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Type I Collagen Stabilization of Matrix Metalloproteinase-2

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The activity of matrix metalloproteinase-2 (MMP-2) is regulated stringently on the posttranslational level. MMP-2 efficiently undergoes autolysis into inactive polypeptides *in vitro,* prompting the hypothesis that MMP-2 autolysis may function as an alternative mechanism for posttranslational control of MMP-2 *in vivo.* Moreover, MMP-2 binds to intact type I collagen fibrils; however, the functional consequences of this interaction have not been fully elucidated. To test the hypothesis that MMP-2 binding to type I collagen functions as a positive regulator of MMP-2 proteolytic potential, the effect of type I collagen on MMP-2 activity, inhibition by tissue inhibitor of metalloproteinase-2 (TIMP-2), and enzyme stability was examined. Here, we report that purified MMP-2 binds but does not cleave intact type I collagen. The presence of type I collagen affects neither enzymatic activity against a quenched fluorescent peptide substrate nor the kinetics of inhibition by TIMP-2. However, MMP-2 is stabilized from autolysis in the presence of type I collagen, but not by elastin, fibrinogen, or laminin. These data provide biochemical evidence that MMP-2 exosite interactions with type I collagen may function in the posttranslational control of MMP-2 activity by reducing the rate of autolytic inactivation. © 2001 Academic Press

Key Words: **type I collagen; matrix metalloproteinase-2; gelatinase A; proteinase.**

Matrix metalloproteinase-2 (MMP-2,[2] gelatinase A) is a member of a family of zinc-dependent metalloendopeptidases that functions in the degradation of collagen types IV, V, VII, X, and XIV, gelatins, elastin, fibronectin, and aggrecan (1–3). The proteinase is composed of five structural domains including an amino-terminal propeptide, a zinc-binding catalytic domain, fibronectin type II (FNII) repeats, a hinge region, and a carboxyl-terminal hemopexin-like domain (1–3). Both the fibronectin type II repeats and the hemopexin-like domain have been implicated in substrate recognition and targeting (4, 5). Unike other MMP family members, MMP-2 production is largely refractory to stimulation by most biologic agents and is constitutively expressed by numerous cell types. Thus, the activity of MMP-2 is stringently regulated predominantly on the posttranslational level and many studies have focused on zymogen activation and enzyme-inhibitor binding as primary mechanisms for control of MMP-2-mediated proteolysis (reviewed in 1–3, 6). Activation of secreted proMMP-2 occurs at the cell surface via formation of a ternary complex between proMMP-2, tissue inhibitor of metalloproteinase-2 (TIMP-2) and a transmembrane MMP designated membrane type 1-MMP (MT1-MMP) (7–10). Pericellular TIMP-2 plays a dual role, as it is critical to promote ternary complex formation for zymogen activation, but can also function to inhibit either MMP-2 or MT1-MMP catalytic activity via formation of a 1:1 noncovalent inactive enzyme–inhibitor complex (7–10).

Active MMP-2 undergoes concentration-dependent autolysis at Pro^{394}–Ile^{395}, resulting in cleavage of the hemopexin-like domain from the catalytic domain (11). This observation suggests that the active state of MMP-2 is unstable in the absence of substrate and indicates that autolytic inactivation may function as an alternative mechanism for posttranslational control of MMP-2 activity (11). This hypothesis is supported by the observation that a hemopexin-like domain fragment of MMP-2 can be recovered from tumor tissues (12). Moreover, intermolecular autolytic cleavage of MMP-2 also occurs on the cell surface (13), indicating that a functional consequence of zymogen activation

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[2] Abbreviations used: MMP-2, matrix metalloproteinase-2; FNII, fibronectin type II; TIMP-2, tissue inhibitor of metalloproteinase-2; MT1-MMP, membrane type 1-MMP; APMA, amino-phenylmercuric acid; MMPI, MMP inhibitor; BSA, bovine serum albumin; DMSO, dimethylsulfoxide.

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may be the generation and sequestration of sufficient MMP-2 to support autolytic processing.

In addition to TIMP-2-mediated regulation of zymogen activation and substrate cleavage, exosite interactions between MMP-2 and pericellular macromolecules may also influence enzyme activity. Previous studies have demonstrated that the FNII-like modules inserted within the MMP-2 catalytic domain promote protein–protein interaction between MMP-2 and type I collagen fibrils, gelatins, and insoluble elastin (14–17). While binding to gelatins may be predicted to promote enzyme activity, the significance of type I collagen binding is unclear, as only avian MMP-2 has been shown to function as an interstitial collagenase (18), while MMP-2 from other species is inactive against type I collagen (19–22). However, processing of MMP-2 by neutrophil elastase into inactive products is dramatically slowed in the presence of type I collagen or gelatin, suggesting that exosite interactions between the FNII-like domains of MMP-2 and pericellular matrix proteins such as type I collagen may protect the enzyme from proteolytic degradation (23). This hypothesis is supported by studies showing that addition of soluble type I collagen or gelatin to purified MMP-2 decreased the rate of autolysis (10). Furthermore, cellular MT1-MMP-mediated proMMP-2 activation is enhanced in the presence of exogenous recombinant FNII-like domain, suggesting that disruption of the proMMP-2/pericellular collagen interaction may promote filtration of proMMP-2 into the MT1-MMP activation pathway (5). Together, these data suggest that MMP-2 binding to type I collagen functions as a positive regulator of MMP-2 proteolytic potential. To advance this hypothesis, the effect of type I collagen on MMP-2 activity, inhibition by TIMP-2, and enzyme stability was examined. These data indicate that collagen binding does not alter net MMP-2 enzymatic activity, but functions to significantly reduce the rate of autolysis.

MATERIALS AND METHODS

Materials. Purified human MMP-1, MMP-2 (TIMP-2-free), and TIMP-2 were generous gifts of Dr. Hideaki Nagase (Kennedy Institute of Rheumatology, Imperial College School of Medicine, United Kingdom). Bovine serum albumin and type I gelatin, amino-phenylmercuric acetate (APMA), dimethyl sulfoxide, and human type I collagen, fibrinogen, elastin, laminin-1, and pepsin were purchased from Sigma (St. Louis, MO). The quenched fluorescent peptide substrate, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, and peptide standard, Mca-Pro-Leu-OH were acquired from BACHEM BioSciences, Inc. (King of Prussia, PA). The serine protease inhibitor aprotinin was purchased from Alexis Biochemicals (San Diego, CA). The broad spectrum hydroxamic acid-based MMP inhibitor designated MMP INH-3850-PI (MMPI) was purchased from Peptides International (Louisville, KY).

Collagenase assays. Cleavage of native or denatured type I collagen was evaluated by incubating type I collagen or gelatin (10 ug) with the indicated concentrations of MMPs in Tris–glucose buffer (50 mM Tris, 200 mM glucose, 200 mM NaCl, pH 7.4) for 18 h (25°C), followed by electrophoretic resolution on 8–15% SDS–polyacrylamide gels (24). Collagen degradation was visualized by staining gels with Coomassie blue. Prior to incubation with collagen or gelatin, MMPs were diluted to a concentration of 1 μM and incubated for 30 min (37°C) with 2 mM APMA to promote enzyme auto-activation. Type I gelatin was prepared by thermal denaturation of human type I collagen (60°C, 20 min).

Collagen binding. Microtiter plates (high binding; Greiner, Lake Mary, FL) were coated overnight by passive adsorption at 4°C in assay buffer (100 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij-35) containing 100 μg of the indicated matrix protein. Nonspecific binding sites on coated wells were blocked by incubation with a 200-μl volume of 3% BSA in assay buffer for 2 h (37°C). To evaluate the structural integrity of immobilized collagen or gelatin, wells were incubated with MMP-2 (~1 nM, 35°C, 18 h) or pepsin (1 unit in 0.1% acetic acid, pH 2.6, 37°C, 15 min) prior to solubilization in reducing Laemmli sample dilution buffer (24) and electrophoresis on 8% SDS–polyacrylamide gels. Protein degradation was visualized by staining gels with Coomassie blue. To evaluate the activity of collagen-bound MMP-2, purified MMP-2 was activated with APMA as described above, diluted to 50 nM in assay buffer, and incubated overnight (4°C) in protein-coated wells in a 50-μl volume. Aliquots (5 μl) were removed and analyzed by gelatin substrate zymography (designated "solution phase") (25). Wells were then washed five times with 50 μl of assay buffer and bound MMP eluted by incubation with 50 μl of nonreducing Laemmli sample dilution buffer (24) for 1 h at 25°C (designated "bound") prior to evaluation by gelatin zymography. The presence of bound MMP-2 was confirmed by incubating wells overnight at 4°C with 10% DMSO to disrupt MMP-2–type I collagen interactions (16). In control experiments, wells were incubated with 50 μl of assay buffer in place of Laemmli buffer.

MMP activity assays. To quantify the activity of bound MMP-2, wells were coated with collagen (100 μg) as described above and incubated overnight (4°C) with a 100-μl volume of APMA-activated MMP-2 (10 nM) containing 1% DMSO. Unbound enzyme was removed and wells washed twice with 100 μl of assay buffer before adding 100 μl of fresh assay buffer. The plate was then switched to 37°C, the quenched fluorescent peptide substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (26) was added to wells (10 μM), and substrate cleavage was evaluated using an excitation wavelength of 326 nm and monitoring emission at 396 nm with a fluorescent plate reader (Molecular Devices, SpectraMAX Gemini). A 1% concentration of DMSO was used to ensure the solubility of the synthetic substrate without affecting the ability of MMP-2 to bind collagen (data not shown; Ref. (16)). Steady-state initial velocities of substrate cleavage were established for solution phase and bound MMP-2 over a period of 15 min. Standard curves were generated by measuring the relative fluorescence emission of the cleavage product Mca-Pro-Leu-OH in solution containing 1% DMSO. Plots were constructed of velocity (fmol/s) vs substrate (μM) using SigmaPlot software, and regression values calculated to produce kinetic constants (V_{max}, k_{cat}, and K_m). Inhibition of MMP-2 was evaluated by adding increasing amounts of TIMP-2 and monitoring initial velocity as described above. Analyses were carried out in triplicate.

Enzyme stability. The stability of bound MMP-2 was determined by incubating APMA-activated MMP-2 in protein-coated microtiter wells overnight (4°C) in the presence of 1 μg/ml aprotinin (although no contaminating serine proteases were detected in the enzyme or matrix protein preparations). The temperature was raised to 37°C for 18 h to induce MMP-2 autolysis. Following incubation, MMP-2 activity in aliquots was analyzed using the quenched fluorescent peptide substrate and by gelatin zymography as described above.

RESULTS AND DISCUSSION

Interaction of MMP-2 with Type I Collagen

To evaluate the role of type I collagen binding in regulation of MMP-2 proteolytic potential, the func-

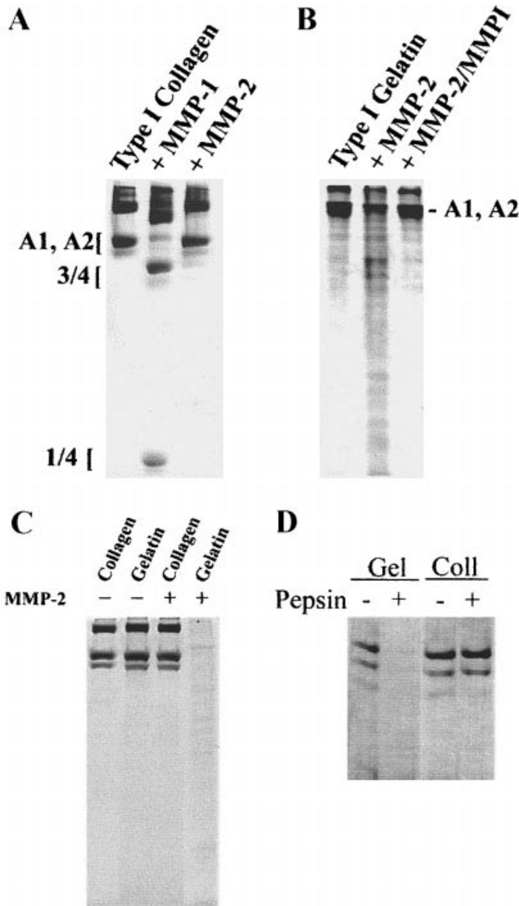


FIG. 1. MMP-2 does not cleave soluble or immobilized type I collagen. (A) Soluble human placental type I collagen (10 μg) was incubated at 25°C for 18 h in the presence of MMP-2 or MMP-1 (50 nM) as indicated. Reaction products were analyzed by electrophoresis on 8–15% gradient SDS–polyacrylamide gels followed by Coomassie blue staining. The migration positions of the $\frac{3}{4}$ and $\frac{1}{4}$ collagenase cleavage products are indicated. (B) Type I gelatin (10 μg) was incubated in the presence or absence of MMP-2 (50 nM) as indicated under conditions as described in (A). Control reactions contained the broad spectrum hydroxamic acid-based inhibitor MMPI (10 μM). (C) Collagen- or gelatin-coated microtiter wells were incubated with MMP-2 (~1 nM) for 18 h at 35°C followed by solubilization and analysis of bound protein by electrophoresis on 8% SDS–polyacrylamide gels and Coomassie blue staining. (D) Collagen- or gelatin-coated microtiter wells were incubated with pepsin (1 unit) for 15 min at 37°C followed by solubilization and analysis of bound protein by electrophoresis on 8% SDS–polyacrylamide gels and Coomassie blue staining.

tional consequences of the MMP-2–collagen interaction were evaluated. Although the gelatinolytic activity of MMP-2 is well established, evidence that MMP-2 can cleave soluble type I collagen is limited to a single report (18). To determine whether MMP-2 functions as an interstitial collagenase, type I collagen was incubated with catalytic amounts of TIMP-2-free MMP-2 or MMP-1 (collagenase-1). While MMP-1 cleaves native type I collagen into 3/4 and 1/4 alpha chain fragments, no collagen cleavage is catalyzed by MMP-2 (Fig. 1A). Equivalent concentrations of MMP-2 efficiently cleave type I gelatin (Fig. 1B), confirming that the purified enzyme is catalytically active. These results support previous conclusions that native type I collagen does not function as substrate for human MMP-2 (19–22). Thus, the interstitial collagenase activity previously attributed to MMP-2 (18) may reflect enzymatic properties unique to the avian enzyme.

As interaction of MMP-2 with type I collagen may function as a mechanism for posttranslational regulation of MMP-2 activity, association of MMP-2 with collagen immobilized on microtiter plates by passive adsorption was evaluated. To first assess the structural integrity of immobilized collagen, microtiter plates coated with collagen or gelatin were incubated with either MMP-2 (Fig. 1C) or pepsin (Fig. 1D). Whereas gelatin was susceptible to cleavage by both MMP-2 and pepsin (Fig. 1C, lane 4; Fig. 1D, lane 2), immobilized collagen was resistant to these gelatinolytic proteinases, indicating that immobilization does not result in collagen denaturation. Microtiter plates coated with type I collagen or BSA by passive adsorption were then incubated with MMP-2 overnight, and a qualitative analysis of collagen-bound MMP-2 obtained by gelatin zymography of solubilized proteins. Bound MMP-2 was detectable only in type I collagen-coated wells, but not in BSA-coated control samples (Fig. 2, lanes 2 and 4). Major autolytic fragments of MMP-2 (43/40K) lacking the hemopexin domain but retaining the FNII repeats (11) were also collagen-associated (Fig. 2). To evaluate the reversibility of binding, collagen-coated wells containing bound MMP-2 were

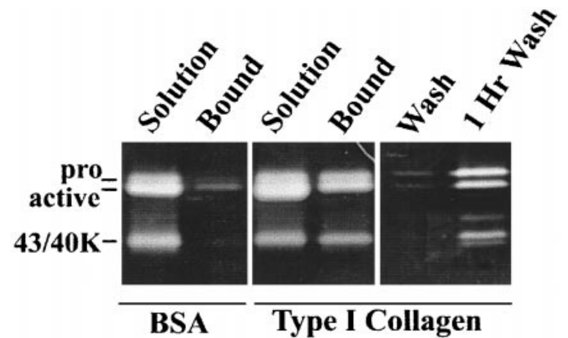


FIG. 2. MMP-2 binds to type I collagen. MMP-2 (50 nM) was preactivated with APMA as described under Materials and Methods and incubated overnight (4°C) in wells coated with type I collagen or BSA (150 μg) as indicated. Soluble enzyme was removed (designated "solution," lanes 1 and 3). After washing wells five times with assay buffer (100 mM Tris–HCl, pH 7.l5, 100 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij-35), bound enzyme was eluted using nonreducing Laemmli sample dilution buffer (50 μl, designated "bound," lanes 2 and 4). To assess the reversibility of binding, collagen-coated wells containing bound MMP-2 were washed with assay buffer as described above (lane 5), incubated for 1 h in assay buffer, and the solubilized enzyme analyzed (designated "1 h wash," lane 6). All samples were evaluated by gelatin zymography on 9% SDS–polyacrylamide gels containing copolymerized gelatin as described under Materials and Methods.

washed free of detectable enzyme, followed by incubation in assay buffer. Within 1 h significant MMP-2 is dissociated from collagen into the solution phase (Fig. 2, lane 6), indicating that MMP-2 interaction with immobilized type I collagen is reversible. These data are in good agreement with previous reports that have estimated that the dissociation constant for MMP-2 binding to type I collagen is in the low micromolar range (16, 17).

To quantify the catalytic activity of bound MMP-2, wells were coated with various matrix proteins and incubated with MMP-2 as described above. Laminin-1 and the fibrous proteins fibrinogen and elastin (27) were utilized to control for matrix specificity and nonspecific enzyme trapping within fibrous matrices. Following incubation, soluble enzyme was removed (solution phase), and the activity of bound MMP-2 evaluated by the addition of a quenched fluorescent peptide substrate (26). Type I collagen- and elastin-coated wells reproducibly retained ~20 and ~8% of total MMP-2 activity, respectively, relative to other control matrix protein surfaces upon which little enzymatic activity was detected (Fig. 3A). Gelatin zymography following solubilization of proteins confirmed the presence of collagen-bound MMP-2 activity (Fig. 3B). These data support previously published results (17) which demonstrate binding of both pro- and active MMP-2 to type I collagen and suggest that collagen binding does not negatively influence enzymatic activity.

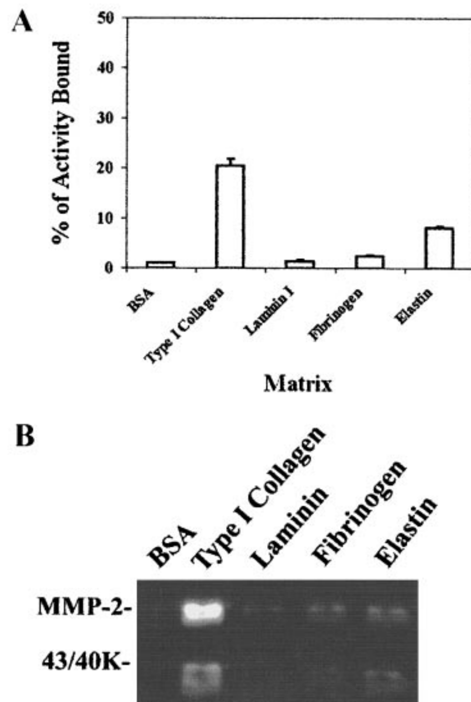
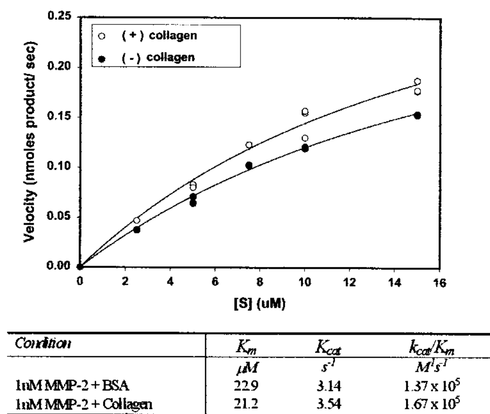


FIG. 3. Quantification of MMP-2 catalytic activity. (A) To quantify the activity of MMP-2 bound to collagen, MMP-2 (10 nM) was incubated overnight (4°C) in wells coated with type I collagen, Laminin-1, fibrinogen, elastin, or BSA as indicated. Unbound enzyme was removed, wells washed five times, and the activity of MMP-2 quantified using the quenched fluorescent peptide substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH2 (10 μM) and monitoring emission at 396 nm using an excitation wavelength of 326 nm. Steady-state velocities were established and compared with the activity of 10 nM soluble MMP-2 (designated 100%) ($n = 3$). (B) Following quantitative analysis, MMP-2 was eluted from wells (coated with specific proteins, as indicated) using nonreducing Laemmli sample buffer and analyzed by gelatin zymography.

MMP-2 Kinetic Activity in the Presence of Type I Collagen

As the FNII-like modules reside within the catalytic domain of MMP-2, enzymatic activity may be influenced by collagen binding. To test this hypothesis, the kinetics of substrate cleavage by MMP-2 were measured in the absence or presence of type I collagen using a quenched fluorescent peptide substrate. Although steady-state velocity was reproducibly higher in the presence of collagen, no significant difference in catalytic efficiency (k_{cat}/K_m) was observed (Fig. 4), indicating that overall enzyme activity is not altered by collagen binding. As MMP-2-collagen binding is of relatively low affinity (16, 17) and likely involves continual exchange of enzyme between the insoluble (bound) and solution phase (Fig. 2), these data demonstrate that transient collagen interactions do not decrease MMP-2 proteolytic potential. It should be noted, however, that minor variations in the activity of bound MMP-2 (relative to solution phase) may not be detected in this system, which evaluates the equilibrium effects of collagen binding on catalytic activity.

In addition to substrate cleavage, interaction of MMP-2 with its primary inhibitor TIMP-2 may also be influenced by collagen binding (28, 29). To address this possibility, MMP-2 was incubated with increasing molar amounts of TIMP-2 in the presence or absence of collagen, and the remaining enzymatic activity evaluated (Fig. 5). TIMP-2 effectively inhibited MMP-2 activity at a MMP-2:TIMP-2 molar ratio of approximately 1.2:1 and this ratio was unaltered by collagen. Further, no differences were observed in the rate of inhibition (not shown), indicating that collagen binding does not restrict access of the inhibitor to the enzyme active site. However, as indicated above, the dissociation of MMP-2 from collagen fibers during the time course of the assay may mask subtle differences in inhibition kinetics. In this regard, it is interesting to note that inhibition of MMP-2 by membrane-localized TIMP-2 is slowed in the presence of soluble type I collagen or gelatin (10). These differences may reflect collagen-induced changes in enzyme stability during the time course of the assay, as stabilization against autolysis would contribute to overall higher levels of catalytic activity.



Condition	K_m	K_{cat}	k_{cat}/K_m
	μM	s^{-1}	$M^{-1}s^{-1}$
1nM MMP-2 + BSA	22.9	3.14	1.37×10^5
1nM MMP-2 + Collagen	21.2	3.54	1.67×10^5

FIG. 4. MMP-2 kinetic activity is not altered by type I collagen. Microtiter plates were coated with type I collagen or BSA (100 μg) as indicated. Wells were loaded with increasing concentrations of quenched fluorescent substrate (0–15 μM, as indicated) and substrate cleavage initiated by the addition of APMA-activated MMP-2 (10 nM). Substrate cleavage was monitored as described in Fig. 3, and kinetic parameters analyzed by nonlinear regression of the velocity data. Kinetic constants of substrate cleavage in type I collagen- or BSA-coated wells are summarized in the inset.

Type I Collagen Stabilizes MMP-2 against Autolysis

Previous studies have demonstrated that MMP-2 undergoes autolytic processing in solution phase, resulting in a truncated enzyme with reduced or abrogated catalytic activity (10, 11). To evaluate the effect of collagen binding on enzyme stability, MMP-2 autolysis was examined in the presence of collagen or control matrix proteins. Binding of activated MMP-2 to immobilized proteins was carried out at 4°C, followed by incubation at 37°C to promote enzyme autolysis. In solution phase, a significant decrease in activity is observed following incubation for 16 h at 37°C (Fig. 6A). However, in the presence of type I collagen, MMP-2 activity is stabilized (Fig. 6B). This effect is specific to type I collagen, as neither laminin-1, fibrinogen, nor elastin enhanced MMP-2 stability (Fig. 6B). This result was confirmed using the quenched fluorescent peptide substrate, demonstrating a statistically significant retention of MMP-2 catalytic activity (Table I). Together these data indicate that although collagen binding has no effect on MMP-2 catalytic activity or TIMP-2 inhibition, proteolytic potential may be en-

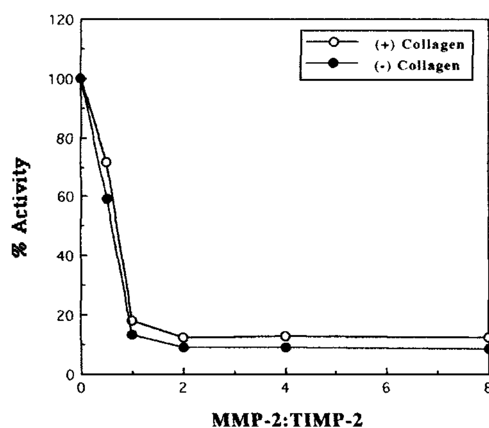


FIG. 5. Effect of type I collagen on MMP-2 inhibition by TIMP-2. MMP-2 (10 nM) was allowed to bind to collagen-coated wells overnight (4°C) before addition of the quenched fluorescent peptide substrate (10 μM) and the indicated molar ratio of TIMP-2. Steady-state velocity was calculated as described under Materials and Methods. Data are plotted as percentage activity, with MMP-2 activity in the absence of TIMP-2 designated as 100%.

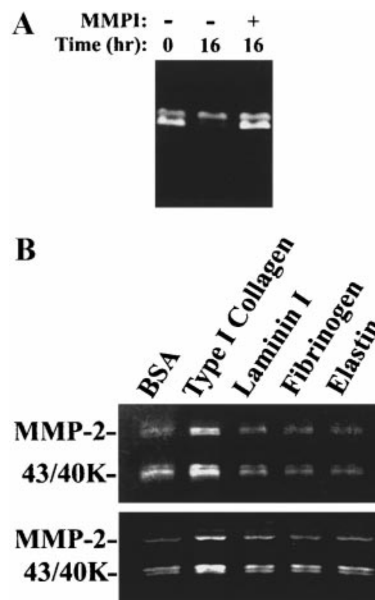


FIG. 6. Collagen stabilizes MMP-2 against autolysis. (A) APMA-activated MMP-2 (10 nM) was incubated for 16 h at 37°C in the absence or presence of the MMP inhibitor MMPI (10 μM) as indicated to prevent autolysis. (B) Microtiter wells were coated with the indicated matrix proteins (100 μg), incubated with MMP-2 (10 nM) overnight (4°C), and shifted to 37°C for 18 h to promote enzyme autolysis. Dimethyl sulfoxide (10% final concentration) was added to wells to elute bound MMP-2 and duplicate samples were analyzed by gelatin zymography (top and bottom).

TABLE I

Type I Collagen Stabilizes MMP-2 Activity[a]

Condition	Velocity (fmol/s)	Product generated (pmol ± SD)
Bovine serum albumin	66.9 ± 1.1	206.9 ± 9.0
Type I collagen	118.5 ± 2.1	340.9 ± 4.3
Laminin I	86.7 ± 3.6	242.8 ± 5.1
Fibrinogen	80.6 ± 2.9	224.1 ± 9.6
Elastin	90.4 ± 3.9	241.5 ± 7.4

[a] Microtiter wells were coated with the indicated matrix proteins (100 μg), incubated with MMP-2 (10 nM) overnight (4°C), and shifted to 37°C for 18 h to promote enzyme autolysis. Substrate (7.5 μM) was added and steady-state reaction velocities and product generation were evaluated for 45 min (N = 3).

hanced in the presence of collagen due to protection from autolytic inactivation.

It has previously been reported that high concentrations (0.5 mg/ml) of soluble type I collagen stabilize MMP-2 activity (10). Our data support these findings and demonstrate that immobilized collagen, which may mimic more closely the insoluble collagen structures found *in vivo,* can also potentiate MMP-2 activity. We hypothesize that as MMP-2 binds to immobilized type I collagen, the concentration of solution-phase enzyme is decreased, subsequently reducing the extent of soluble enzyme autolytic turnover. Further, these data demonstrate that type I collagen may play a dual role in regulation of MMP-2-mediated pericellular proteolysis. Initially, cellular binding to collagen or experimental engagement of collagen binding integrins using immobilized antibodies induces MT1-MMP-mediated activation of proMMP-2 in a variety of cell types (5, 30–35), thus increasing the concentration of active pericellular MMP-2. Subsequently, binding of the active proteinase to this integrin-associated collagen can further potentiate substrate cleavage by stabilizing active MMP-2 against autolytic inactivation. Based on these data, it is interesting to speculate that binding to type I collagen may function as an additional post-translational mechanism for regulation of MMP-2 activity by enhancing enzyme stability as well as increasing the proximity of active proteinase to potential extracellular matrix substrates.

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