Directed Mutagenesis Reveals That Two Histidines in Tissue Inhibitor of Metalloproteinase-1 Are Each Essential for the Suppression of Cell Migration, Invasion, and Tumorigenicity

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Abstract
Tissue inhibitor of metalloproteinases (TIMPs) are secreted proteins that regulate the activity of metalloproteinases, enzymes important in development, tissue remodeling, angiogenesis, and tumorigenesis. To assess the importance of three highly conserved amino acids, His7, Asp16, and His95, in determining the biological properties of mouse TIMP-1, they were mutated into Arg, Tyr, and Arg, respectively. Recombinant vectors constructed to express the wild-type and mutant TIMP-1 proteins under the control of the metallothionein promoter were transfected into mouse melanoma B16F10 cells, which produce very little TIMP-1. Individual clones were isolated and characterized by Southern, Northern, and Western blotting to verify the presence of the TIMP-1 minigene and its expression. Analyses of conditioned media for collagenase-inhibiting activity indicated that both histidine mutants, but not the aspartic acid mutant, were functionally impaired. An investigation of the cell migration, matrix invasion, and tumor formation capabilities of several individual clones representing each of the mutants revealed that the His7Arg and His95Arg mutations, but not the Asp16Tyr mutation, largely abolished the ability of the protein to inhibit all of these activities. These data establish that for B16F10 cells, endogenously generated TIMP-1 is an effective inhibitor not only of matrix invasion and tumorigenicity but also, unexpectedly, of cell motility on plastic. The novel finding that both His7 and His95 are separately essential for significant TIMP-1 activity in vivo provides an important new insight into TIMP-1 function.

Introduction
The MMPs constitute a family of enzymes that includes collagenases, stromelysins, and gelatinases (1, 2). These proteins are important both to the development, differentiation, and growth of the body, and to the tissue remodeling that occurs during normal physiological processes (e.g., bone turnover and breast and uterus involution) and wound healing. They are involved in various pathologies, including metastatic disease, periodontal disease, rheumatoid arthritis, and osteoarthritis (3). Because the MMPs are effective at degrading various components of the ECM, collagen and elastin for example, which are resistant to many proteinases, they are, singly or in combination, important in the penetration of cells through the basement membrane and ECM that separate many tissues (4, 5).

TIMPs are a family of proteins that are capable of inhibiting the activity of the MMPs (6). Three members are known: TIMP-1 (~28 kDa, glycosylated), TIMP-2 (~21 kDa, nonglycosylated), and TIMP-3 (~24 kDa, nonglycosylated). They bind via the N-terminal domain of the inhibitor with high affinity (Kn ~ 1 nM) to the active site of the MMP, and they can be recovered from the complex in an unaltered, fully active form. The three TIMPs differ in that they interact with the MMPs and components of the ECM in different ways. TIMP-1 and TIMP-2 form specific complexes with the proenzyme forms of the 92-kDa (MMP9, gelatinase B) and 72-kDa (MMP2, gelatinase A) type IV collagenases, respectively; formation of the complex involves the C-terminal domains of both the enzyme and the inhibitor and may stabilize the proenzyme, possibly retarding activation and/or inhibiting autodegradation (7–11). TIMP-3 has a strong affinity for components in the ECM (12, 13).

TIMP-1 was first purified both as a collagenase inhibitor from human amniotic fluid (14) and as EPA, a protein purified from medium conditioned by a T-lymphoblast cell line infected with HTLV-II (15). Both TIMP-1 and TIMP-2 have the ability to stimulate the growth of erythroid progenitors (or burst-forming units-erythroid) and mature erythroid precursors (colony-forming units-erythroid; Refs. 16, 17). They can also stimulate the proliferation of various other cell types (18, 28).

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3 The abbreviations used are: MMP, matrix metalloproteinase; ECM, extracellular matrix; TIMP, tissue inhibitor of metalloproteinases; EPA, erythroid potentiating activity; HTLV-II, human T-cell lymphotropic virus type II; wt, wild type.
TIMP-1 transcription is enhanced by serum-stimulation of quiescent fibroblasts (20) and in cells infected by HTLV [the latter because of the stimulation of transcription of the TIMP-1 gene by the viral transactivator Tax (21)]. It is possible that enhanced EPA/TIMP-1 expression may contribute to the pathology of HTLV infection, adult T-cell leukemia, via its ability to stimulate cell proliferation. TIMP-3 can accelerate the development of the transformed phenotype of chick embryo fibroblasts infected with a temperature-sensitive Rous sarcoma virus and stimulate the proliferation of nontransformed cells under growth-limiting conditions (22). The ability of all the TIMPs to inhibit all the MMPs makes them effective inhibitors of the invasive ability of cells, because invasion entails the MMP-facilitated penetration of basement membranes and ECM (23, 24). The invasive activity of cells is determined, in part, by the balance between the metalloproteinase levels and the TIMP levels. TIMP-1 and TIMP-2 can also act as tumor suppressors, blocking the development of a tumor, possibly by inhibiting angiogenesis (24–29).

The three members of the TIMP family have a highly conserved structure with significant regions of substantial amino acid homology and six disulfide bonds that define two domains in the protein, each consisting of three disulfide closed loops (30, 31). Three of the disulfide bonds (see Fig. 1) define an N-terminal ½ segment, which has the metalloproteinase inhibitor activity that is determined, in part, by a highly conserved N-terminal sequence, amino acids 1–22 (32, 33). The purpose of the present study was to alter certain of the conserved amino acids (His7, Asp16, and His95) in mouse TIMP-1 and ascertain the consequences of those mutations on the various biological activities of TIMP-1 when expressed in the B16F10 melanoma cells. In the N-terminal domain, His7 is absolutely conserved among the 12 TIMPs whose sequences have been determined, Asp16 is replaced by Glu in ovine TIMP1, and His95, although totally conserved in the nine TIMP-1 and -2 sequences, is Tyr in the three sequenced TIMP-3 species (6, 13, 34–36).

**Results**

**Characterization of wt and Mutant TIMP Clones.** The mouse B16F10 melanoma cell line was transfected with the mammalian expression vector pNMH encoding either wt mouse TIMP-1 or a mutated single amino-acid substituted TIMP-1 designated H7R, D16Y, and H95R in which the histidine, aspartic acid, and histidine residues at positions 7, 16, and 95 had been changed to arginine, tyrosine, and arginine, respectively, as shown in Fig. 1. B16F10 cells, which are highly metastatic and tumorigenic (37), were chosen for this study because previous research had shown that elevation of the otherwise low expression of TIMP-1 by means of a recombinant expression vector could reduce their tumorigenicity, but, interestingly, not their ability to extravasate (26, 27, 38). Thus, B16F10 would appear to be a suitable cell line for investigating differences in the biological activity of mutated forms of TIMP.

After transfection of B16F10 cells with wt and mutant TIMP expression vectors, 10 individual neomycin-resistant clones originating from cells transfected with pNMH alone (controls) or with each of the constructs expressing either wt or mutant...
TIMP-1 sequences were screened for TIMP-1 mRNA expression by Northern blotting. Two or three clones of each construct were selected as suitable transfectants based on their consistently elevated levels of TIMP-1 mRNA and, in most cases, protein. Many clones exhibited a constitutively enhanced increase in TIMP-1 expression, and a few could be further up-regulated by Cd²⁺. In the experiments reported below, the cells were exposed to 1 µM CdSO₄; treatment with CdSO₄ did not affect the behavior of the B16F10 cells or the endogenous TIMP-1 mRNA level.

Fig. 2 shows Northern, Southern, and Western blot analyses of the clones. The pNH1 clones are B16F10 cells transfected with the plasmid vector DNA alone; they exhibit (Fig. 2A) a single species of TIMP-1 mRNA at ~0.9 kb at a level similar to that in the parental cells. Lanes labeled H7R, D16Y, and H95R contain RNA from individual clones transfected with the designated mutant, either induced (+) or not induced (−) with Cd²⁺. The two wt clones are B16F10 cells expressing a nonmutated TIMP-1; wt 4 was generated as part of our work, whereas wt 6–5 was provided by R. Khokha (Ontario Cancer Institute, University of Toronto, Toronto Ontario, Canada) (26). The increased size of the TIMP-1 transcripts in the two clones illustrated here and which was also apparent in Khokha et al. (26) presumably is the consequence of the process by which the transfected DNA was concatemerized and integrated into the cellular DNA; other wt clones generated transcripts of the expected size. The appearance of RNA species at higher molecular weights was reproducible within any one clone, but variable among the clones. The absence of the endogenous 0.9-kb TIMP-1 mRNA species in these wt clones suggests the intriguing possibility that expression from the recombinant minigene is suppressing expression of the endogenous TIMP-1 gene by an autoregulatory mechanism.

All of the clones were characterized by Southern analysis (Fig. 2B) of an EcoRI digest of the genomic DNA. The EcoRI fragments corresponding to the endogenous gene are approximately 8 and 3.1 kb in size, whereas the rearranged expression cassette is 2.3 kb. At least one copy, and in most cases multiple copies, of the expression cassette was found in each of the clones studied. H95R 2 had a large number of copies, whereas the H7R 6 clone had only a few copies. There was no evident correlation between the apparent genomic copy number and the level of TIMP-1 mRNA or protein, suggesting again the existence of a mechanism limiting TIMP-1 expression.

The fully glycosylated wt TIMP-1 seen in the Western blots, indicated in Fig. 2C (arrow), migrated in our 12.5% SDS-PAGE system with an apparent molecular weight of ~31 kDa. (The signal seen at ~43 kDa appears unrelated to TIMP-1.) Most of the clones secreted TIMP-1 in amounts sufficient for it to be detected, and in a few cases the level was increased with cadmium induction. The presence of multiple bands, e.g., in the wt 6–5 and H7R 6 clone, likely reflects variable glycosylation, which has been reported previously and is suggested to be the result of variations in Golgi trafficking and posttranslational modification (33). The media from the D16Y mutants gave a particularly weak signal despite the fact that the cells possessed respectable amounts of mRNA and, as shown below, exhibited phenotypes consistent with the production of active TIMP-1.

Samples of the conditioned medium similar to those shown in Fig. 2C were analyzed for their ability to inhibit collagenase activity. We chose representatives of each mutant that by Western blot analysis appeared to produce the largest amount of TIMP-1 protein. To estimate TIMP activity in the conditioned medium, samples normalized to the same protein concentration, which correlated well with the number of cells, were analyzed for their ability to inhibit collagenase as revealed by the extent of cleavage of the β, α₁, and α₂ subunits of tritiated type I collagen. In preliminary experiments, the concentration of collagenase just sufficient to cleave fully the [³H]collagen substrate was established, thus maximizing the sensitivity of the assay for TIMP inhibition. This is confirmed by the two lanes labeled 0.25 and 1.0 collagenase in Fig. 3 that show complete cleavage by 1.0 µg of collagenase (in 100 µl) but incomplete cleavage by 0.25 µg.

Analysis of the autoradiograms shown in Fig. 3 indicated that collagen cleavage (by 1.0 µg of collagenase) was inhibited to approximately the extent shown below each lane in reactions containing equivalent amounts of conditioned medium. Media conditioned by the two clones expressing wt TIMP-1 (6.5 and 4) and the D16Y mutant were more effective at inhibiting collagen cleavage than were media from the mutant H7R 1 and the controls (B16F10 and the vector control pNH1.2). Very little inhibitory activity was detected in medium conditioned by H95R 2 despite the strong signal seen in the Western blot analysis in Fig. 2C. These results suggest that both the H7R and H95R mutations significantly impair the collagenase-inhibiting capacity of TIMP-1. The absence of any inhibition attributable to the wt TIMP-1 gene in the B16F10 cells may be, as discussed above, because overexpression of the mutant minigene has suppressed expression from the endogenous gene.

Migration Ability of the Mutant TIMP-1 Clones. The ability of the cells to migrate on "conditioned" plastic was assessed by quantifying the extent to which they could emigrate from a monolayer into an adjacent area from which the cells had been removed by scraping. Pictures typical of the patterns seen with the different clones are shown in Fig. 4A, and the data from a typical experiment are summarized in Fig. 4B. The distance migrated was determined as that distance from the scraped edge of the monolayer that included ~90% of the migrating cells. (In other experiments, we established that the growth rates of cells expressing the different mutants did not differ appreciably from wt TIMP-1; there were also no obvious differences in their ability to adhere to plastic.) These data show that B16F10 cells expressing either wt TIMP-1 or the D16Y mutant were significantly impaired in their ability to migrate into the denuded area compared with the vector control clones and the H7R and H95R mutants.

Invasive and Tumorigenic Ability of the Mutant TIMP-1 Clones. The invasiveness of the transfected cells was measured by plating [³H]thymidine-labeled cells on a Matrigel-coated membrane in a transwell chamber. The invasion index for the individual clones was determined from the percentage of the total radioactivity that was found in the
**Fig. 2.** Northern, Southern, and Western blot analyses of selected clones of B16F10 cells. A, total RNA, isolated from B16F10 cells, from cells transfected with vector alone (pNMH) and with vectors expressing wt (WT) or mutant TIMP-1 constructs, cultured in the presence (+) or absence (−) of 1 mM CdSO₄, was electrophoresed, blotted, and probed for TIMP-1 mRNA. Approximate sizes of the RNAs in kilobases are indicated on the right. The Northern blot was also probed for actin to confirm the quality of the RNA and the uniformity of loading. B, for this Southern blot, DNA purified from each clone was cleaved with EcoRI, electrophoresed, blotted, and probed with a TIMP-1 cDNA. Lambda HindIII molecular weight markers in kilobase pairs are shown on the right. The endogenous TIMP-1 bands are at 8.0 and 3.1 kbp; the unarranged EcoRI cassette containing the TIMP-1 cDNA is at 2.3 kb (24, 43). C, samples of serum-free media conditioned by the cells in the presence of 1 mM CdSO₄ were concentrated ×30 for this Western blot analysis. Equal amounts of protein were electrophoresed, blotted, and probed with mouse TIMP-1 antiserum. The arrow marks the position of the largest and most abundant of the TIMP-1 species seen in these blots. The location of molecular weight markers in kDa is shown on the right.
Fig. 3. Analysis of collagenase-inhibitor activity present in media conditioned by wt and mutant TIMP-1 clones. The medium in each of the seven lanes on the left is indicated at the top, and each reaction had 1.0 μg of activated collagenase per 100 μl. The three rightmost lanes contain [3H]collagen alone and [3H]collagen with 1.0 and 0.25 μg of collagenase. Reactions were performed and analyzed as described in "Materials and Methods." The positions of the major collagen species (β, α1, and α2) are indicated on the left, and the major cleavage products (α1B, α1A, α2, and α2A) are indicated on the right. Densitometric analysis indicated the relative amount of radioactivity in each of these species in the gel, and this allowed the approximate extent of degradation of each of the chains (β, α1, and α2) to be determined: from an average of these numbers, the extent of inhibition of collagenase was estimated and is recorded at the bottom of each lane.

lower chamber after 72 h. Data from a typical experiment are illustrated in Fig. 5A. The level of invasion for B16F10 is defined as 100% to facilitate comparison among experiments. The control pNH clones, as well as the H7R and H95R mutants, were as invasive as the B16F10 cells, whereas clones expressing wt TIMP-1 and the D16Y mutant were significantly less invasive. The invasion indices were reproducible in independent experiments, each time done in duplicate.

Fig. 5B shows that all the clones were capable of forming tumors in the syngeneic C57BL/6 mice, but with differing efficiencies. Most significantly, cells expressing either the H7R or H95R TIMP-1 mutation consistently formed large tumors as efficiently as B16F10 cells alone or transfected with the vector control. The D16Y TIMP-1 mutant was able to retard the growth of a tumor to about the same extent as wt TIMP-1. In this particular series of experiments, the wt 6–5 clone was found to be more tumorigenic than expected from past studies, both ours and Khokha et al. (1993), a result we attribute to prolonged passage of these cells in culture, possibly with a concomitant reduction in expression of the transgene.

**Discussion**

The goal of the research described here was to use site-directed mutagenesis to investigate the significance of specific highly conserved amino acids in the TIMP-1 molecule in determining certain biological properties of cells expressing that protein. This information not only will be useful in elucidating aspects of the TIMP-MMP interactions, but also will allow a determination of whether a particular activity of TIMP-1 (e.g., suppression of tumor growth or its EPA) is in fact due to metalloproteinase inhibition and not some other function (signaling via a receptor, for example). Despite the complications inherent in this approach (unpredictable effects on protein folding, glycosylation, secretion, and stability), it can provide important insights as to how the structure of the protein relates to its function.

We chose not to mutate conserved C or P residues because of concern that the structure of the protein would be severely disrupted. Instead, we selected residues that were likely to be on the surface of the protein and possibly critical for complexing with the essential Zn²⁺ in the active site of the MMP. When we began this work, H7, D16, and H95 appeared to us to be the most promising candidates for coordinating with the zinc. H7 is conserved in all 12, and D16 in 11 (it is E in ovine TIMP-1), of the TIMP cDNAs that have been sequenced, suggesting that they are of crucial importance for some aspect of TIMP-1 function. H95 is conserved in the nine sequenced TIMP-1 and TIMP-2 cDNAs, but in the three TIMP-3 sequences it is Y. This suggests that an H in this position is important for some aspect of TIMP-1 and TIMP-2 function, but that for TIMP-3, a Y is preferred, a provocative conclusion given our finding that the H95R mutation resulted in a loss of activity.

**Consequences of the Mutational Changes.** In assessing the phenotypic consequences for the B16F10 host cell of these mutations in the expressed recombinant TIMP-1, it was important to exclude the possibility that the observed differences were simply a reflection of the clonal variation inherent in the tumor cell. To do this, we selected about 10 independent G418-resistant clones resulting from the initial transfection. These were then screened by Northern and Western blots to identify clones expressing the recombinant TIMP-1 mRNA and protein; Southern blots were used to confirm the presence of the expression cassette, to determine the approximate copy number of the integrated DNA, and to verify the uniqueness of the clones. Only then were the biological activities of the clones assessed and an attempt made to determine the efficiency with which each of the mutant proteins could inhibit collagenase.

To estimate the amount of metalloproteinase inhibitor activity produced by representative mutant clones, we sought to quantify the collagenase-inhibiting activity in concentrated serum-free media conditioned by the cell lines. To do this, [3H]collagen was reacted with human interstitial collagenase (just sufficient to cleave all the collagen in the reaction) that had been preincubated with equivalent amounts of medium conditioned by the various cell lines. This assay gives us a measure of the total free TIMP activity in the medium. Assuming the absence of clonal variations, we interpret the measurements to reveal differences in the amount of TIMP-1 activity secreted by the different clones. The results were in harmony with what we observed in the assessment of the biological activities; that the D16Y mutant encoded a fully active TIMP-1, whereas both the H7R and H95R mutants encoded a TIMP-1 protein that was much less active than wt TIMP-1. It is interesting that the Western blot analysis of media conditioned by both the D16Y mutants (Fig. 2C) gave the weakest signal of all the mutants, whereas substantial...
Fig. 4. Motility on plastic of selected clones of stably transfected B16F10 cells on plastic. A, representative clones showing the migration of cells from a monolayer wounded by scraping away neighboring cells as described in "Materials and Methods." B, quantitation of the migratory capacity of the individual cell lines. Each bar on the histogram represents a single experiment; similar results were obtained in a duplicate experiment. A statistical comparison of the data for the wt and D16Y clones with the pNMH clones using the unpaired t test yielded Ps of <0.0001 and <0.0008, respectively. According to this test, the H7R and H95R measurements did not differ significantly from the pNMH measurements.
The antiserum does not recognize the mutant protein very efficiently. Because TIMP-1 is a highly conserved plasma protein, we believe that the antiserum likely recognizes a very limited repertoire of epitopes, perhaps in part involving D16. The weakness of the antiserum necessitated that we use a high concentration to get a good signal, unfortunately giving rise to significant background noise that we were unable to reduce by various strategies.

Zymograms of the conditioned media from all the cell lines revealed similar amounts of two gelatinolytic activities present to about the same extent in each case (not shown). Their apparent molecular weights suggested they corresponded to the 92-kDa (MMP 9, gelatinase B) and 72-kDa (MMP2, gelatinase A) type IV collagenases. There was insufficient TIMP-1 activity in the concentrated (serum-free) conditioned media from any of these cell lines to permit estimation of its amount in reverse zymograms.

Cells expressing TIMP-1 with the D16Y mutation were very similar to cells expressing wt TIMP-1 with regard to their limited ability to migrate on “conditioned” plastic, and also their reduced invasiveness and tumorigenicity. Thus, as far as these criteria are concerned either a D or a Y is equally permissible at position 16. Cells expressing TIMP-1 bearing either the H7R or the H95R mutation yielded TIMP-1 protein that was a less effective inhibitor than wt TIMP-1 (or the D16Y mutant) in inhibiting collagenase, in blocking migration of cells on plastic, and in suppressing matrix invasion and tumorigenicity. We conclude that histidines in positions 7 and 95 are preferable to arginines if TIMP-1 is to be able to function as an inhibitor of these activities. Inhibition of migration may reflect a requirement for metalloproteinase activity in the breakdown of the focal adhesions via which the cells attach to the plastic (39). Inhibition of invasion is consistent with the known involvement of metalloproteinases in the breakdown of the ECM. Because the development of a full-fledged tumor is believed to depend on angiogenesis, and because metalloproteinase activity is involved in angiogenesis, we suggest that suppression of tumor development by TIMP-1 reflects its anti-angiogenic activity (29, 40).

TIMP-1 has, in certain circumstances, a growth-promoting activity: the EPA. A number of cell types have been shown to respond to TIMP-1 with increased proliferation (18, 19). Measurements of the growth curves of the cell lines expressing the different TIMP-1 mutants did not differ significantly from the parent cell (data not shown). Thus, at least for
B16F10 cells, TIMP-1 does not appear to influence the proliferative ability of the cells. It remains to be seen whether a cell known to exhibit increased proliferation in response to TIMP-1 will respond differently to any of the TIMP-1 mutants.

Comparison with Other Studies. The current paradigm is that occupation of the active site in the MMP by TIMP entails multiple contacts between the two proteins and the formation of a coordination complex with the Zn$^2+$, most likely involving the highly conserved region between C3 and C13 of the TIMP protein. Bodden et al. (41) investigated the ability of peptides representing different segments of the TIMP-1 protein to compete with the binding of TIMP-1 to collagenase, and although long peptides (20–34 aa) implicated much of the TIMP-1 protein in binding, short peptides (7–10 aa) revealed that the central region encompassing the C3-C9, C13-C124, and C127-C173 disulfide linkages (see Fig. 1) was particularly important in binding and also contained sequences able to inhibit collagenase.

O’Shea et al. (33) studied the effects of certain amino acid changes (H7A, H7Q, H7E, Q9A, R20A, K22A, E82D, Y38V, K41A, H74A/H77A, D81A/D82A, H95A, H95Q, and W105A) on the ability of TIMP-1 to inhibit stromelysin-1 (MMP3) and putative uterine metalloproteinase (matrilysin, MMP7). Of the 14 mutations studied, which included all the histidines in the N-terminal domain conserved between TIMP-1 and TIMP-2, only those affecting H7 or Q9 caused a reduction (15–41%) in specific inhibitory activity. This was the result of an increase in the rate of dissociation of enzyme-inhibitor complexes giving rise to a higher apparent $K_i$. These mutants were also the most poorly expressed in terms of secreted protein. These data established the importance of the C3-C13 segment and revealed further that changes in any single amino acid, even the rigorously conserved H7, had a relatively minor effect on TIMP-1 activity, as assessed in vitro; each mutant was still an efficient metalloproteinase inhibitor.

This contrasts with our $in vivo$ studies, which reveal a substantial role for H7 in endowing TIMP-1 with the ability to inhibit the invasive, migratory, and tumorigenic properties of B16F10 cells.

The function of the H95 moiety is intriguing. In the test tube, both the H95Q and H95A TIMP-1 mutants were functional inhibitors, suggesting that those particular amino acid changes, at least, had no effect on the kinetics of TIMP inhibition of collagenase or putative uterine metalloproteinase (33). Yet, Williamson et al. (42), using diethylpyrocarbonate to derivatize the histidines in TIMP-1, obtained evidence that H95 and also H144 and H164 in the C-terminal domain, but not H7, were essential for MMP inhibition. H95 and H7 are separated by eight amino acids, including two prolines and the two cysteines that form the disulfide bond that links them most directly. It will be fascinating to discover how each of these two highly conserved histidines functions and what the consequences are of a histidine to tyrosine change. To do this, we will express these mutant proteins in E. coli to obtain sufficient protein to characterize biochemically.

Materials and Methods

Construction and Expression of Mutant TIMPs. The single amino acid substitutions H7 to R, D16 to Y, and H95 to R were made using the Altered Sites in vitro Mutagenesis System (Promega, Madison, WI). Briefly, the TIMP cDNA insert containing the signal sequence was cloned into the pSELECT-1 plasmid, which encodes resistance to both ampicillin and tetracycline. Two oligonucleotides, one that restores ampicillin resistance and one that introduces a mutation at the desired site (H7, D16, or H95) in the insert, were annealed to the single-stranded DNA template. Each mutant oligo (25–26 nt) contained random nucleotides at each position in the target codon. Subsequent transformations into E. coli allowed the selection of mutant clones, which were then sequenced around the target codon to ascertain the nature of the mutation. After choosing for each mutant a plasmid that contained a structurally compatible amino acid substitution, the entire TIMP-1 cDNA insert was sequenced to verify that the coding sequence was otherwise unaltered. The full-length TIMP-1 cDNA was then excised and cloned into the BamHI site downstream of the mouse metallothionein I promoter of the mammalian expression vector pNMH, which is inducible by cadmium sulfate (43). The parental cell line used was the tumorigenic and metastatic mouse melanoma B16F10. The cells were transfected by the calcium-phosphate method, and G418-resistant clones were isolated and expanded. Cells were routinely grown in DMEM (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Mediatech, Herndon, VA) and 1 mM sodium pyruvate.

RNA and DNA Analysis. Total RNA was prepared from cells in exponential growth, maintained in the absence or presence of 1 mM CdSO4 for 18 h, using TRI-Reagent (Molecular Research Center, Cincinnati, OH). RNA (10 μg) was electrophoresed on a 1% agarose gel containing 6.8% formaldehyde and transferred to GeneScreen Plus (DuPont New England Nuclear, Boston, MA). Genomic DNA was prepared by lysing the cells with 0.1 M NaCl, 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 0.5% SDS, and incubating overnight at 50°C with 0.1 mg/ml proteinase K. DNA was precipitated with 2 volumes of 100% ethanol. DNA (10 μg) was digested with EcoRI, electrophoresed on a 1% agarose gel in the presence of 0.1 mg/ml ethidium bromide, and transferred to GeneScreen Plus. Blots were hybridized with 10$^6$ cpn/ml of 32P-oligolabeled TIMP cDNA insert. The levels of mRNA were corrected for loading differences by comparing them to the level of the control β-actin mRNA.

Western Blot and TIMP Activity Analyses. Cells were grown to 80% confluence, and conditioned medium was collected after incubating the cells for 24 h in serum-free DMEM with or without 1 mM CdSO4. The conditioned medium was dialyzed (molecular weight cutoff of 12–14 KDa) against 5 mM Tris-HCl (pH 7.5) and 5 mM NaCl. The dialyzed samples were then lyophilized and resuspended in water at 1:30 the original volume. Equal amounts of protein were electrophoresed on a 12% SDS-PAGE minigel and transferred to nitrocellulose (Bio-Rad, Rockville Center, NY). The membrane was blocked overnight in 5% nonfat dry milk in TBS (0.1% Tween 20, 0.1% Tris-HCl (pH 7.5), and 0.9% NaCl) at 4°C. The primary antibody, made in rabbits against a recombinant baculovirus-produced mouse TIMP-1 (44), was incubated with the membrane at 1:50 in 2% milk in TBS at 4°C for 18 h. The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG, used at 1:3000 in 2% milk in TBS for 1–2 h at room temperature. The antibody signal was detected by enhanced chemiluminescence (Amersham, Chicago, IL).

To detect TIMP activity, samples of the 3×10$^5$ concentrated, conditioned medium (diluted 1:5 in the reaction mix, consisting of 50 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 5 mM CaCl$_2$, and 0.05% Brij-35) were incubated for 30 min at room temperature with 10 μg/ml of p-aminophenyl mercuroic acid-activated human interstitial collagenase (generously provided by Dr. Howard Weilgis, Jewish Hospital, Washington University, St. Louis, MO; 3.3 × 10$^4$ cpn of 3H-labeled rat tail type I collagen (a kind gift from Dr. Richard Berg, Collagen Corp., Palo Alto, CA) was then added, and the incubation was continued for 16 h. The samples were then diluted into ×2 Laemmli buffer, boiled for 15 min, and electrophoresed on a 7.5% SDS-polyacrylamide gel. The gels were then impregnated with PPO in DMSO and exposed to Kodak XAR-5 film for 1 month. Percent inhibition of collagenase cleavage was estimated from an average amount of cleavage of the three major forms (β, α1, and α2) as determined from the densities of the autoradiographic bands quantified by densitometric anal-

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4 Substitution of R for H and Y for D at surface locations are not expected to alter the protein conformation significantly.
ysis of the autoradiogram using an LKB Ultrascan XL Densitometer (LKB Instruments, Gaithersburg, MD).

Migration Assays. Migration of the mutant and wt cells was measured by the wounding method (45). Cells (4 × 10^6) were plated in 6-well plates in triplicate and allowed to grow for 24 h. One-half of the confluent monolayer was scraped away with a plastic cell scraper (Fisher Scientific, Springfield, NJ), the wells were washed 2 times with PBS, and the cells were incubated in serum-free DMEM containing 1 μM CaSO_4 for 40 h. Photographs were taken of the cells at the line of scraping to determine the distance the cells had migrated as measured by a micrometer ruler photographed at the same magnification.

Matrigel Transwell Invasion Assay. The cell lines were analyzed for their invasive properties using 24-well Matrigel basement membrane matrix chambers (Collaborative Research/Becton Dickinson, Bedford, MA) using a procedure adapted from Imamura et al. (46). Each chamber insert consisted of an 8-μm polyethylene terephthalate membrane coated with 100 μg/cm^2 of Matrigel. Subconfluent cultures of each cell line tested were first prelabeled with 10 μCi/ml ^3H-thymidine for 24 h. Medium (500 μ) conditioned by 373 cells was used in the bottom well as a chemotactic agent, and 1 × 10^5 labeled cells in 500 μl of serum-containing DMEM were plated on the Matrigel membrane. After incubation for 18 h at 37°C in humidified 5% CO_2, the medium in the upper well was replaced with medium containing 1 μM CaSO_4, and the incubation was continued for an additional 48 h. The medium was then removed from the upper and lower wells. The cells from each side of the membrane were trypsinized and added to the medium from their respective well. The invasion index was calculated from the amount of radioactivity in the lower well, expressed as a percentage of the sum of radioactivity in both wells:

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\% \text{ invasion} = \frac{L}{L + U} \times 100
\]

where L and U represent the amount of radioactivity in the lower and upper wells, respectively.

Tumor Formation. Male C57BL/6 mice (6–8 weeks old) were injected s.c. on both the right and left thigh with 1 × 10^6 cells that had been cultured in the presence of 1 μM CaSO_4 for 18 h. Animals were monitored for primary tumor growth at the site of injection every 2 days beginning 7 days after injection. When the tumors reached ~10 mm in diameter on the control animals, all the animals were sacrificed, and the tumor size was measured after dissection using vernier calipers.

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References


