Spontaneous Propeptide Processing of Mini-Stromelysin-1 Mutants Blocked by APMA ((4-Aminophenyl)mercuric Acetate)†

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ABSTRACT: Human stromelysin-1 (SL-1) is a member of the stromelysin subfamily of matrix metalloproteinases (MMPs). The MMPs play a major role in the degradation of the extracellular matrix (ECM) during normal and pathological conditions. SL-1 like the other MMPs can be activated in vitro by the stepwise removal of the propeptide that contains a single unpaired cysteine which coordinates the active site zinc. Other residues in the propeptide also play a role in maintaining the latency of the enzymes. Deletion mutants and single-site amino acid replacements within the propeptide of a carboxyl-terminally truncated stromelysin-1 (mini-SL-1) were constructed and expressed in *Escherichia coli* to further examine what amino acids within the propeptide of SL-1 are important for maintaining latency. While the natural enzyme displayed some limited tendency to spontaneously (autolytically) convert to lower M_r in a stepwise manner and finally to the fully processed form, all of the truncation mutants of more than 19 amino acids generated in *E. coli* showed greatly accelerated self-cleavage indicative of diminished stability and/or resistance to proteolysis of the residual propeptide. Mutant Δ63 as well as other mutants in which most of the propeptide had been deleted no longer responded to exposure to the organomercurial APMA by accelerated autolytic processing. Rather, APMA inhibited the autolytic processing in these mutants, further confirming the complexity of the action of this organomercurial in the activation of pro-MMPs.

The matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that play a major role in both the physiological and pathological remodeling of the extracellular matrix. Human stromelysin-1 (SL-1, MMP-3)[1] is a member of the stromelysin subfamily of MMPs and, like the other MMPs, is composed of a number of distinct structural and functional domains (as reviewed by Woessner (*1*) and Birkedal-Hansen (*2*)). The human SL-1 protein (*3*, *4*) consists of 477 amino acid residues and has an apparent M_r of 55 000. The short signal sequence is followed by the propeptide which is essential for maintaining latency and the catalytic domain which contains the active zinc-binding site. A short proline-rich hinge region joins the catalytic portion of the molecule to a carboxyl-terminal hemopexin-like domain.

SL-1 like most of the other MMPs can be activated in vitro by treatment with organomercurials or proteolytic enzymes which initiate a stepwise autolytic excision of the propeptide (*5*−*7*). Intermediate forms generated by treatment with proteinases (M_r 53 000) or organomercurials (M_r 46 000) precede the generation of the stable, fully processed active form (M_r 45 000). The activation of SL-1 by various reagents can be explained by the "cysteine switch" model in which the latency is maintained via the coordination of the active site zinc by the cysteine residue in the highly conserved amino acid sequence PRCGVPDV of the propeptide (*8*, *9*). This model proposes that "activation" defined as the acquisition of catalytic activity is a result of dissociation of the propeptide cysteine sulfhydryl group from the active site zinc and its replacement with a water molecule that plays a role in catalysis. Although the propeptide cysteine sulfhydryl group appears to play a significant role in immobilizing the propeptide in its "locked position" and in maintaining catalytic latency, recent studies have shown that other residues surrounding this cysteine as well as the 35 amino-terminal amino acids of the propeptide are also involved in maintaining the latency of the MMPs (*10*−*14*).

Matrilysin, an MMP that lacks the hemopexin domain (*15*), retains catalytic function, suggesting that the catalytic domain of the MMPs is necessary and sufficient for proteolytic

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[1] Abbreviations: α_2M, α_2-macroglobulin; APMA, (4-aminophenyl)-mercuric acetate; BSA, bovine serum albumin; IPTG, isopropyl β-D-thiogalactoside; mAb, monoclonal antibody; MMP, matrix metalloproteinase; mini-SL-1, carboxyl-terminal truncated stromelysin-1; pAb, polyclonal antibody; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SL-1, stromelysin-1, MMP-3; SL-2, stromelysin-2, MMP-11.

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activity. Studies on recombinant MMPs in which the hemopexin domain had been deleted confirmed this conclusion (*10*, *16*−*21*). It has also become clear that the hemopexin domain is not required for maintenance of MMP latency, for activation with organomercurials, or for binding of inhibitors to the active site (*16*, *21*). Therefore, hemopexin-domain-deleted SL-1 (mini-SL-1) was used in this study to further study the role played by the propeptide in the latency and activation of the enzyme. Mini-SL-1 propeptide deletion or point mutations gave rise to enzymes with greatly accelerated spontaneous autolytic processing of the propeptide. Some of these mutants showed a greatly altered response to exposure to APMA ((4-aminophenyl)mercuric acetate) in that precursor processing was inhibited rather than accelerated.

MATERIALS AND METHODS

Plasmid Construction. The expression vector pGXSL-1 (*14*) was used to construct an expression plasmid for carboxyl terminal-truncated SL-1 (mini-SL-1) as previously described (*21*) utilizing site-directed mutagenesis (*22*). Briefly, plasmid pGXSL-1 single-stranded DNA and the oligonucleotide 5′ GGAGATATACATATGTTATCCGCTGGATGGAGCT-GC 3′ were used to introduce a ribosomal binding site and an initiation codon. The single-stranded DNA of the resulting plasmid ($pGXSL_{Mut8}$) and oligonucleotide 5′ TATGGAC-CTCCCCCGGATCCCTGATAGAGATATGTAGAAG 3′ were used to introduce a stop codon after amino acid 253 to delete the hemopexin-like domain of SL-1 (plasmid pGXmini-SL-1).

Construction of Mini-SL-1 Mutants. Amino-terminal deletion mutants of the propeptide sequence of mini-SL-1 were constructed by site-directed mutagenesis using single-stranded plasmid pGXmini-SL-1 DNA and 30−40 residue oligonucleotides as primers. The following oligonucleotides were designed so that each mutated protein had a translation start methionine that was either part of the native sequence or synthetically added:

Mut Δ1−14, 5′ GAAGGAGATATACATATGAACCT-TGTTCAGAAATATCTAG 3′; Mut Δ1−19, 5′ GAAG-GAGATATACATATGTATCTAGAAAACTAC-TACGACC 3′; Mut Δ1−26, 5′ GAAGGAGATATACATAT-GCTCAAAAAAGATGTGA 3′; Mut Δ1−47, 5′ GAAG-GAGATATACATATGCGAGAAATGCAGAAGTTCCTTG 3′; Mut Δ1−49, 5′ GGAGATATACATATGCAGAAGT-TCCTTGGATTGGAGGTGACG 3′; Mut Δ1−52, 5′ AG-GAGATATACATATGTTCCTTGGATTGGAGGTG 3′; Mut Δ1−58, 5′ AGGAGATATACATATGACGGGGAAGCTG-GACTCCGAC 3′; Mut Δ1−59, 5′ AGGAGATATACAT-ATGGGGAAGCTGGACTCCGAC 3′; Mut Δ1−63, 5′ AG-GAGATATACATATGTCCGACACTCTGGAGGTG 3′; Mut Δ1−72, 5′ GAAGGAGATATACATATGCCCAGGTGTG-GAG 3′; Mut Δ1−82, 5′ GAAGGAGATATACATATGT-TCAGAACCTTTCCTG 3′.

Site-directed mutagenesis was also used to obtain point mutations at Thr59, Gly60, and Asp63 using the following oligonucleotides (changed nucleotides in italics): 5′ ATTG-GAGGTG*G*CGGGGAAGCTGGAC 3′ (Thr59Ala); 5′ AT-TGGAGGTGACGG*CA*AAGCTGGAC 3′ (Gly60Ala); 5′ ACGGGGAAGCTGG*C*CTCCGACACTC 3′ (Asp63Ala).

The sequence identity of all the plasmids was verified by double-stranded DNA sequencing using Sequenase Version 2, as described by the manufacturer (United States Biochemical; Cleveland, OH).

Expression and Purification of Recombinant SL-1 Protein from E. coli. Plasmid DNA for mini-SL-1 wild-type or mutants was transformed into BL-21 (DE3) *E. coli* cells (*23*), which contains a genomic copy of the T7 RNA polymerase gene. Cells were grown to a density of 10^6−10^8 cells/mL and then induced with 1 mM IPTG (isopropyl β-D-thiogalactoside; Sigma; St. Louis, MO) for another 2−3 h. The cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl, pH 7.5, with 0.2 M NaCl. Cell suspensions were passed through a French press at 10 000−15 000 psi in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma) and centrifuged to separate the supernatant and the pellet. The supernatant was diluted 10-fold with 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$ (buffer A) and passed over a monoclonal antibody affinity column prepared by coupling anti-SL-1 mAb IID_4 to CNBr-activated Sepharose 4-B (*24*). Alternatively, the pellet was extracted with 6 M urea in buffer A, diluted, and dialyzed against buffer A to remove the urea prior to passage over the antibody affinity column. The bound material was eluted from the column with 6 M urea in buffer A and dialyzed against buffer A to remove the urea. Protein concentrations were determined by the Bradford method (*25*), using bovine serum albumin (BSA; Sigma) as a standard.

Treatment of Mini-SL-1 Mutants with Thiol Reagents. Samples of antibody affinity purified wild-type and mini-SL-1 mutants were incubated for various time intervals at 37 °C with 1 mM APMA or 2 mM iodoacetamide. Companion samples were incubated without thiol reagents under the same conditions. Samples were resolved by SDS−PAGE, transferred to nitrocellulose membrane, and probed with anti-SL-1 mAb IIB_2 (5 μg/mL).

Antibodies. Native human SL-1 was purified from human gingival fibroblast conditioned media by affinity chromatography (*24*) and used to make monoclonal antibodies according to previously described methods (*26*). Anti-SL-1 mAb IID_4, mAb IIB_2, and mAb $IIID_3$ were utilized because of their reactivity with the catalytic domain of SL-1.

Electrophoretic Methods. Samples were separated on 12−15% SDS−PAGE gels according to the methods of Laemmli (*27*). Proteins were detected by staining with Coomassie blue or transferred to nitrocellulose membranes (BioRad; Melville, NY) by electroblotting for Western blot analyses. The membranes for the Western blots were blocked with 5% dry milk in 50 mM Tris-HCl, pH 7.5, and incubated with primary antibody (5 μg/mL) for 14−16 h at 4 °C. Alkaline phosphatase-conjugated goat antimouse IgG (Bethesda Research Labs; Bethesda, MD) was used as the secondary antibody and developed with 4-nitro blue-tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim Corp.; Indianapolis, IN).

Casein Zymography. The ability of the enzymes to cleave casein was assessed by resolving samples in a 12% SDS−PAGE gel copolymerized with 1 mg/mL casein. 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

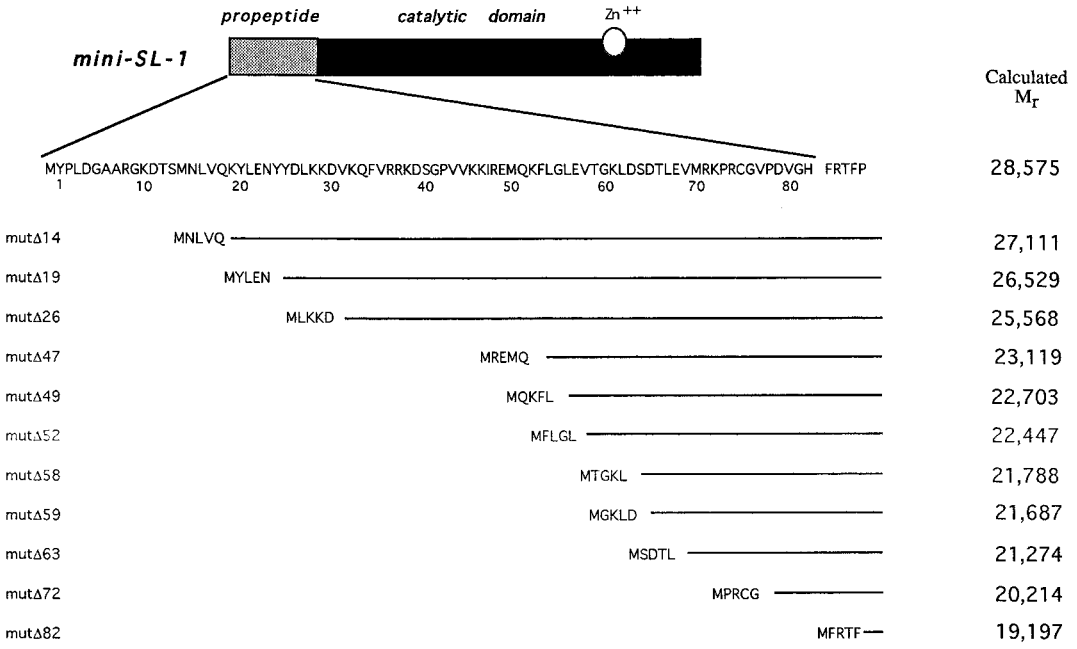

FIGURE 1: Schematic representation of the mutant forms of mini-SL-1. Schematic diagram of a series of mutants constructed using site-directed mutagenesis by deletion of amino acid residues from the amino-terminal region of the propeptide. The molecular weight of the mini-SL-1 mutants calculated from their amino acid sequences are shown.

$ZnCl_2$ 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 fragments generated by spontaneous autolytic processing, samples were resolved in 15−20% SDS−PAGE gels and transferred to Immobilon-P paper (Millipore; Bredford, MA) as described by the manufacturer (*28*). The protein bands were detected by staining with Coomassie blue, excised, and sequenced on a Porton P12050E peptide microsequencer.

α_2M Capture Assay. Wild-type and mini-SL-1 mutants were incubated in the presence or absence of 1.5 mg/mL α_2M isolated from outdated human plasma by the method of Sottrup-Jensen (*29*) in 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 5 mM $CaCl_2$, and 1 μM $ZnCl_2$. The reaction mixtures were incubated for up to a maximum of 30 min at 23 °C. Samples were then mixed with an equal volume of electrophoretic sample buffer, resolved by SDS−PAGE, and analyzed by Western blots using anti-SL-1 monoclonal antibodies (IID_4 and $IIID_3$).

RESULTS

Expression of Mini-SL-1 and Amino-Terminal Mutants. To examine the role played by the SL-1 propeptide in the maintenance of latency and in APMA activation, we analyzed a progressive series of amino-terminal deletion mutants (Δ14, Δ19, Δ26, Δ47, Δ49, Δ52, Δ58, Δ59, Δ63, Δ72, and Δ82) expressed in *E. coli* (Figures 1 and 2A). The mutant proteins were refolded by dilution and purified by affinity chromatography using anti-stromelysin-1 mAb IID_4. The purified mutant preparations consisted of a series of nascent proteins of decreasing M_r (commensurate with the deletions) and of a number of smaller processing products formed during manipulation and/or storage of the samples. Amino-terminal sequencing permitted us to identify the processing fragments (A−C) as detailed in Table 1. Fragment A gave three amino-terminal sequences (Table 1) indicating cleavage of Glu^{34}-Phe^{35}, Val^{36}-Arg^{37}, and Asp^{40}-Ser^{41}. This same region (Phe^{34}-Val-Arg-Arg-Lys-Asp^{39}) is also the site of the initial cleavage when intact proSL-1 is activated by other proteinases (*5*, *30*). Fragment B arose from cleavage of Glu^{68}-Val^{69} (Table 1), the bond previously identified by Nagase et al. (*5*) as the site of the initial autolytic attack following APMA-induced self-cleavage. Fragment C (Phe^{83}-Arg-Thr-Phe-Pro^{87}) represented the fully processed, active SL-1 resulting from

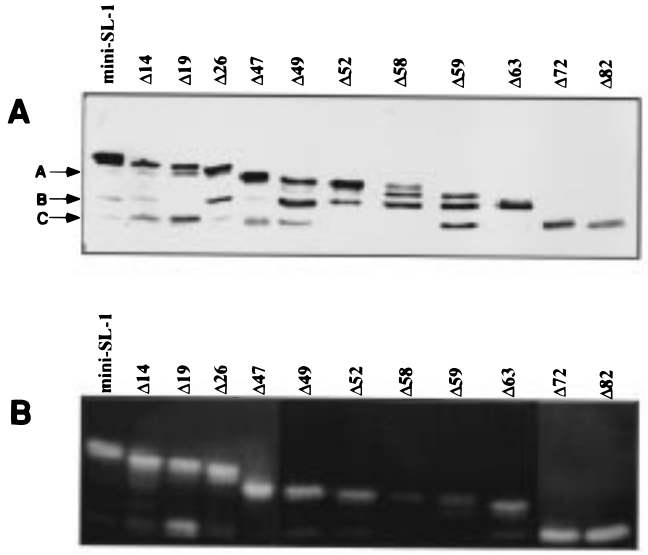

FIGURE 2: Expression of the mini-SL-1 mutants was analyzed by Western blots and casein zymography. (A) Wild-type and amino-terminal deletion mutants of mini-SL-1 purified by affinity chromatography were subjected to SDS−PAGE and analyzed by Western blots probed with anti-SL-1 mAb IID_4 (5 μg/mL) and mAb $IIID_3$ (5 μg/mL). Some of the spontaneous conversion fragments are indicated by letters. (B) Wild-type and amino-terminal deletion mutant samples were resolved in a 13% SDS−PAGE gel copolymerized with 1 mg/mL of casein under nondenaturing conditions, incubated at 37 °C for 16 h, and stained with Coomassie blue to visualize lytic bands.

Table 1: Amino-Terminal Sequence Analyses of the Major (A−C) and Minor (X, Y) Spontaneous Generated Fragments of Wild-Type and Mutants of Mini-SL-1

fragment	amino-terminal sequence	calcd M_r
A	Phe[35]-Val-Arg-Arg-Lys[39]	24 598
	Arg[37]-Arg-Lys-Asp-Ser[41]	24 352
	Ser[41]-Gly-Pro-Val-Val[45]	23 796
B	Val[69]-Met-Arg-Lys-Pro[73]	20 598
C	Phe[83]-Arg-Thr-Phe-Pro[87]	19 066
X	Met[50]-Gly-Lys-Phe-Leu[54]	22 703
	Phe[53]-Leu-Gly-Leu-Glu[57]	22 316
	Leu[54]-Gly-Leu-Glu-Val[58]	22 169
	Gly[55]-Leu-Glu-Val-Thr[59]	22 055
Y	Thr[59]-Gly-Lys-Asp-Ser[63]	21 657
	Gly[60]-Lys-Asp-Ser-Asp[64]	21 556
	Ser[64]-Asp-Thr-Leu-Val[68]	21 143
	Thr[66]-Leu-Val-Met-Arg[70]	20 941

cleavage of His[82]-Phe[83] which marks the transition between the propeptide and the catalytic domain (*5*, *30*). All of the various M_r forms identified by Western analysis were catalytically competent as indicated by casein zymography after exposure to SDS (Figure 2B). While this method does provide evidence for the catalytic potential of each band, it does not discriminate between spontaneously active or latent forms or forms which have acquired only partial activity. In addition to these species, two barely visible bands were occasionally observed between fragments A and B (data not shown), one (X) with sequences indicative of cleavage of Glu[49]-Met[50], Lys[52]-Phe[53], Phe[53]-Leu54, and Leu[54]-Gly[55] (Table 1) and another (Y) with sequences resulting from cleavage of Val[58]-Thr[59], Thr[59]-Gly[60], Asp[63]-Ser[64] and Asp[65]-Thr[66] (Table 1).

Activation of Amino-Terminal Mutants of Mini-SL-1. A body of evidence has shown that exposure to organomercurials results in stepwise autolytic conversion of full-length proMMP to the fully processed, catalytically active form (*1*, *5*, *8*, *9*, *30*−*34*). The ability of the mini-SL-1 mutants containing varying length propeptide segments to undergo autocatalytic conversion to lower M_r forms spontaneously or in response to exposure to APMA was examined (Figure 3 and data not shown). Mini-SL-1 and Δ19 showed little or no spontaneous processing during 16 h of incubation, and both were fully converted to the active form when treated with APMA (fragment C), Δ19 perhaps somewhat faster than full-length mini-SL-1. Amino-terminal deletion of 26 or more amino acids, however, gave rise to a drastically altered pattern and/or time course of autolytic conversion (Figure 3). Mutants Δ26, Δ49, and Δ59 (Figure 3) each converted from the intermediate and fully processed forms so quickly that it was difficult to determine whether APMA exerted any accelerating effect on this process. In mutants with deletions of 26 or more amino acids from the amino-terminus, APMA appeared to retard and inhibit conversion rather than to accelerate it. Mutants Δ26, Δ49, and Δ59 converted to the fully processed form (M_r 19 900) more slowly in the presence than in the absence of the organomercurial. This observation suggested that the organomercurial inhibited rather than accelerated this conversion. This behavior is most evident in Δ63, the conversion of which is more or less completely blocked by APMA. Interestingly, 2 mM iodoacetamide (in lieu of APMA) did not inhibit the spontaneous conversion process (Figure 4).

α2M Capture. To determine whether the various mutants and their progressive processing products (fragments A−C) displayed spontaneous catalytic activity (without further activation), an α2M capture technique was used. This method is based on the rapid entrapment and covalent linkage of proteases which cleave a bond in the promiscuous bait region (*12*, *29*). It readily discriminates between catalytically active and latent forms of proteinases (Figure 5). The M_r 20 500 fragment B and the fully processed M_r 19 900 fragment C of the wild-type and mutated enzymes were captured by α_2M. The slightly larger intact Δ59 mutant protein (M_r 21 687) was captured only partially or not at all as seen by the presence of the faint band of unactivated Δ59 in the control and α_2M treated samples. These results indicate that fragments generated by cleavage of Glu[68]-Val[69] (fragment B) and His[82]-Phe[83] (fragment C) possessed spontaneous enzymatic activity, whereas the intact Δ59 mutant did not. Fragments generated by cleavage within the Glu[34]-Phe-Val-Arg-Arg-Lys-Asp-Ser[40] sequence also were not captured and therefore were deemed devoid of overt proteolytic activity (data not shown). This analysis permits us to determine the boundaries for autolytic processing that results in generation of spontaneous catalytic activity and to define the minimal propeptide required for maintenance of catalytic latency. It is interesting to note that fragment B, which retained 14 amino acids of the propeptide including Cys75, was fully captured by α2M and therefore apparently contained a completely opened "cysteine switch".

Expression and Activation of Point Mutations of Mini-SL-1. Comparison of the peptide sequence K[52]FLGEVT-GKLDSDTLE[68] in SL-1 with the propeptide domain sequence from different human MMPs (Table 2) showed that Thr59, Gly60, and Asp63 are almost totally conserved. The presence of these conserved amino acids suggests that these residues may play a role in latency or activation of the enzyme. To test this hypothesis, three point mutations in mini-SL-1 were constructed where Thr59, Gly60, and Asp63 were each replaced with Ala residues (Figure 6). The T59A mutant was rapidly processed from high molecular weight form to the 20 500 intermediate form in the presence of APMA but slowly, if at all, to the fully processed M_r 19 900 form. As demonstrated in Figure 6, the G60A and D63A mutants also underwent APMA-induced M_r conversions. The conversion of these two mutants was slower than T59A yet faster than wild-type mini-SL-1. In all three cases the replacement of Thr59, Gly60, and Asp63 by Ala accelerated autolytic conversion and increased the rate of APMA-induced activation (2 h; Figure 6) as compared to wild-type mini-SL-1 (>12 h; Figure 3). APMA treatment of the mutants resulted in an increase in activation and not the APMA-induced inhibition of conversion seen with the deletion mutants in this region.

DISCUSSION

Previous studies utilizing chemical modification (*34*) or replacement by site-directed mutagenesis of Cys-75 (*14*) have demonstrated that reaction of APMA with the propeptide cysteine (Cys-75) sulfhydryl group is not required for M_r conversion and catalytic activation of MMP precursors. It seems plausible that the initial interaction with APMA results in changes that destabilize the Cys−Zn bond. While it is still possible that APMA can react with Cys-75 as it

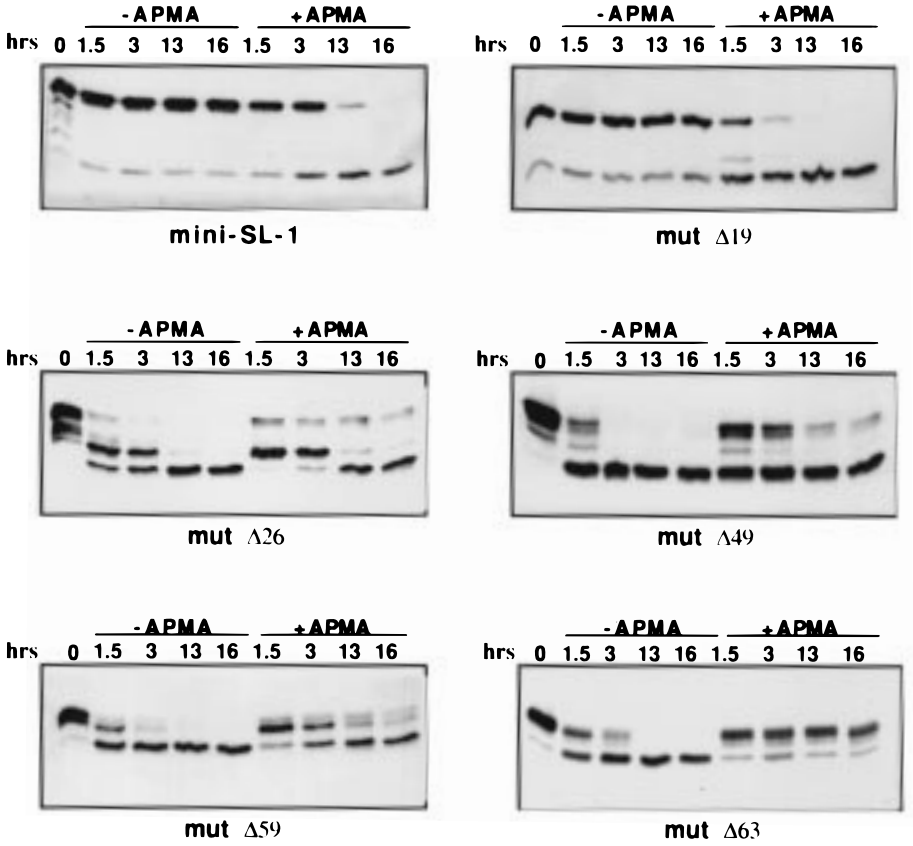

FIGURE 3: APMA-induced activation of wild-type amino-terminal deletion mutants of mini-SL-1. Samples of the purified enzymes were incubated with and without 1 mM APMA for 0, 1.5, 3, 13, and 16 h at 37 °C. The reaction products were resolved in SDS−PAGE gels and analyzed by Western blots using anti-SL-1 mAb IIB_2 (5 μg/mL).

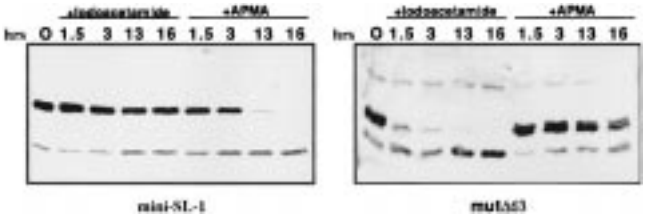

FIGURE 4: Treatment of Δ63 mutant and wild-type mini-SL-1 with thiol reagents. Purified Δ63 and wild-type protein were incubated with 2 mM iodoacetamide or 1 mM APMA for the indicated time periods at 37 °C. Samples were resolved by SDS−PAGE, transferred to nitrocellulose, and stained with anti-SL-1 mAb IIB_2 (5 μg/mL).

dissociates from the Cys−Zn coordinate bond, this interaction is neither a necessary nor sufficient condition for activation. We have previously shown that mutants in the critical salt bridge between R74 and D79 which loops the cysteine toward the active site Zn and presents the sulfhydryl group for bonding (*35*) fail to respond to APMA by activation or M_r conversion, suggesting that this salt bridge plays an important structural or functional role in APMA activation (*14*). In this study we observed that APMA inhibited activation and/or spontaneous conversion of mutant Δ63 and to a lesser extent that of several other truncation mutants with deletions of at least 26 amino acids. Iodoacetamide which reacts readily with free thiol groups could not substitute for APMA in blocking activation/conversion, suggesting that ability of APMA to inhibit the propeptide processing is not mediated merely by blocking the free, reactive thiol group of Cys-75. In the aggregate, these findings showed that deletion mutants lacking 26 or more residues from the amino terminus behave very differently and respond differently to APMA than did the wild-type and Δ19 proteins, in that they rapidly activate autolytically in the absence of the organomercurial and that addition of the organomercurial inhibits or blocks terminal processing, the more effectively the shorter the propeptide. This finding was unexpected and suggests that APMA may exert both positive and negative influences on the activation process.

In agreement with our findings, Freimark et al. (*13*) previously noted that truncation of 20 or more amino acids from the amino-terminal region of the propeptide of SL-1 results in spontaneous activation. These studies showed that removal of 20−26 (or more) residues from the amino-terminal region of propeptide from mini-SL-1 dramatically altered proenzyme processing kinetics to the fully "active" M_r 19 900 form both in the presence and absence of APMA. Although the deletion mutants processed autolytically at a greatly accelerated rate, they were not spontaneously active as determined by α2M capture. A similar pattern (rapid spontaneous autolytic conversion but no capture by α2M) has previously also been observed with a (C73S) mutant of MMP-1 (*12*). Spontaneous processing may result from a change in the rate of "switch" opening/closing, rather than from full and irreversible dissociation of the propeptide from its properly docked position. In the Δ59 deletion mutant, a small amount of intact nascent, unprocessed protein was not captured and therefore had remained latent (Figure 5). However, fragment B (Figure 5) starting with Val69 was captured and therefore had acquired at least partial catalytic activity.

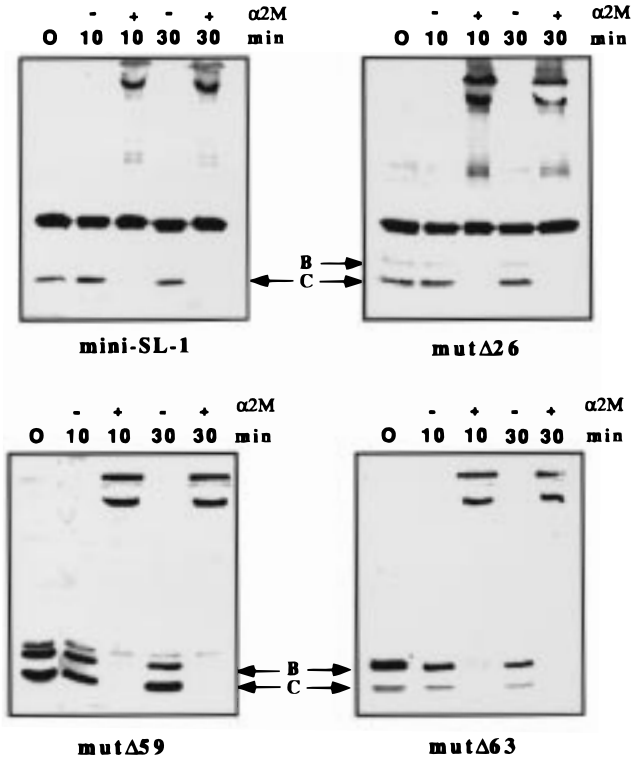

FIGURE 5: Interaction of the wild-type and mutant forms of mini-SL-1 with α_2M. The wild-type and Δ26, Δ59, and Δ63 mutants of mini-SL-1 were incubated with or without 1.5 mg/mL α_2M for 10−30 min at 23 °C. Samples were subjected to SDS−PAGE and then analyzed by Western blots using anti-SL-1 mAb IID_4 and mAb $IIID_3$ (5 μg/mL). Disappearance of reactivity at the M_r of the protein indicates catalytic competency and results in the formation of high-M_r covalent complexes with the α_2M.

Taken together these findings suggest that the amino acids between Thr59 and Val69 play a major role in maintaining latency.

It should be noted that fragment B that was captured by the α2M still contained the propeptide Cys, suggesting that when the other, secondary interactions that play a role in maintaining latency are removed, the Cys−Zn bond alone is not sufficient to maintain latency. This observation lends further support to the notion that the propeptide is locked in its position in part by an entropy-effect (multiple intraprotein interactions). Other studies (*36*, *37*) have made similar observations. These studies showed that the activation of the proSL-1 (F83R) mutant by proteinases or APMA and the activation of proMMP-9 by APMA resulted in seemingly "active" intermediates which retained 13−15 residues of the propeptide including the conserved cysteine. The amino acids between Thr59 and Val69 may serve as the last domain required to maintain latency after the other domains that play a role in maintaining latency have been removed by deletion or proteolytic cleavage. Individual amino acid contacts in this region may play an important role in determining the rate of APMA-mediated activation and/or of switch opening. When individual amino acid replacements were made at Thr59, Gly60, or Asp63, the mutated proteins could undergo APMA-induced activation within 2 h (as judged by M_r conversion) as opposed to the 12 h required for APMA-induced activation of wild-type mini-SL-1. This observation suggested that the rate of APMA-induced activation of the mutated SL-1 proteins were increased dramatically. These replacements may each lower the stability of the "switch-closed" latent conformation in these mutated proteins, thereby altering the rate of activation.

Table 2: Comparison of Amino Acid Sequence of Human SL-1 from Lys52 to Glu68 in the Propeptide with the Comparable Region of Other MMPs[a]

Enzyme	Sequence
	53 55 56 59 60 63 66
Human SL-1	KFLGLEVTGKLDSDTLE
Human SL-1	KFLGLEVTGKLDTDTLE
Rabbit SL-1	KFLGLEVTGKLDSNTLE
Mouse SL-1	KFLGLEMTGKLDSMTME
Rat SL-1	KFLGLKMTGKLDSNTME
Rat SL-2	KFLGLEMTGKLDSNTVE
Human FIB-CL	EFFGLKVTGKPDAETLK
Rabbit FIB-CL	EFFGLKVTGKPDAETLK
Pig FIB-CL	QFFGLKVTGKPDAETLN
Rat FIB-CL	SFFGLDVTGKLDDPTLD
Human PMN-CL	RFFGLNVTGKPNEETLD
Mouse MME	QFFGLEATGQLDNSTLA
Human PUMP-1	KFFGLPITGMLNSRVIE
Human Mr 72 GL	KFFGLPQTGDLDQNTIE
Human Mr 92 GL	KQLSLPETGELDSATLK

[a] Highly conserved residues are shown in bold, and the amino acid numbers shown are for human SL-1.

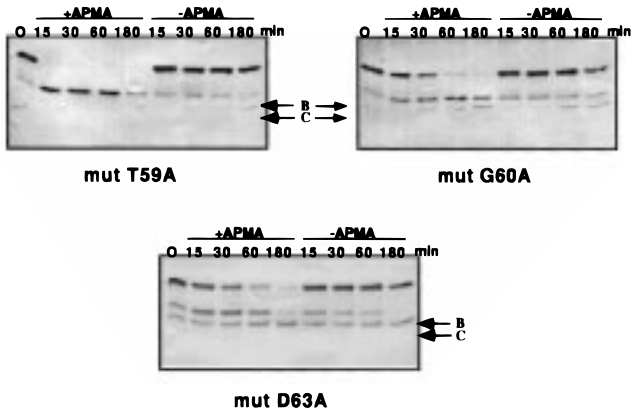

FIGURE 6: APMA-mediated activation and processing of the single amino acid mutations of the mini-SL-1. Purified T59A, G60A, and D63A mutants of mini-SL-1 were incubated at 37 °C in the presence or absence of 1 mM APMA for the indicated time period. Samples were then resolved by SDS−PAGE, transferred to nitrocellulose, and stained with anti-SL-1 mAb IIB_2 (5 μg/mL).

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