic diseases. To determine whether GVSK was capable of degrading fibrotic collagen, frozen sections from a human keloid were treated with either 10 mM Ca\(^{2+}\) buffer or GVSK in 10 mM Ca\(^{2+}\) buffer. In the section treated with GVSK, there was a dramatic decrease in the intensity of the H&E staining, indicating that most of the collagen had been degraded by the enzyme (Fig. 7B). The smaller collagen bundles in the hypodermis were completely digested, and the majority of the larger bundles of collagen deep within the keloid were also digested as compared with buffer treatment alone (Fig. 7, A and B, inset). These results indicate that GVSK is capable of digesting collagen from fibrotic tissue.

**DISCUSSION**

Through the use of comprehensive mutagenesis, we generated a library of MMP-1 mutants with the potential to be conditionally active in vivo based on a physiological parameter. After an initial screen based on differential activity as a function of temperature, a number of mutants were generated that had mutations at or adjacent to amino acids critical for the coordination of the Ca\(^{2+}\) ions in the catalytic domain. A representative mutant, GVSK, which has comparable kinetic parameters to MMP-1, was characterized and found to have catalytic activity that was dependent on the concentration of Ca\(^{2+}\) and temperature. At 37 °C in low Ca\(^{2+}\) (1 mM), GVSK activity was reduced over 10-fold as compared with its activity at high Ca\(^{2+}\) (10 mM). This loss of activity was not observed at 25 °C (data not shown). Activity loss occurred rapidly and correlated with the degradation of the full-length protein.

Ca\(^{2+}\) ions are absolutely required for the activity of MMPs (24, 25). Although Ca\(^{2+}\) does not directly participate in proteolysis, it is integral in the stabilization of the tertiary structure of the enzyme (12). In fact, circular dichroism analysis of human MMP-1 revealed that in the absence of Ca\(^{2+}\), the protein is in a catalytically inactive and partially unfolded state with native secondary structure but altered tertiary structure (14). Over time, in the absence of Ca\(^{2+}\), the enzymatic loss is irreversible, and in the case of MMP-3, it results in autolysis (13).

In the GVSK mutant, amino acids 159 (Gly to Val) and 208 (Ser to Lys) in the catalytic domain have been mutated. Gly\(^{159}\) is highly conserved among MMP proteins (11). Gly\(^{159}\) is located between β-sheet strands III and IV in the S-loop, and by coordinating the Calcium 3 (18) calcium molecule along with five other amino acids (Asp\(^{156}\), Gly\(^{157}\), Asn\(^{161}\), Asp\(^{179}\), and Glu\(^{182}\)), it facilitates the stabilization of the S-loop to the underlying β-sheet strand IV (11, 18) which, in turn, is critical for tertiary structure stabilization. Hence, Gly\(^{159}\) plays a critical role in Ca\(^{2+}\) binding, and it is not surprising that mutations at that position would have an effect on Ca\(^{2+}\) binding and affinity. It is possible that the substitution of the larger, hydrophobic side chain of valine for the hydrogen molecule of glycine disrupts the structure of the Ca\(^{2+}\) binding pocket such that its affinity for Ca\(^{2+}\) is altered. At higher concentrations of Ca\(^{2+}\), the tertiary structure is stabilized, but as the concentration decreases, Ca\(^{2+}\) does not bind as efficiently. Concomitant with decreased Ca\(^{2+}\) binding would be the loss of tertiary structure and the subsequent inactivation and autolysis of the protein that we observed. Interestingly, a number of other mutants that emerged from our screen had mutations in the other amino acids involved in the coordination of this Ca\(^{2+}\) molecule (Asp\(^{156}\), Asp\(^{179}\), and Glu\(^{182}\) (data not shown)).

Although GVSK was highly dependent on the Ca\(^{2+}\) concentration for activity, the concentration of Zn\(^{2+}\) had no apparent effect. Ser\(^{208}\) is located adjacent to His\(^{209}\), which is directly involved in the binding of the catalytic Zn\(^{2+}\) molecule. Although His\(^{209}\) is strictly conserved among the MMP proteins, Ser\(^{208}\) is not even highly conserved (11). It is not surprising then that a mutation at this position would not affect the activity of GVSK as a function of Zn\(^{2+}\) because the position does not appear to be important based on sequence homology among the family members. It is not possible, however, to rule out the fact that this mutation may have an indirect effect on the Ca\(^{2+}\) sensitivity.
Although MMP-1 and GVSK were active in vivo at both the low and the high calcium concentrations, GVSK formed higher molecular weight complexes with α2-macroglobulin at a faster rate as compared with MMP-1. The formation of these higher molecular weight complexes in vitro correlated with the loss of enzymatic activity. This inactivation of GVSK occurred much faster in low calcium as compared with high calcium. It is possible that the structure of GVSK both in vitro and in vivo in low calcium is altered such that it is more susceptible to α2-macroglobulin recognition and subsequent inactivation. The inactivation of GVSK and MMP-1 may be one of the primary mechanisms of MMP-1 regulation in the extracellular matrix of the skin. The regulation of MMP-1 by α2-macroglobulin in acute and chronic skin wounds has previously been proposed (26).

Excessive deposition of extracellular matrix proteins is the predominant characteristic of many fibrotic diseases. The range of affected organs and tissues is large and includes a host of diseases with high unmet medical needs that range from pulmonary, liver, and kidney fibrosis to localized scleroderma, Peyronie disease, Dupuytren contracture, hypertrophic scarring, and keloids. The key molecule involved in the pathogenesis of tissue fibrosis in fibrotic diseases is TGF-β (27, 28).
signaling in fibroblasts results in the production of collagens and other extracellular matrix proteins. Concomitant with the increase in extracellular matrix proteins is the decrease in the synthesis of MMPs and the increase in their inhibitors, the tissue inhibitors of metalloproteinases, or TIMPs (29). This decrease in the expression of MMP-1 may be a major contributing factor that allows for the establishment and progression of fibrosis, and it follows that MMP-1 treatment could result in the attenuation of fibrosis.

The potential ability of MMP-1 to treat fibrotic diseases was first demonstrated in an animal model of liver fibrosis in which adenovirus-driven expression of the proMMP-1 was able to decrease fibrosis (30). Subsequently, recombinant human MMP-1 protein was shown to decrease fibrosis in an animal model of muscle injury when directly injected into the damaged area (31, 32).

In this study, we have added to the evidence supporting the possible use of MMP-1 in the treatment of fibrotic diseases by showing that keloidal collagen can be degraded with GVSK. Keloids may represent an ideal treatment scenario that could benefit from such a conditionally active protein. For example, when injected into a keloid, the activity of GVSK in a high Ca²⁺ buffer would be restricted to the desired treatment area because diffusion of the enzyme from this area would cause it to encounter the lower, physiological Ca²⁺ concentration and be inactivated. This would be a distinct advantage over MMP-1 whose activity would not be inhibited by physiological Ca²⁺ levels and which upon diffusion could cause collagen degradation peripheral to the desired treatment area. With the availability of these and other models of fibrotic diseases (33–37), there are many potential opportunities to determine whether a conditionally active form of human MMP-1, such as GVSK, whose activity can be tightly regulated with Ca²⁺, will prove to be an effective treatment for therapeutic applications including fibrotic diseases.

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