Autolytic Activation of Recombinant Human 72 Kilodalton Type IV Collagenase†

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ABSTRACT: Human 72 kDa type IV collagenase (gelatinase A, MMP-2) was expressed in a baculovirus/insect cell system. The enzyme was produced in the wild-type form and in two mutant forms, where the active site Glu_{375} was substituted by Asp or Gln. The mutated proteins had strongly reduced or no detectable activity, respectively, allowing detailed analysis of rapid autoactivation reactions. MMP-2 was readily degraded to a proenzyme form lacking the first four amino acid residues. This cleavage was shown to be an autolytic process, although enzyme activity was apparently not affected by this truncation. Conversion to the active enzyme form was achieved without external activator in a concentration-dependent manner at 37 °C. The activation of MMP-2 was shown to be a stepwise process, probably via a Δ_{1-50} form as a highly unstable intermediate. The C-terminal hemopexin-like domain is removed rather early at two cleavage sites, and degradation within the Zn-binding site inactivates the enzyme. The fibronectin- and hemopexin-like domains are stable, although the autodegradation pattern did not show any sequence specificity, except for charged residues in the P_1' position. The results indicate that a specific activator may not be essential for MMP-2.

Connective tissue turnover in physiological and pathological conditions is highly dependent on the action of matrix metalloproteases (MMPs). Matrix metalloproteases are a family of homologous extracellular enzymes which are secreted in latent forms and which have a Zn atom at the catalytic site (Matrisian, 1992). The matrix metalloproteinases can degrade various components of the extracellular matrix, and they have been subclassified on the basis of their substrate specificity as determined *in vitro* into interstitial collagenases, type IV collagenases/gelatinases, stromelysins, matrilysin and metalloelastase, all of which have been cloned from several species (Woessner, 1991; Matrisian, 1992; Shapiro et al., 1992, 1993). The metalloproteases are multidomain proteins, all members having homologous catalytic and N-terminal domains. This basic structural motif is represented by matrilysin, the smallest matrix metalloprotease which has a broad substrate specificity. Stromelysins 1, 2, and 3 and the two interstitial collagenases have an additional C-terminal domain, which shares some homology with hemopexin. The hemopexin domains of the enzymes have been proposed to modulate substrate specificity (Murphy et al., 1992a; Sanchez-Lopez et al., 1993; Schnierer et al., 1993), although the hemopexin domain of type IV collagenases has been reported to serve other functions such as binding of specific inhibitors (TIMP1 and TIMP2)[1] (Howard & Banda, 1991; Fridman et al., 1992; Kleiner et al., 1992; Nguyen et al., 1994) and putative cell surface receptors (Strongin et al., 1993; Murphy et al., 1992b). In contrast, a type IV collagenase-specific domain homologous to domain II of fibronectin has been reported to be responsible for substrate binding (Banyai & Patthy, 1991; Collier et al., 1992, Banyai et al., 1994).

There are two genetically distinct forms of type IV collagenases, a 72 kDa enzyme (MMP-2, gelatinase A) and a 92 kDa form (MMP-9, gelatinase B) (Collier et al., 1988; Wilhelm et al., 1989). The latter has a short additional sequence of unknown function between the catalytic and C-terminal domains (Wilhelm et al., 1989). Both enzymes cleave the native form of type IV and V collagens within their triple-helical domains, and they also have high activity against denatured collagen (gelatin) and fibronectin (Collier et al., 1988; Wilhelm et al., 1989; Okada et al., 1990). Since type IV collagen is a major component of the basement membranes, type IV collagenases have been thought to be key enzymes in the normal turnover of basement membranes and in pathological processes which involve the penetration of these matrices by cells, such as invading tumor cells (Stetler-Stevenson, 1990; Stetler-Stevenson et al., 1993). All matrix metalloproteases are secreted as latent forms, but the mechanisms of their activation *in vivo* are still some of the major unanswered questions regarding these enzymes. The current hypothesis is that the latent enzyme forms are maintained by a conserved cysteine residue of the N-terminal domain which is coordinated to the Zn atom of the catalytic site (Springman et al., 1990). According to the "cysteine switch" model, activation of the matrix metalloproteinase involves the removal of this cysteine residue from the active

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[1] Abbreviations: APMA, (4-aminophenyl)mercuric acetate; AcNPV, *Autographa californica* nuclear polyhedrosis virus; HPLC, high-performance liquid chromatography; MOI, multiplicity of infection; P_1', protease substrate nomenclature according to Schecter and Berger (1967); PCR, polymerase chain reaction; RP, reversed phase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SF, *Spodoptera frugiperda*; TIMP, tissue inhibitor of metalloproteinases.

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center followed by autolytic degradation of the N-terminal domain (Van Wart & Birkedal-Hansen, 1990). Such activation can be achieved *in vitro* by different reagents, including proteases, mercurial compounds, detergents, or some oxidizing substances, many of them without any physiological relevance. In some cases, however, there are activators of physiological relevance like the plasminogen/plasmin system for interstitial collagenase (Werb et al., 1977; He et al., 1989), or oxidative substances generated during oxidative burst for the activation of neutrophil collagenase. The 72 kDa type IV collagenase is unique in that respect, since no serious candidates for physiological activation have convincingly been demonstrated so far, although it has been shown that some cell lines are capable of specifically activating extracellular MMP-2; these results narrow the potential activator to a membrane protein, either a specific binding protein or a metalloprotease (Brown et al., 1990, 1993; Ward et al., 1991; 1993; Monsky et al., 1993; Strongin et al., 1993). Activation *in vitro* has been achieved with stromelysin, but whether this is close to physiological activation remains unclear since those experiments were carried out in the presence of APMA, a known activator of matrix metalloproteases and with rather high stromelysin concentrations (Miyazaki et al., 1992). Although the mechanism of *in vivo* activation is still an open question, it is likely that autolytic reactions are involved. Knowledge of the autoprocessing pathway could be useful for understanding how the actions of matrix metalloproteases are regulated in the extracellular space.

In the present study, we report on detailed analysis of the autoactivation pattern of 72 kDa type IV collagenase using recombinant enzyme expressed in a baculovirus/insect cell system. The results show that the autoactivation of the enzyme has a stepwise mechanism by which the proenzyme is processed through cleavage at specific sites to the active 62K form. Other autodegradation sites, which lead to the removal of the C-terminal domain and further truncation of the N-terminus, were also identified. Self-inactivation of the enzyme occurs from cleavage sites within the Zn-binding motif.

EXPERIMENTAL PROCEDURES

Materials. Full-length 72 kDa type IV collagenase cDNA, cloned in p16SPT19−1, has been described elsewhere (Kleiner et al., 1993). SF9 insect cells, baculovirus transfer vector pVL1393, and AcNPV were a kind gift from M. Summers (Summers, 1986). TNM-FH insect cell medium and gelatin−agarose were from Sigma, fetal calf serum from GIBCO, and Brij 35 "for membrane research" from Boehringer Mannheim. All other chemicals were of analytical grade. The HPLC system (Waters, Model 625) was equipped with a Waters 991 diode array detector.

Cloning of 72 kDa Type IV Collagenase cDNA into Baculovirus. The 72 kDa type IV collagenase cDNA was subcloned into the *Sma*I site of the pVL1393 transfer vector. Plasmid DNA amplified in *Escherichia coli* strain MC 1061 was transferred into the AcNPV genome by homologous recombination *in cellulo* so that SF9 cells were transfected with transfer vector and AcNPV DNA using the calcium phosphate method. Infected cells were screened for recombinant virus by dot blot hybridization. Positive virus clones were harvested from the culture medium, and the DNA was characterized by sequencing and Southern blotting.

Site-Directed Mutagenesis. Mutations (Glu_{375}→Asp and Glu_{375}→Gln) were introduced by PCR using primers coding for the mutated amino acid residue according to a procedure described earlier (Landt et al., 1990).

Production of Recombinant 72 kDa Type IV Collagenase. SF9 insect cells were grown at 27 °C in TNM-FH medium containing 10% fetal calf serum to a density of (1−2) × 10^6/mL. For infection, cells were centrifuged for 5 min at 500*g* (room temperature), resuspended in serum-free medium at 10^7cells/mL, and incubated for 1 h at room temperature with virus at 3−5 MOI. The cells were precipitated as before and resuspended in serum-free medium to a give a density of 10^6 cells/mL. The cells were cultivated for 96 h, and precipitated at 4 °C, 500*g*, for 15 min. The supernatant was clarified by centrifugation at 4 °C, 25000*g*, for 1 h, made up to 1 M NaCl/0.05% (w/v) Brij 35, and pumped over a gelatin−Sepharose column (5 mL) at 50 mL/h (4 °C). The column was washed with 50 mM Tris, pH 7.3, 1 mM $CaCl_2$, 0.5 M NaCl, and 0.05% Brij 35. Bound protein was eluted with a DMSO gradient up to 10% (v/v) in 500 mL. For further purification, the protein was concentrated by ultrafiltration, transferred to 20 mM Tris, pH 8.0, 1 mM $CaCl_2$, and 1% DMSO (protein concentration about 0.5 mg/mL), and subjected to anion-exchange chromatography (room temperature) using a Waters Protein Pak Q 8 HR (5 mm x 50 mm) column. The NaCl gradient was from 40 to 150 mM in 24 min, flow rate 0.75 mL/min; 72 kDa type IV collagenase was eluted at 50−60 mM.

Electrophoresis and Zymography. SDS−PAGE was carried out using 10% gels with a discontinuous buffer system (Laemmli, 1979), followed by silver staining (Dammerval et al., 1987). For zymography, the gels were polymerized with 0.1% (w/v) gelatin. Following electrophoresis, the gels were incubated for 1 h in 50 mM Tris, pH 7.5, 10 mM $CaCl_2$, 0.01 mM $ZnCl_2$, and 2.5% Triton X100, followed by incubation for 12 h at 37 °C in the same buffer containing 1% Triton X-100. Gels were stained with Coomassie Brilliant Blue. Electrophoresis samples were prepared by incubating for 15 min at room temperature with an equal volume of 125 mM Tris, pH 6.8, 4% (w/v) SDS, and 20% (v/v) glycerol containing 10% (v/v) β-mercaptoethanol as an option.

Digestion of Type IV Collagen. The activity of the wild-type recombinant 72 kDa type IV collagenase against native type IV collagen was determined by a procedure described earlier (Fessler et al., 1984). Briefly, tritium-labeled type IV collagen, purified from EHS tumor, was dissolved in 50 mM Tris pH 7.5, 10 mM $CaCl_2$, and 0.2 M NaCl and incubated with recombinant wild-type MMP-2 (0.5 μg/mL) for 24 h at 27 °C. The digestion products were electrophoresed on 6% SDS−PAGE and analyzed by fluorography.

Autoactivation Experiments. Purified 72 kDa type IV collagenase was incubated at 37 °C with or without 2 mM APMA for different time periods. Autoactivation was terminated by adding EDTA to 10 mM (HPLC samples) or by adding electrophoresis sample buffer. Autodigestion products were analyzed by electrophoresis or subjected to RP-HPLC

Separation of Autoactivation Products by RP-HPLC. Samples containing 20 μg of 72 kDa type IV collagenase (300 pmol) were run on a Waters Delta Pak C_{18} 300 Å column [2 mm (program 3) or 3.9 mm (programs 1, 2) × 150 mm]. The liquid phase was 0.100% TFA in H_2O (A)

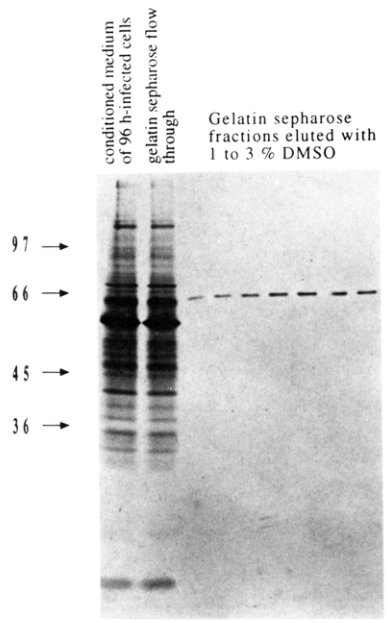

FIGURE 1: One-step purification of MMP-2. SF9 cells were infected in serum-free medium. After 96 h, the medium was collected, adjusted to 1 M NaCl/0.05% Brij 35, and pumped over a gelatin−agarose column (25 mm x 30 mm, flow 100 mL/ h). Bound protein was eluted with 50 mM Tris, pH 7.5, 1 mM $CaCl_2$, 0.5 M NaCl, and 0.05% Brij 35 using a DMSO gradient up to 10% over 500 mL. Fractions were analyzed by SDS−PAGE (unreduced samples). The position of molecular weight standards is indicated on the left.

and 0.092% TFA in acetonitrile (B) with the following gradient programs: 0−60% B, linear gradient in 60 min, flow 0.8 mL/min (program 1); 5−57% B in 60 min, flow 0.5 mL/min (program 2); and 5−57% B in 65 min, flow 0.1 mL/min (program 3). Fractions were collected manually, concentrated by vacuum evaporation to about 30 μL, and subjected to N-terminal sequencing.

N-Terminal Sequencing. N-terminal sequencing of enzyme peptides was carried out with an ABI 477 pulsed liquid sequencer using standard sequencing chemistry as recommended by the manufacturer. Samples from RP-HPLC were applied directly; otherwise, samples were concentrated in a mini ultrafiltration device with a 10 kDa cutoff membrane (Millipore UFC3 LGC), where an Immobilon membrane (Millipore), 5 mm in diameter, was placed on top of the UF membrane. The Immobilon membrane with bound protein was then washed with 50% methanol and sequenced.

RESULTS

Production and Characterization of Recombinant Protein. Human 72 kDa type IV collagenase was expressed in the baculovirus/insect cell system and purified from the conditioned medium of cells infected for 96 h. In principle, the enzyme could be purified in one step by a gelatin affinity column (Figure 1), but HPLC anion-exchange chromatography was required as a second step to ensure the removal of minor (autoactivation) products. The final yield was 1−4 mg/L of culture medium (10^9 cells).

In addition to the wild-type enzyme, two mutant forms were produced, where the Glu_{375}, essential for catalytic activity, was substituted by Asp or Gln. Both mutant proteins showed identical behavior on chromatography, but the enzymatic activity of the Asp variant was strongly decreased (Figure 2), and the Glu→Gln mutant had no activity when analyzed under the same conditions (not shown). The wild-

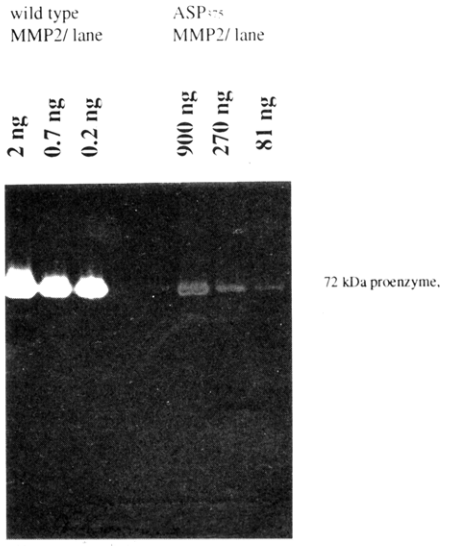

FIGURE 2: Analysis of wild-type MMP-2 and its Glu_{375}→Asp mutant by gelatin zymography. Different amounts of enzyme were analyzed by gelatin zymography as given under Experimental Procedures (unreduced samples).

type recombinant 72 kDa type IV collagenase showed the same cleavage pattern of native type IV collagen into 1/4- and 3/4-size fragments (not shown) as has been reported for enzyme purified from mammalian tissues (Fessler et al., 1984). When the three different recombinant enzymes were sequenced, only the mutant forms had the full-length N-terminus, whereas the wild-type enzyme lacked the first four amino acids. However, when the wild-type enzyme was prepared for sequencing from a diluted solution directly after elution from the gelatin affinity column, the original N-terminus could be found as well.

Autoactivation. When purified 72 kDa type IV collagenase was incubated at concentrations as high as 5 μM at 37 °C, it was readily processed in a concentration dependent manner (Figure 3), resembling the products reported in earlier studies (Howard et al., 1991) where APMA was used as activator. When a larger amount of the purified proform of recombinant 72 kDa type IV collagenase was analyzed with the highly sensitive gelatin zymography procedure, it became clear that a low amount of active enzyme was present in this preparation, although only as a minute fraction. This fraction could not be detected with silver staining or lower concentrated enzyme, but it cannot be excluded that it is sufficient to trigger an autoactivation cascade if the 72 kDa type IV collagenase is concentrated. Partially in contrast to what has been reported in earlier studies using our recombinant enzyme (Keski-Oja et al., 1992), we could not activate it with the proteases urokinase, plasmin, trypsin, or chymotrypsin (not shown).

Autolytic Processing. The studies described here were carried out with APMA-activated wild-type 72 kDa type IV collagenase if not stated otherwise. We also examined the activation of wild-type 72 kDa type IV collagenase merely incubated at 37 °C and that of APMA-activated Glu_{375}→Asp mutant and found basically the same pattern of processing for both forms. The activation rate was so slow, however, that degradation peptides were generated in too low amounts to allow for a detailed study of the final degradation products. Therefore, the APMA-activated wild-type 72 kDa type IV collagenase was chosen as a model to determine the

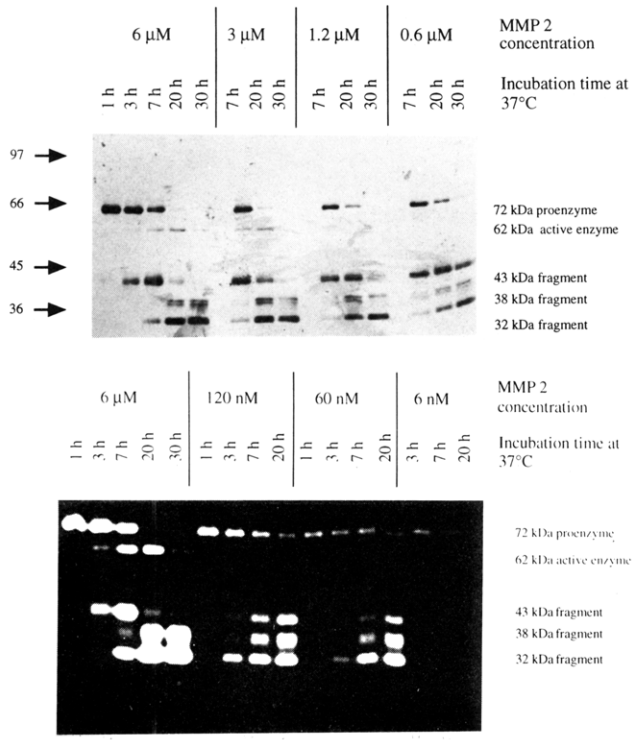

FIGURE 3: Concentration dependency of MMP-2 autoactivation. Different concentrations of MMP-2 were incubated at 37 °C and analyzed by SDS−PAGE (unreduced samples). (Top) Silver-stained gel (160 ng/lane). (Bottom) Zymogram (3 ng/lane).

autoactivation cleavage sites. The results are given in Table 1.

The first step in the activation process is cleavage of the Asp_4−Ile_5 bond. The resulting peptide could only be found from the Glu_{375}→Asp mutation; the purified and concentrated wild-type enzyme was already digested at this bond, apparently without effect on the latency of the enzyme.

The next steps are rapid and not easy to follow. We observed two peptides coming up at the same time; both of them are immediately further degraded. These peptides are probably the processing products of the second activation peptide resulting from cleavage at Lys_{50}−Phe_{51}. The resulting Ile_4−Lys_{50} could not be identified, owing to its short lifetime and the fact that it can be expected to coelute with the protein components of the reaction mixture. We cold not find any indication for a one-step processing of the initial latent form to an active 62 kDa enzyme as has previously been assumed (Stetler-Stevenson et al., 1989). There was no Ile_4−Asn_{80}-peptide, nor could we find the degradation products which are expected if that peptide is processed too quickly. Instead, the two peptides downstream of Phe_{51} were found to be released clearly after the first activation products mentioned above. The N-terminus is further processed at several cleavage sites to Ala_{165}, which seems to be stable against further cleavage.

Interestingly, a rather early event is the digestion in the C-terminal end of the catalytic domain at two cleavage sites, which result in the removal of the hemopexin domain and probably initiate inactivation of the enzyme, which is completed later by splits right in the Zn-binding motif. We did not find cleavage products from the hemopexin domain, indicating that this part of the enzyme is resistant to autodegradation. From these data, we suggest an autoactivation pathway as shown in Figures 4 and 5.

DISCUSSION

Human 72 kDa type IV collagenase was expressed in a baculovirus/insect cell expression system. This expression system provided the enzyme in native form, free of TIMP-2 which usually is associated with the enzyme when isolated from tissues or cultured cells. Purification of the enzyme to homogeneity could be achieved with at most two chromatographic steps. Chromatographic properties and catalytic activity of the recombinant enzyme were identical to those of enzyme isolated from natural material, indicating that this expression system provided correctly folded enzyme. A recombinant protein expression system like this also provides the access to mutated forms that may be useful to study specific properties of the enzyme. Here, we substituted Glu_{375}, which is essential for the catalytic mechanism of metalloproteases, by Asp or Gln, alternatively. As expected, the two mutated forms exhibited strongly reduced or no activity, respectively. The mutants proved to be valuable tools in studies of the autoprocessing of 72 kDa type IV collagenase.

Surprisingly, our sequencing analyses showed that the wild-type proenzyme purified according to standard procedures was already a truncated form, lacking the first four amino acid residues (Ala-Pro-Ser-Pro) of the propeptide preceding Ile_5. This truncation was not seen in the two mutated proteins which were made using the same basic cDNA. However, when the Glu_{375}→Asp mutant which had very weak enzymatic activity was treated with APMA, loss of the same four amino acid residues could be observed. We conclude that this early processing is not an artifact of the expression system, as the same type of truncation has been observed in 72 kDa type IV collagenase isolated from human A2058 melanoma cells (Bergmann et al., unpublished observations; Howard et al., 1991) or from a different expression system (Crabbe et al., 1993). Together, these results prove that this truncation is a normal step in the autolytic process. The significance of the four-residue cleavage is obscure, but it has no significant effect on the latency of the enzyme. At present, the tertiary structure has not been reported for any latent mammalian matrix metalloproteinase, but the homologue astacin has the N-terminus buried within the active site (Bode et al., 1992). If the same situation applies for 72 kDa type IV collagenase, it is also possible that the N-terminus is involved in regulating the latent structure and that its removal is a prerequisite, although not a sufficient condition for activation. It would be interesting to examine if mutation of the Pro_4−Ile_5 site inhibits activation of the enzyme.

The present results showed that the recombinant wild-type 72 kDa type IV collagenase was readily degraded without exogenous activation, the autodegradation being concentration-dependent. This observation has been reported earlier (Crabbe et al., 1993) and is consistent with intermolecular processing as the rate-limiting step. There is, however, evidence demonstrating that activation in the presence of APMA is independent of enzyme concentration, indicating intramolecular processing (Okada et al., 1990). This leads to the conclusion that activation is initiated by an intermolecular reaction, causing a structural rearrangement allowing further processing which then may involve intramolecular steps. The triggering event can be provided by a reaction with reagents such as APMA or by concentrating the enzyme.

Table 1: Characterization of Autocleavage Products of Recombinant 72 kDa Type IV Collagenase

retention time (min)	HPLC program[a]	N-terminal sequence[b]	remarks
		Peptides Identified after 2 min Activation	
11.32	1	LNT	peptide characterized by exaustive sequencing
15.55	2	APSP	peptide characherized by exaustive sequencing found only on Asp mutant
36.46	3	IIKFPGDVAPKXDKELAVQYLN	peptide characterized by exhaustive sequencing
39.72	3	FYGXPXESXNLFV....	
35.00	3	IIKFPGDVAPKXDKELAVQY	peptide characterized by exhaustive sequencing
35.00	3	LFVLKDTLXK	peptide characterized by exaustive sequencing coeluting with IIKF..QY
24.57	3	FYGXPKESXN	peptide characterized by exhaustive sequencing
		New Peptides Identifed after 8 min Activation	
28.3	1	IYTYTK...	
13.7	1	LEXSQDP...	
19.7	1	QTGDL...	
26.4	1	QTGDLD...	
34.4	1	FFGLP	peptide characterized by exhaustive sequencing
42.7	1	XXXPGT...	
49.3	1	XNFFPR...	
		New Peptides Identified after 17 min	
27.4	1	LYGAS...	
29.6	1	YNFFP...	
47.3	1	YNFFP...	
		New Peptides after 90 min	
13.1	1	LVAA...	
15.6	1	no sequence	
17.9	1	IYTYTKN...	
24.7	1	WEHGDGYPFD...	
26.0	1	XNFF...	
33.2	1	IGYTPDLDPE...	
37.1	1	YNFFP...	
37.1	1	LFVLKD...	coeluting with YNFFP...
		After 15 h Incubation	
22.7	1	KTDKE...	
23.2	1	VXXDVT...	
29.5	1	FQVWSDVTP...	coeluting with YNFF
31.0	1	NFFPR...	
32.6	1	IMINFG...	
32.9	1	SXIXDG...	
41.7	1	AFAPG...	

[a] See Experimental Procedures. [b] X, unidentified residue.

In the latter case, activation can be due either to a contamination with minute amounts of active enzyme, or a yet unknown activating factor, or to a nonproteolytic intermolecular structural rearrangement, provoked by the high enzyme concentration.

The cleavage sites used in the autodegradation of 72 kDa type IV collagenase were characterized in detail, using the recombinant wild-type and mutant enzymes. The cleavage following the Pro_4−Ile_5 split leads to rapid activation of the enzyme resulting in the metastable 62 kDa active form, which is usually referred to as the active form. In contrast to a one-step process as suggested earlier, our results indicated that there are intermediate forms. This controversy can be explained by the high reaction rate of the first processing steps, which does not allow for isolation and sequencing of the intermediate forms. These steps might be candidates for the intramolecular activation as discussed before (Okada et al., 1990). From the point of view of the cysteine-switch mechanism, this implies that Cys_{73} in the propeptide is coordinated very weakly to the active site and requires the propeptide to be kept in its position, since the intermediate form, beginning with Phe_{51}, appears to be highly active. Similar conclusions were drawn from experiments with stromelysin 1 (MMP-3), where the activation is triggered by a structural rearrangement within the precursor molecule, rather than by a disruption of the corresponding Cys−Zn bond (Chen et al., 1993). For the 92 kDa type IV collagenase, the active form still contains the corresponding Cys residue (Triebel et al., 1992).

The present results provided information on further processing of 72 kDa type IV collagenase showing that it autodegrades in a relatively regular manner into numerous fragments (Figures 3 and 4), some of which have been described earlier (Howard et al., 1991).

It is likely that the cleavage sites described here can be combined in different ways to give rise to a rather large number of active and inactive 72 kDa type IV collagenase fragments; the exact composition of the mixture is probably influenced by the activation rate, which itself is dependent on external parameters such as enzyme concentration, buffer composition, and temperature. The autocleavage sites do not reveal any remarkable sequence specificity, confirming results with synthetic peptides published earlier (Netzel-Arnett et al., 1993). However, the fibronectin domain and probably the hemopexin domain are stable against autodegradation since no peptides derived from those domains were identified. This implies sterical restrictions for substrate specificity. The crystal structure obtained for the catalytic domain of some MMPs suggests that the P′ site of the substrate has to be in an extended (β sheet) conformation (Bode et al., 1994; Lovejoy et al., 1994; Borkakoti et al., 1994; Stams et al., 1994; Gooley et al., 1994); the fibronectin

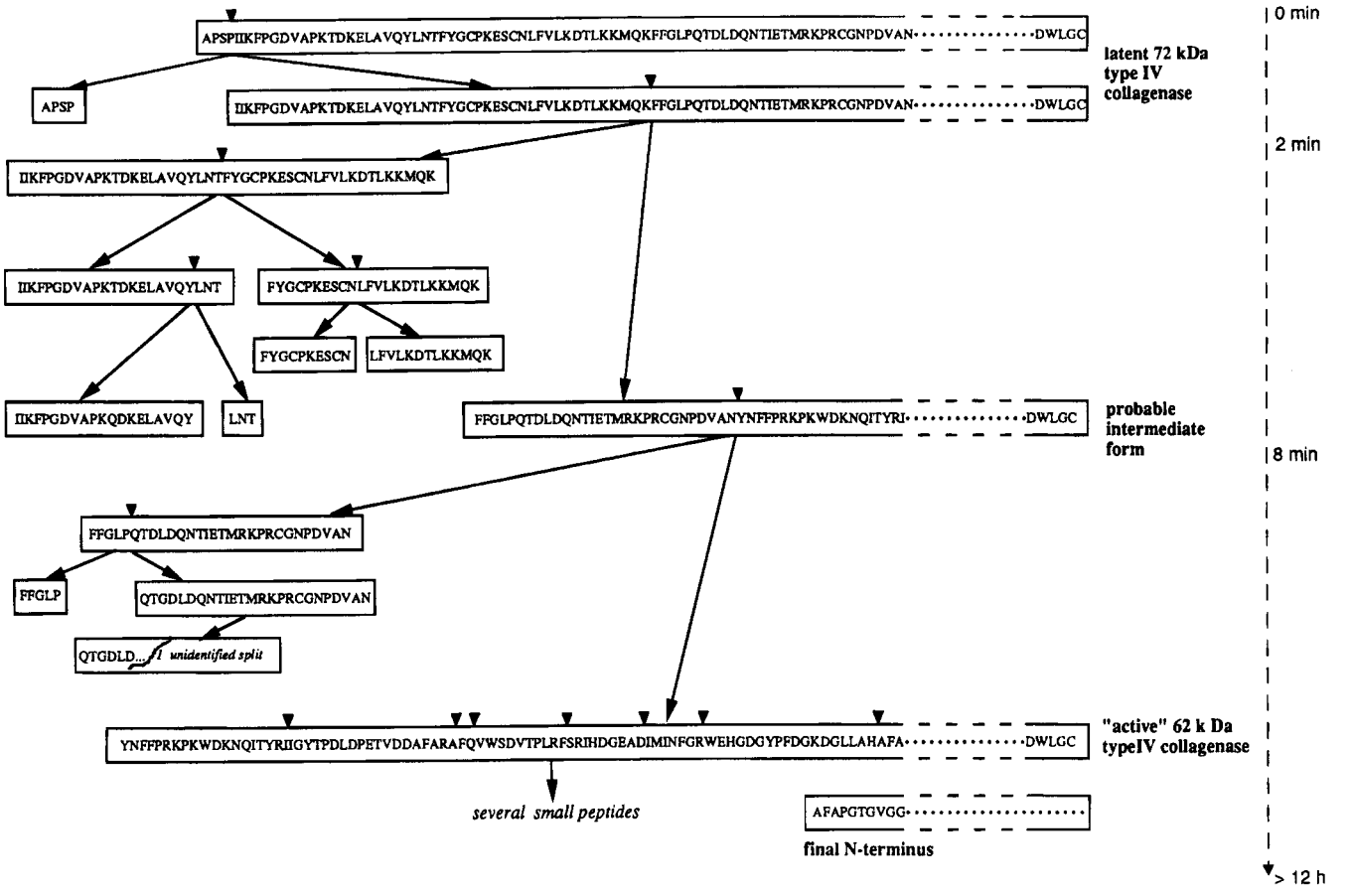

FIGURE 4: Autolytic truncation of the N-terminal domain of MMP-2. Fragments formed through processing of the N-terminal domain of MMP-2 following APMA activation according to the results shown in Table 1. The 72 and 62 kDa polypeptides corresponding to the bands in Figure 3 are indicated.

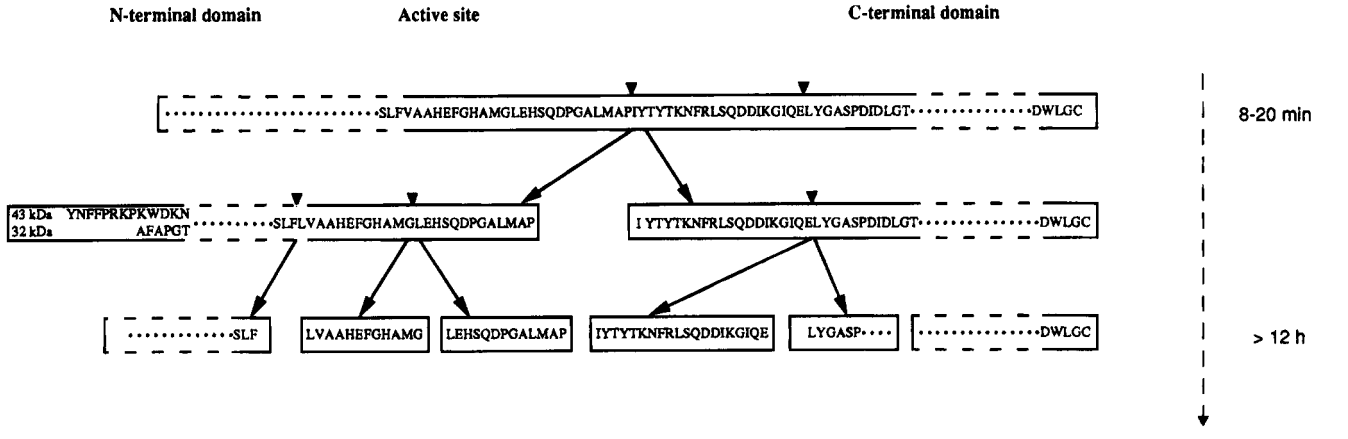

FIGURE 5: Autolytic cleavage sites in the catalytic domain of MMP-2. Fragments formed through processing of the catalytic domain following APMA activation according to the results shown in Table 1. The N-termini of the two major fragments are indicated and marked according to the bands shown in Figure 3. The 38 kDa fragments seen in Figure 3 are probably more unstable intermediates which occur in too small amounts to be characterized in detail.

and hemopexin domains are probably sterically restricted and cannot adopt this conformation, while other parts of the enzyme have an open structure which can be readily autodigested.

Although the autoactivation pathway described here for 72 kDa type IV collagenase was obtained by *in vitro* analysis, it is likely that the physiological activation of this enzyme shares some of its features. A processed form of 72 kDa type IV collagenase starting at Leu_{38} has recently been found after activation with a still uncharacterized membrane activator (Strongin et al., 1993). We have identified the same cleavage site, but in our case, it is clearly a secondary split, since the first activation peptide spans this region (Table 1).

Future work may be directed toward the question whether the secondary cleavages carry any physiological function. In principle, some of the early activation peptides could serve as cytokine-like messengers in order to influence the cellular activation of matrix metalloproteases.

The tight control of activation and degradation which is required to restrict the proteolytic effect of 72 kDa type IV collagenase *in vivo* is quite remarkable. It is known that TIMP-2 when associated with latent 72 kDa type IV

collagenase can decrease proteolytic activity and autodegradation *in vitro*, although the N-terminal conversion is still possible (Howard et al., 1991; Kleiner et al., 1992, 1993). Obviously, TIMP-2 protects cleavage at Pro_{394}–Ile_{395} and Glu_{414}–Leu_{415}, which confirms that the inhibitor has binding sites close to the catalytic domain (Kleiner et al., 1993). As mentioned above, no physiological activator candidates have yet been reported to activate latent 72 kDa type IV collagenase, and the ease with which 72 kDa type IV collagenase is autoactivated questions the need for a stoichiometric external activation, since raising the local concentration could be a major contribution toward activation. There is some evidence for the existence of a membrane-bound protein with affinity for the hemopexin domain which may be involved in cellular activation. Our results support the idea that the *in vivo* activation could be enhanced in that way, by merely concentrating the enzyme onto a surface.

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