APMA (4-Aminophenylmercuric Acetate) Activation of Stromelysin-1 Involves Protein Interactions in Addition to Those with Cysteine-75 in the Propeptide†

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ABSTRACT: Matrix metalloproteinases (MMPs) can be activated *in vitro* by multiple mechanisms such as treatment with proteases, organomercurials, oxidants, and detergents. The proposed cysteine switch model for activation suggests that these multiple methods for activation cause the dissociation of the single cysteine residue in the propeptide from the active site zinc. In particular, it has been suggested that organomercurials such as 4-aminophenylmercuric acetate (APMA) work by directly reacting with the sulfhydryl group of this cysteine residue, resulting in its displacement from the active site. However, recent data by Chen et al. [(1993) *Biochemistry 32,* 10289−10295] demonstrated that modification of this cysteine residue in the propeptide of stromelysin-1 by sulfhydryl reagents did not result in an active enzyme as predicted. To investigate the roles that this cysteine residue and the propeptide salt bridge (R74 to D79) might play in the APMA-induced activation of stromelysin-1, we have changed these residues by site-directed mutagenesis. Wild-type stromelysin-1 and the mutants were all expressed at detectable levels using a recombinant vaccinia virus system and determined to be catalytically competent by zymography. The wild-type stromelysin-1 and the cysteine mutants (C75S and C75H) underwent APMA-induced activation as determined by the characteristic reduction in molecular weight associated with activation and by their ability to cleave casein only when activated. On the other hand, mutants R74K, D79A, and C75H/D79A did not undergo APMA-induced activation. These results demonstrate that APMA-induced activation of stromelysin-1 involves protein interactions in addition to those with cysteine-75 in the propeptide and also suggest that the R74 to D79 salt bridge may play a role.

The matrix metalloproteinases (MMPs)[1] are a family of zinc-dependent endopeptidases that participate in extracellular matrix degradation and remodeling as it occurs during normal and pathological conditions. The family includes the collagenases, stromelysins, gelatinases, matrilysin, membrane-type MMPs, and metalloelastase. The MMPs share certain structural and functional characteristics that include a signal sequence, an amino-terminal propeptide domain, a catalytic zinc binding domain, a proline-rich hinge region, and a carboxyl-terminal hemopexin-like domain (Woessner, 1991; Birkedal-Hansen et al., 1993).

The propeptide domain of all the MMPs contains the highly conserved amino acid sequence PRCGVPDV with a single cysteine residue which appears to be critical for maintaining the latency of the enzymes. This cysteine residue forms a bond with the zinc atom located within the active site of the catalytic domain and is the basis for the proposed "cysteine switch" model of activation for the MMPs (Springman et al., 1990; Van Wart & Birkedal-Hansen, 1990). Activation of latent MMPs is believed to occur by dissociation of the sulfhydryl group of the cysteine from the active zinc site and its replacement with a water molecule that plays a role in catalysis (Van Wart & Birkedal-Hansen, 1990). Disruption of this Cys−zinc bond can be attained *in vitro* by sulfhydryl reagents such as heavy metal ions, oxidants, organomercurials, or iodoacetamide (Murphy et al., 1980; Weiss et al., 1985; Lindy et al., 1986; Nagase et al., 1990; Okada et al., 1990; Springman et al., 1990; Van Wart & Birkedal-Hansen, 1990; Woessner, 1991; Chen et al., 1993). Latent MMPs can also be activated by conformational changes of the polypeptide chain induced by detergents or chaotropic agents (Birkedal-Hansen & Taylor, 1982; Springman et al., 1990) and also by limited cleavage of the propeptide by proteolytic enzymes such as trypsin or chymotrypsin (Stricklin et al., 1983; Nagase et al., 1990; Okada et al., 1990). Mutational analyses have shown that not only is the Cys residue in the PRCGVPDV sequence of the propeptide critical but several other residues in this area also play a role in maintaining latency (Sanchez-Lopez et al., 1988; Park et al., 1991; Windsor et al., 1991; Freimark et al., 1994). Amino acids R74 and D79 in the PRCGVPDV

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[1] Abbreviations: ABTS, diammonium 2,2′-azinodi(3-ethylbenzthiazoline sulfonate); APMA, 4-aminophenylmercuric acetate; BUdR, 5-bromodeoxyuridine; DMEM, Dulbecco's modified Eagle's medium; mAb, monoclonal antibody; MMP, matrix metalloproteinase; pAb, polyclonal antibody; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; STI, soybean trypsin inhibitor; SL-1, stromelysin-1, MMP-3 [nomenclature according to Nagase et al. (1992)]; SL-2, stromelysin-2, MMP-10; SL-3, stromelysin-3, MMP-11; TPCK, *N*-tosyl-L-phenylalanine choromethyl ketone.

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sequence have been observed in the crystal of truncated stromelysin-1 to form a salt bridge (J. J. Birktoft, personal communication); whether or not this salt bridge plays a role in maintaining latency is unclear.

The stromelysins include stromelysin-1 (SL-1), stromelysin-2 (SL-2), and stromelysin-3 (SL-3). SL-3 was initially grouped with the other stromelysins; however, it seems that it is quite different from SL-1 and SL-2 (Basset et al., 1990; Murphy et al., 1991), especially in regard to substrate specificity (Pei et al., 1994) and its ability to be activated by furin (Pei & Weiss, 1995). SL-1 like most of the other MMPs can be activated *in vitro* by treatment with proteolytic enzymes, by organomercurials, or by heat (Gruber et al., 1989; Okada et al., 1989; Nagase et al., 1990; Koklitis et al., 1991). The activation of SL-1 by proteinases and organomercurials has been shown to occur in a stepwise process which generates intermediate forms before the stable active form is apparent (Okada et al., 1989; Nagase et al., 1990). The proteolytic enzymes cleave within a susceptible region of SL-1 involving residues Phe34-Asp39, while the organomercurials induce an initial autocleavage between residues Glu68 and Val69. Therefore, exposure to either proteinases or organomercurials results in conversion of SL-1 to intermediate forms of M_r 53 000−46 000 before the generation of the "active" M_r 45 000 form that results from an autocatalytic cleavage between residues His82 and Phe83. Recent evidence suggests that the organomercurial 4-aminophenylmercuric acetate (APMA) activation of SL-1 may not only be a result of its binding to the cysteine as predicted by the cysteine switch model but rather that APMA first induces a conformational change in the propeptide that is then followed by APMA binding to the propeptide cysteine (Chen et al., 1993). It was also reported that chemical modification of this cysteine in the propeptide of SL-1 by various sulfhydryl reagents did not produce an active enzyme and that these modified enzymes could still be activated by APMA (Chen et al., 1993).

In this paper, we examine the involvement of the propeptide cysteine residue and the R74 to D79 salt bridge in the APMA-induced activation of SL-1. To accomplish this, we have expressed human SL-1 in a recombinant vaccinia virus vector system and introduced a number of mutations at Cys75 and the salt bridge in the propeptide. Our results demonstrate that APMA-induced activation of stromelysin-1 involves protein interactions in addition to those with the cysteine residue in the PRCGVPDV sequence of the propeptide. We also show that the salt bridge formed between R74 and D79 might play a role in APMA-induced activation.

MATERIALS AND METHODS

Expression System. The SL-1 cDNA from plasmid $pBSL_5$ (Whitham et al., 1986) was subcloned into the plasmid pUC119 (Vieira & Messing, 1987) between the *Eco*RI and *Bam*HI sites of the polylinker. The resulting plasmid pUCSL was filled in to make blunt-ends with Klenow (New England Biolabs; Beverly, MA), and a new *Bam*HI site was created by insertion of a *Bam*HI linker (5′ CGCGGATCCGCG 3′). The SL-1 cDNA was excised from the resulting plasmid as a 2.3 kilobase pair *Bam*HI fragment and then ligated into the *Bam*HI site of the expression vector pGEMEX-I (Promega; Madison, WI) to form plasmid pGXSL-1.

Single amino acid mutations (R74K, C75S, C75H, and D79A) in the PRCGVPDV sequence in the propeptide

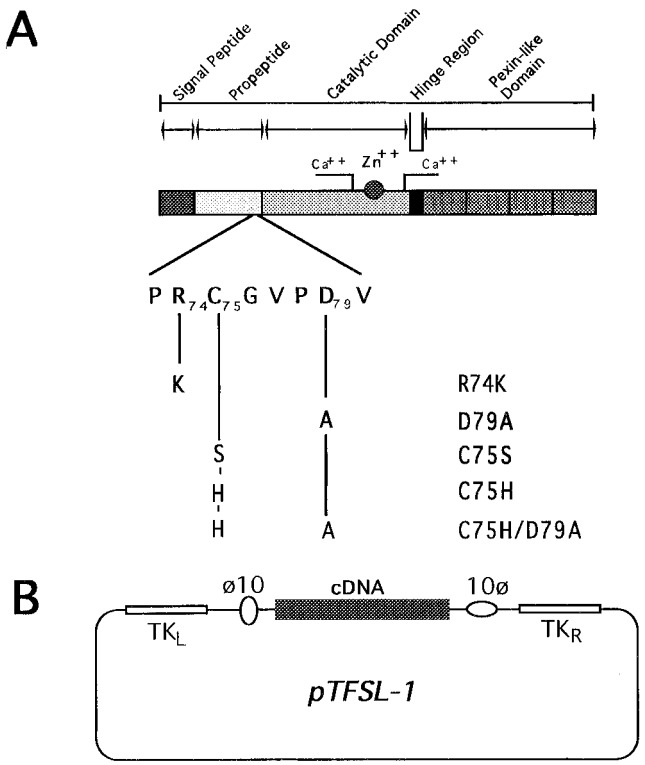

FIGURE 1: (A) Linear model of human SL-1. Mutations in the propeptide are indicated. (B) The expression plasmid. The full-length cDNA for SL-1 or SL-1 mutants (indicated by the stippled box) was placed between the bacteriophage T7 promoter ($\phi 10$) and terminator (10ϕ) and flanked by the left (TK_L) and right (TK_R) parts of the TK vaccinia gene (indicated by the open boxes) of the vaccinia vector pTF7.5.

domain of SL-1 (Figure 1A) were generated by site-directed mutagenesis (Zoller & Smith, 1983) using pGXSL-1 single-stranded DNA and the following oligonucleotide primers:

5′ GATGCGCAAACCACAATGTGGAGTTCCT 3′ (R74K)

5′ CAAGCCCAGGTCTGGAGTTCCTGA 3′ (C75S)

5′ CAAGCCCAGGCACGGAGTTCCTGA 3′ (C75H)

5′ CAGGTGTGGAGTTCCTGCTGTTGGTCACTT 3′ (D79A)

The resulting plasmids (pGXSL-1 mutants) were verified for the presence of each mutation by double-stranded DNA sequencing using Sequenase Version 2 as described by the manufacturer (United States Biochemical; Cleveland, OH). During sequence analysis, a plasmid containing a double mutation within the PRCGVPDV sequence of SL-1 was also identified (C75H/D79A).

The *Bam*HI fragment from pGXSL-1 or pGXSL-1 mutants was cloned into the *Bam*HI site of the vaccinia virus vector pTF7.5 (Fuerst et al., 1987) to create expression plasmids pTFSL-1 and pTFSL-1 mutants (Figure 1B). This resulted in the cDNA for SL-1 or SL-1 mutants being inserted into pTF7.5 between the bacteriophage T7 promoter ($\phi 10$) and terminator (10ϕ), which caused it to be flanked by the left (TK_L) and right (TK_R) vaccinia virus thymidine kinase gene sequence necessary for homologous recombination with wild-type vaccinia virus. Plasmids pTFSL-1 and pTFSL-1

mutants were confirmed by restriction enzyme digests and double-stranded DNA sequencing.

Plasmids pTFSL-1 and pTFSL-1 mutants were tested for expression of recombinant SL-1 or SL-1 mutants by transient expression assays (Fuerst et al., 1986). The plasmids (4 μg of DNA per 100 mm plate) were transfected by a calcium phosphate precipitation method (Chen & Okayama, 1987) into HEp2 cells (human epidermoid carcinoma cells) infected with the recombinant vaccinia virus vTF7.3 (which expresses the T7 RNA polymerase) (Fuerst et al., 1986). At 36−48 h after infection, the medium was screened for the presence of SL-1 or SL-1 mutants by Western blot analyses.

Cell Culture and Recombinant Vaccinia Viruses. HeLa S3 and HEp2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL; Gaithersburg, ME) containing 5% bovine calf serum (HyClone; Logan, UT). Human thymidine kinase minus (TK^-143) cells (Santoro et al., 1988) were grown in DMEM with 5% fetal bovine serum (HyClone) and 1% bovine calf serum, supplemented with 5-bromodeoxyuridine (BUdR, 25 μg/mL).

Plasmids pTFSL-1 and pTFSL-1 mutants were used to transfer the TK^- insertion into the genome of the wild-type vaccinia virus by *in vivo* homologous recombination. Monolayers of HEp2 cells were infected with wild-type vaccinia virus in serum-free DMEM. Transfection was performed 1.5 h later with 20 μg of pTFSL-1 or pTFSL-1 mutant DNA per 100 mm plate by the calcium phosphate precipitation method (Chen & Okayama, 1987). The cells were harvested at 24 h, and the cell lysates were screened for TK^- recombinants by plaque assay on human TK^-143 cells in the presence of BUdR (25 μg/mL). Virus plaques were then grown in small monolayers of TK^- cells under selective conditions. To test for their ability to express SL-1 or SL-1 mutants, HeLa S3 cells were coinfected with recombinant viruses vTFSL-1 or a vTFSL-1 mutant and recombinant virus vTF7.3. After 36 h, proteins secreted into the media were resolved by SDS−PAGE, transferred to nitrocellulose, and stained with anti-SL-1 mAb IID_4 (5 μg/mL) (Windsor et al., 1993). Viruses positive for expression of recombinant SL-1 or SL-1 mutants were then plaque purified once more in TK^- cells. To ensure that each of the recombinant viruses indeed had the expected insert and to confirm the presence of the mutations, the vaccinia virus DNA was isolated, digested with *Bam*HI, and analyzed by DNA sequencing. To accomplish this, confluent plates of HeLa S3 cells were infected with recombinant vTFSL-1 or a vTFSL-1 mutant and then the DNA was extracted by a modified procedure of Esposito et al. (1981). The sequence of the DNA, which was inserted into the vaccinia virus DNA, was analyzed by the dideoxynucleotide chain termination method using Ampli Taq DNA polymerase (Perkin Elmer Cetus; Norwalk, CT) and a sequencing primer which was 5′-end labeled with [γ^{32}P]ATP (Amersham; Arlington Heights, IL).

Expression and Purification. Fifteen 100 mm plates of HeLa S3 cells grown in DMEM supplemented with 5% bovine calf serum were transferred to a spinner flask. The HeLa cells were then infected with recombinant vaccinia virus vTFSL-1 or a vTFSL-1 mutant and then coinfected with recombinant vaccinia virus vTF7.3. At 2 h postinfection, serum-free DMEM was added to a final volume of 1 L. At 36−48 h postinfection, the medium was cleared by centrifugation and passed over an antibody affinity column prepared by coupling anti-SL-1 mAb IID_4 to CNBr-activated Sepharose 4-B (Windsor et al., 1993). The enzyme was eluted with 6 M urea in buffer (50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$) and dialyzed against the buffer to remove the urea. For other assays, 100 mm plates of HeLa S3 cells were infected with either vTFSL-1 or a vTFSL-1 mutant and coinfected with vTF7.3 in 5 mL of serum-free DMEM. After 36−48 h, the media was cleared by centrifugation.

Activation of SL-1 and SL-1 Mutants. Activation of the recombinant wild-type and mutant forms of SL-1 was accomplished by treatment with either APMA or TPCK-trypsin (Sigma; St. Louis, MO). For APMA activation, media containing the wild-type SL-1 or SL-1 mutants were incubated with a final APMA concentration of 1 mM for 12−16 h at 37 °C. Samples of the wild-type SL-1, mutant C75S, and mutant C75H were also incubated with 1 mM APMA at 37 °C for 12−16 h with and without 2 mM 1,10-phenanthroline (Sigma). For trypsin activation, media containing the wild-type SL-1 or SL-1 mutants were incubated with TPCK-trypsin at 25 °C. The trypsin reaction was then stopped by the addition of a 10-fold molar excess of soybean trypsin inhibitor (Sigma) and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma). The samples were resolved by SDS−PAGE, transferred to nitrocellulose, and probed with anti-SL-1 mAb IID_4 (5 μg/mL).

Electrophoretic Methods. Samples were separated on 10% or 12% SDS−PAGE gels according to the methods of Laemmli (1970). Proteins were detected by staining with Coomassie Blue or transferred to nitrocellulose paper (Bio-Rad; Melville, NY) by electroblotting and probed with anti-SL-1 mAb IID_4 (5 μg/mL). Alkaline phosphatase-conjugated goat anti-mouse IgG (Bethesda Research Labs; Bethesda, MD) was used as the secondary antibody and detected with 4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim Corporation; Indianapolis, IN).

Casein Zymography. The casein-cleaving ability of the enzymes was assessed by resolving samples in a 10% SDS−PAGE gel copolymerized with 1 mg of casein/mL. The gel was then washed for 20 min in 50 mM Tris-HCl, pH 7.5, and 2.5% Triton X-100; 20 min in 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, and 2.5% Triton X-100; and 20 min in 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$. The gel was then incubated in 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$ for 16 h at 37 °C and stained with Coomassie Blue to visualize the lytic bands.

Amino-Terminal Sequence Analysis. To determine the amino-terminal sequence of the APMA-activated forms of wild-type SL-1 and of the mutants at C75, culture media were incubated with 1 mM APMA at 37 °C for 12−16 h and then concentrated using Centriprep columns (Amicon, Inc.; Beverly, MA). The samples were then resolved on a 12% SDS−PAGE gel and transferred to Immobilon-P paper (Millipore; Bedford, MA) as described by the manufacturer (LeGendre, 1990). The proteins bands were detected by staining with Coomassie Blue, excised, and sequenced on a Porton P12050E peptide microsequencer.

Two-Site ELISA. The protein concentrations of wild-type SL-1 and SL-1 mutants in conditioned media were estimated by ELISA (enzyme-linked immunosorbent assay). Ninety-six-well microtitration plates were coated with 100 μL of anti-SL-1 monoclonal antibody mAb IID_4 (3 μg/mL) in

sodium borate buffer, pH 8.5, at 23 °C for 2 h. After the wells had been blocked with 300 μL of 1% dry milk and 2% Tween 20 in borate saline for 2 h at 37 °C, the wells were incubated with conditioned media for 16 h at 23 °C followed by incubation for 4 h at 23 °C with biotinylated anti-SL-1 polyclonal antibody 1817[2] (100 μL of 5 μg/mL in borate saline buffer with 1% dry milk and 2% Tween 20). Biotinylation of pAb 1817 was carried out with *N*-hydroxysuccinimide biotin in 0.1 M sodium borate buffer, pH 8.8, using 25 μg of ester/mg of pAb essentially as described by Bayer and Wilchek (1980). Incubation of biotinylated pAb 1817 was followed by the incubation with 100 μL of 0.4 μg/mL streptavidin−horseradish peroxidase in borate saline buffer with 1% dry milk and 2% Tween 20 for 2 h at 23 °C. The plates were then developed by incubation with 0.03% H_2O_2 in citrate buffer, pH 4.0, containing 0.3 mg of ABTS/mL. Absorbance values were determined at 405 nm. Protein concentrations of wild-type SL-1 and the SL-1 mutants were determined from a standard curve constructed using native SL-1 purified from human gingival fibroblast conditioned media (Windsor et al., 1993) by affinity chromatography as described above.

Casein Cleavage Rates. APMA-activated and unactivated samples (0.2−1 μg/mL) of wild-type SL-1 or SL-1 mutants were incubated with 1.25 mg of β-casein/mL at 30 °C (Windsor et al., 1991, 1994). Samples were periodically removed, and the reaction was stopped by addition of 1,10-phenanthroline to a final concentration of 10 mM. The samples were analyzed by SDS−PAGE and the rate of disappearance of the M_r 21 000 β-casein band was quantified by densitometry using a Hoeffer model GS 300 densitometer (Hoeffer Scientific; San Francisco, CA).

RESULTS

Expression. Recent studies have suggested that the highly conserved propeptide sequence PRCGVPDV is involved in maintaining the latency and in the activation of the matrix metalloproteinases (MMPs) (Sanchez-Lopez et al., 1988; Springman et al., 1990; Van Wart & Birkedal-Hansen, 1990; Park et al., 1991; Windsor et al., 1991; Chen et al., 1993). To determine what roles the cysteine and the R74 to D79 salt bridge plays in the APMA-induced activation of human SL-1, a series of SL-1 mutants were constructed by site-directed mutagenesis (Figure 1A) and expressed in a vaccinia virus system. This process resulted in the generation of recombinant viruses vTFSL-1 and vTFSL-1 mutants by the introduction of SL-1 or SL-1 mutant DNA into wild-type vaccinia virus by homologous recombination (Figure 1B). HeLa S3 cells, which failed to express detectable levels of SL-1 (Figure 2A), were coinfected with recombinant vaccinia virus carrying the SL-1 construct (wild-type or mutants) and with a recombinant virus vTF7.3, which results in synthesis of bacteriophage T7 RNA polymerase in the cytoplasm of infected cells (Fuerst et al., 1986). This system led to expression of SL-1 or SL-1 mutants in cells coinfected with the SL-1 recombinant viruses and vTF7.3 but not in uninfected cells or in cells infected with only vTF7.3 (Figure 2A). The presence of the mutations in the recombinant virus was verified by DNA sequencing of the recombinant viruses

[2] L. J. Windsor, D. L. Steele, S. LeBlanc, and K. Taylor. "Catalytic Domain Comparisons of Fibroblast-Type Collagenase, Stromelysin-1, and Matrilysin" (submitted).

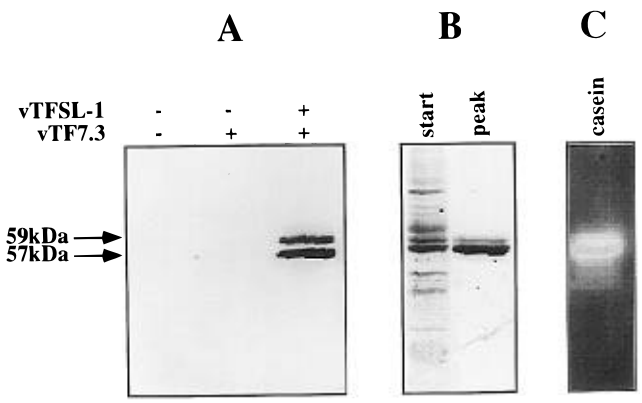

FIGURE 2: Expression of the recombinant human SL-1. Wild-type SL-1 was isolated by affinity chromatography from serum-free media conditioned by HeLa S3 cells coinfected with recombinant vaccinia virus vTFSL-1 and recombinant vaccinia virus vTF7.3. (A) Concentrated media from uninfected HeLa cells, cells infected with a recombinant virus vTF7.3, and cells coinfected with both recombinant viruses vTFSL-1 and vTF7.3 were analyzed by Western blot using anti-SL-1 mAb IID_4 (5 μg/mL). (B) Concentrated media and purified SL-1 were resolved by SDS−PAGE and stained with Coomassie Blue. (C) Affinity-purified wild-type SL-1 was resolved in a 10% SDS−PAGE gel copolymerized with 1 mg of casein/mL and lytic bands were visualized by Coomassie Blue.

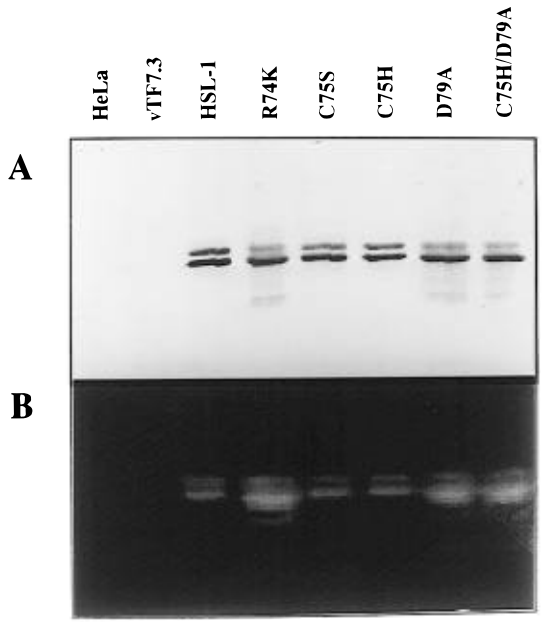

FIGURE 3: Expression of the SL-1 mutants was analyzed by Western blot and casein zymography. HeLa S3 cells were coinfected with recombinant vaccinia viruses vTFSL-1 or vTFSL-1 mutants and with a recombinant vaccinia virus vTF7.3. (A) Samples of conditioned media from HeLa cells coinfected with recombinant vaccinia viruses vTFSL-1 or vTFSL-1 mutants and vTF7.3 were resolved by SDS−PAGE, transferred to nitrocellulose, and probed with anti-SL-1 mAb IID_4 (5 μg/mL). (B) Companion samples of culture media were resolved in a 10% SDS−PAGE gel copolymerized with 1 mg of casein/mL, and the lytic bands were visualized by staining with Coomassie Blue.

(data not shown). Recombinant SL-1 and SL-1 mutants (Figure 3A) were secreted as a M_r 59 000/57 000 doublet (glycosylated and unglycosylated forms) (Wilhelm et al., 1987; Nagase et al., 1990). The amino-terminal sequence of the secreted recombinant wild-type SL-1 was Tyr-Pro-Leu-Asp-Gly-Ala-Ala-Arg-Gly-Glu, which was identical to that previously reported for the natural enzyme (Whitham et al., 1986; Wilhelm et al., 1987; Nagase et al., 1990). The

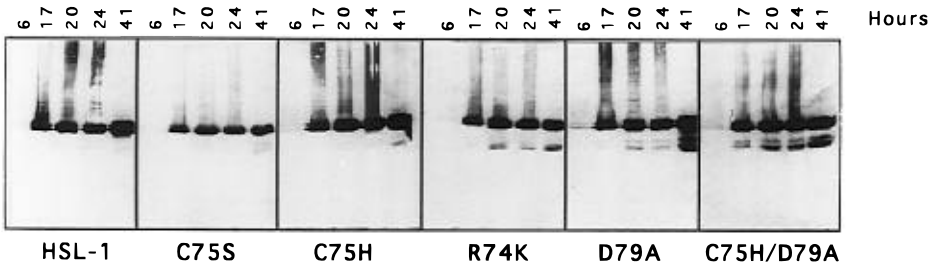

FIGURE 4: Spontaneous conversion of SL-1 mutants to lower molecular weight forms. Conditioned media from HeLa cells coinfected with recombinant vaccinia viruses vTFSL-1 or vTFSL-1 mutants and vTF7.3 were collected 6−41 h after infection. The media were concentrated, resolved by SDS−PAGE, transferred to nitrocellulose, and stained with anti-SL-1 mAb IID_4 (5 μg/mL).

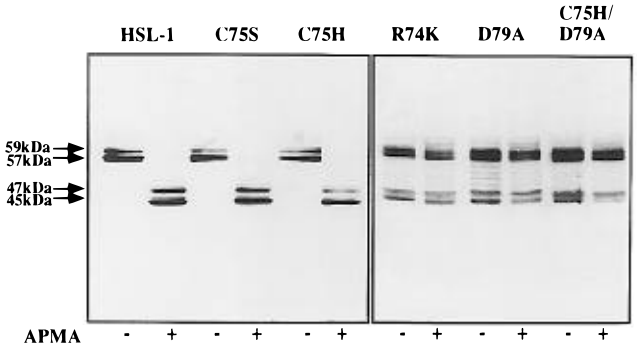

FIGURE 5: APMA-induced activation of SL-1 and SL-1 mutants. Conditioned media containing the wild-type SL-1 and SL-1 mutants were incubated with (+) and without (−) 1 mM APMA at 37 °C for 12−16 h, resolved by SDS−PAGE, and analyzed by Western blots probed with anti-SL-1 mAb IID_4 (5 μg/mL).

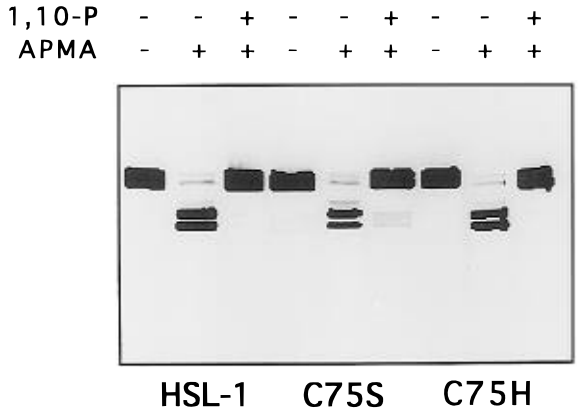

FIGURE 6: Inhibition of APMA-induced activation of wild-type SL-1 and SL-1 mutants by 1,10-phenanthroline. Conditioned media containing the wild-type SL-1, mutant C75S, and mutant C75H were treated with 1 mM APMA for 12−16 h at 37 °C in the presence (+) or absence (−) of 2 mM 1,10-phenanthroline (1,10-P). Samples were resolved by SDS−PAGE, transferred to nitrocellulose, and stained with anti-SL-1 mAb IID_4 (5 μg/mL).

recombinant enzymes (SL-1 and SL-1 mutants) were catalytically competent, as determined by casein zymography (Figure 3B), which demonstrated that none of the mutations had destroyed the catalytic machinery of the enzyme. Affinity purification of the mutants using an anti-SL-1 mAb IID_4 affinity column, a technique that was used successfully for the isolation of wild-type recombinant SL-1 (Figure 2B), proved to be less useful with the mutants because the mutants rapidly converted to lower M_r forms during purification (data not shown). Conversion to lower M_r forms by some of the mutants was observed in culture medium before any manipulation (Figure 4). These initial observations suggest that mutants carrying substitutions in the PRCGVPDV sequence were considerably more unstable than wild-type as has been previously observed (Sanchez-Lopez et al., 1988; Park et al., 1991; Windsor et al., 1991; Freimark et al., 1994).

APMA-Induced Activation of the SL-1 and SL-1 Mutants. A body of evidence suggests that the cysteine plays an important, but not yet adequately defined role in the activation of the MMPs. The "cysteine-switch" model suggests that there is an equilibrium between a "switch-open" and a "switch-closed" form and that reaction of the free thiol group in the switch-open form with organomercurials drives the equilibrium toward the open form. The open, organomercurial-reacted form subsequently undergoes autolytic conversion to active form. The ability of the SL-1 mutants to undergo this autolytic conversion to lower M_r forms in response to exposure to organomercurials was examined by incubation with 1 mM APMA for 12−16 h at 37 °C (Figure 5). The conversion was monitored by Western blot analyses of freshly harvested culture media to minimize processing due to manipulation. This experiment gave the somewhat unexpected result that the two Cys75 mutants (C75S and C75H) underwent APMA-induced M_r conversion as well as the wild-type SL-1, whereas the other mutants (R74K, D79A, and C75H/D79A) no longer responded to APMA (Figure 5). The observed conversion of wild-type SL-1, C75S, and C75H was blocked by the presence of 1,10-phenanthroline in the reaction mixture during APMA treatment, indicating that it was metalloproteinase mediated (Figure 6). The amino-terminal sequence of the wild-type, C75S, and C75H enzymes generated by incubation with APMA was Phe-Arg-Thr-Phe-Pro, which indicates that the cleavage had occurred between the His82 and Phe83 residues as previously reported for APMA-induced activation of native SL-1 (Nagase et al., 1990; Rosenfeld et al., 1994).

Table 1: Catalytic Activities of SL-1 and SL-1 Mutants with (+) and without (−) APMA Determined on β-Casein (in mol mol^{-1} h^{-1}).

	− APMA	+ APMA
SL-1	0	520 ± 87
C75S	56 ± 36	560 ± 57
C75H	0	544 ± 116
R74K	0	0
D79A	0	0
C75H/D79A	0	0

β-Casein Cleavage Rates. To further examine the ability of these mutants to undergo APMA-induced activation, the concentrations of SL-1 and SL-1 mutants in conditioned media were estimated by two-site ELISA, and the rate of casein cleavage was calculated using these estimations. SL-1 and all the SL-1 mutants in the absence of APMA displayed little to no activity (Table 1). However, the C75S and C75H displayed casein cleaving ability when APMA-activated at

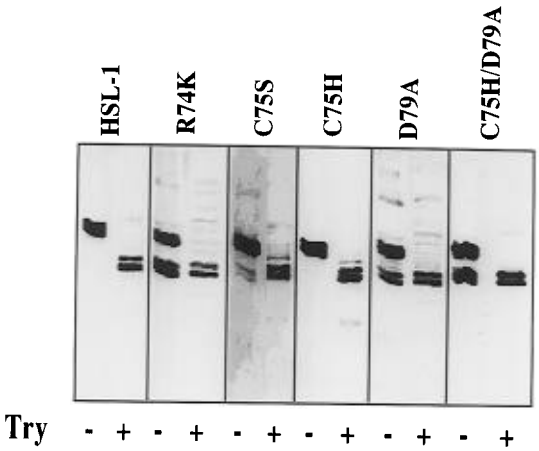

FIGURE 7: Trypsin activation of wild-type SL-1 and SL-1 mutants. Conditioned media containing wild-type SL-1 or SL-1 mutants were incubated with trypsin at 25 °C, and the reaction was stopped by addition of STI and PMSF. Samples were resolved by SDS−PAGE, transferred to nitrocellulose, and stained with anti-SL-1 mAb IID_4 (5 μg/mL).

560 and 544 mol mol^{-1} h^{-1}, respectively (Table 1). Their activity was almost identical to the activity of APMA-activated wild-type SL-1 (520 mol mol^{-1} h^{-1}). In the absence or presence of APMA, mutants R74K, D79A, and C75H/D79A displayed no activity on casein (Table 1).

Activation of SL-1 and SL-1 Mutants by Trypsin. Previous studies have demonstrated that incubation of SL-1 with trypsin, chymotrypsin, HNE (human neutrophil elastase), or plasmin results in the stepwise conversion of the latent form to active form (Okada et al., 1988; Nagase et al., 1990). The ability of the latent forms of the mutants of SL-1 to convert to lower M_r forms in response to protease treatment was examined by incubation with trypsin at 25 °C (Figure 7). This analysis showed that all mutants of SL-1 and the wild-type SL-1 were processed by exposure to trypsin to a lower molecular weight form. It is also apparent that some of these mutants were already partially converted, suggesting that they were less able to maintain latency.

DISCUSSION

The cysteine switch model for MMPs activation proposes that the single cysteine residue in the propeptide sequence PRCGVPDV interacts with the active site zinc and that disruption of this interaction results in activation of the enzymes. The interaction of this cysteine with the active site zinc has been demonstrated by spectroscopic evidence of sulfur ligation to the active site metal (Salowe et al., 1992) and by extended X-ray absorption fine structure spectroscopy (Holz et al., 1992). Mutational analyses have suggested that other residues in this sequence stabilize the cysteine−zinc interaction in that mutants in this region have an increased tendency to undergo spontaneous activation and/or degradation (Sanchez-Lopez et al., 1988; Park et al., 1991; Windsor et al., 1991). A recent report by Chen et al. (1993) demonstrated that chemical modification by sulfhydryl reagents of this conserved cysteine in SL-1 was not sufficient to produce an active enzyme. Chen et al. (1993) also reported that their modified stromelysin-1 could still be activated by APMA. It has previously been shown that a mutation at this cysteine residue in fibroblast-type collagenase resulted primarily in M_r 28 000 and 18 000 species (Windsor et al., 1991) and that the M_r 28 000 species containing the mutated propeptide failed to react with α_2M-macroglobulin, indicating that it was still latent (Windsor et al., 1991). However, it was not reported whether this mutant fibroblast-type collagenase could be activated by APMA. Therefore, we have in this study expressed wild-type SL-1 and mutants of SL-1 at the propeptide cysteine and at the R74 to D79 salt bridge to determine whether they are critical for APMA-induced activation of SL-1. This study has clearly shown that APMA interacts with other regions of the protein in addition to its binding to the propeptide cysteine in the activation of stromelysin-1, since mutants of stromelysin-1 that have eliminated this cysteine still undergo APMA-induced activation. It has also been shown that the ability of APMA to induce activation is eliminated by disrupting the salt bridge between R74 and D79.

The wild-type SL-1 and SL-1 mutants were expressed at detectable levels in a recombinant vaccina virus system. It was determined by casein zymography that all of the mutants were catalytically competent. The initial observations that some of the mutants formed lower M_r forms before any manipulation suggested that mutants carrying substitutions in the PRCGVPDV sequence were considerably more unstable than wild-type as has been previously observed (Sanchez-Lopez et al., 1988; Park et al., 1991; Windsor et al., 1991; Freimark et al., 1994).

It is interesting to note that the two Cys75 mutants (C75S and C75H) when treated with APMA yielded a large M_r 47 000/45 000 doublet like the wild-type SL-1. Also each of the APMA-activated forms of C75S and C75H mutants (560 and 544 mol mol^{-1} h^{-1}, respectively) had wild-type proteolytic activity (520 mol mol^{-1} h^{-1}) against the casein substrate. Since the C75S and C75H mutants responded just as well as wild-type to exposure to APMA, yet contain no thiol group at position 75, it is evident that direct binding of APMA to the thiol in itself does not explain the APMA-induced activation reaction in SL-1. These data demonstrate that the APMA-induced activation of SL-1 involves protein interactions other than those with the propeptide free thiol group. Chen et al. (1993) arrived at a similar conclusion based on chemical modification of Cys75 in wild-type SL-1.

The mutants R74K, D79A, and C75H/D79A gave interesting results in that they neither underwent APMA-induced activation and molecular weight conversion nor displayed any caseinolytic activity when treated with APMA. Therefore, it appears that disruption of the R74 to D79 salt bridge in SL-1 has eliminated the ability of APMA to induce activation. The exact role this salt bridge plays in the APMA-induced activation of SL-1 is still unclear at this time. Further characterization of these mutants has been somewhat limited due to the spontaneous breakdown of these mutants.

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