Biochemistry

 Volume 30, Number 33 August 20, 1991

Accelerated Publications

The N-Terminal Domain of Tissue Inhibitor of Metalloproteinases Retains Metalloproteinase Inhibitory Activity†

Gillian Murphy,*,‡ Annick Houbrechts,§,∥ Mark I. Cockett,§ Richard A. Williamson,⊥ Mark O'Shea,‡ and Andrew J. P. Docherty§

Strangeways Research Laboratory, Cambridge CB1 4RN, U.K., Celltech Ltd., 216 Bath Road, Slough SL1 4EN, U.K., and Biological Laboratory, University of Kent, Canterbury CT2 7NJ., U.K.

Received May 10, 1991; Revised Manuscript Received June 10, 1991

ABSTRACT: Recombinant tissue inhibitor of metalloproteinases (TIMP-1) and a truncated version containing only the three N-terminal loops, $\Delta_{127-184}$TIMP, have been expressed in myeloma cells and purified by affinity chromatography and gel filtration. $\Delta_{127-184}$TIMP was found to exist as two main glycosylation variants of molecular mass 24 kD and 19.5 kDa and an unglycosylated form of 13 kDa. All forms of the truncated inhibitor were able to inhibit and form complexes with active forms of the matrix metalloproteinases, indicating that the major structural features for specific interaction with these enzymes resides in these three loops. Stable binding of $\Delta_{127-184}$TIMP to pro 95-kDa gelatinase was not demonstrable under the conditions for binding of full-length TIMP-1.

Two distinct tissue inhibitors of metalloproteinases, TIMP-1[1] and TIMP-2, that are synthesized and secreted by many cell types have been isolated and biochemically characterized. Both TIMPs have essentially similar properties, specifically inhibiting enzymes of the matrix metalloproteinase family (Murphy et al., 1981; Cawston et al., 1983; Stricklin & Welgus, 1983; Stetler-Stevenson et al., 1989; Goldberg et al., 1989; Wilhelm et al., 1989; Ward et al., 1991), and are thought to be of great importance in the maintenance of connective tissue integrity. TIMP-1 is also known to possess growth factor activity and is identical with erythroid potentiating activity. TIMP-1 and TIMP-2 have similar primary structures from cDNA cloning (Docherty et al., 1985; Boone et al., 1990), including 12 conserved cysteines. The assignment of six disulfide bond pairs has been made for TIMP-1 and delineates what appears to be a two-domain structure (Williamson et al., 1990). However, little has yet been described of the mechanism of TIMP binding and inhibition of MMPs, although TIMP-1 has been shown to interact noncovalently with active MMPs to yield complexes of very low dissociation constant (Cawston et al., 1983; Welgus et al., 1985; Murphy et al., 1989). As part of a program to determine the mechanism of TIMP action by mutagenesis, we have examined the role of the N-terminal domain. This paper presents data which indicate that the properties of binding and inhibition of active MMPs are contained within this domain. However, the unique property of TIMP-1 binding to the pro form of 95-kDa gelatinase (Wilhelm et al., 1989) is not maintained or is severely weakened in this truncated structure.

Experimental Procedures

Expression of $\Delta_{127-184}$TIMP-1 and TIMP-1. The TIMP cDNA was subcloned into pSP64 (Melton et al., 1984) between the *Hin*dIII and *Bam*HI sites of the polylinker. This was achieved by using oligonucleotide adapters to convert the

† G.M. is supported by the Arthritis and Rheumatism Council, U.K. R.A.W. is supported by a cooperative project grant to R. B. Freedman from the SERC and Celltech Ltd.

* To whom correspondence should be addressed.
‡ Strangeways Research Laboratory.
§ Celltech Ltd.
∥ Current address: Department of Biochemistry, Université de Liége, Inst. Chimie (B6) Sart Tilman, B-4000 Liége 1, Belgium.
⊥ University of Kent.

[1] Abbreviations: APMA, (4-aminophenyl)mercuric acetate; IgG, immunoglobulin G; MMP, matrix metalloproteinase; PAGE, polyacrylamide gel electrophoresis; PUMP, putative (punctuated) metalloproteinase; SDS, sodium dodecyl sulfate; TIMP, tissue inhibitor of metalloproteinases.

0006-2960/91/0430-8097$02.50/0

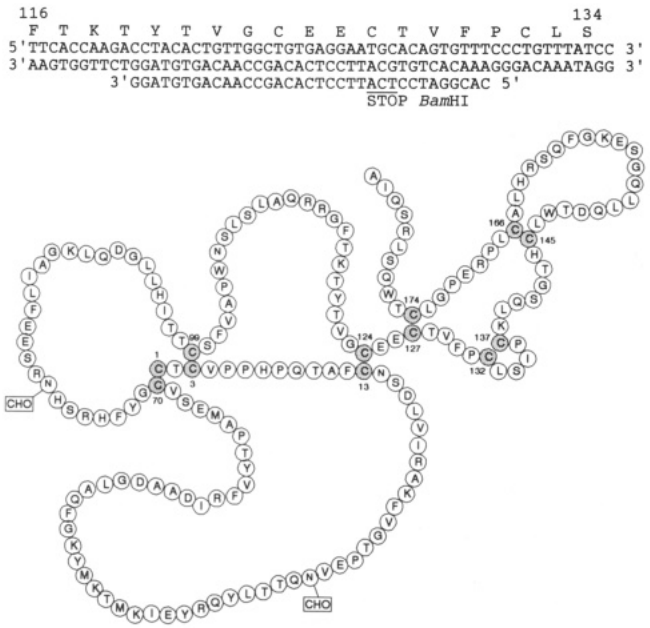

FIGURE 1: (Top) TIMP cDNA sequence encoding amino acids 116–134 and the mutagenic oligonucleotide. (Bottom) Two-dimensional representation of the structure of TIMP-1 showing the disulfide bond assignments of Williamson et al (1990).

5′ *Nco*I site adjacent to the ATG start codon (nucleotides 62–67; Docherty et al., 1985) into a *Hin*dIII site and to convert the 3′ *Hin*fI site adjacent to the TIMP stop codon (nucleotides 686–690) into a *Bam*HI site. The conversion of C_{127} to a stop codon followed by a *Bam*HI site was achieved by using this template DNA and the polymerase chain reaction (Mullis & Faloona, 1987). The DNA was subjected to 20 rounds of amplification with *Taq* polymerase in the presence of an oligonucleotide that primes from within the SP6 promoter and a second mutagenic oligonucleotide with the sequence shown in Figure 1 (top). The amplified product was digested with *Hin*dIII and *Bam*HI before agarose gel electrophoresis and glass milk extraction (Vogelstein & Gillespie, 1979). The shortened version of the TIMP coding sequence (referred to as $\Delta_{127-184}$TIMP) was reassembled in pSP64. Its sequence was shown by the dideoxy chain termination method with a series of oligonucleotide primers (Sanger et al., 1977) to correspond to that of wild-type TIMP except for the C-terminal deletion achieved by insertion of the stop codon described above.

The 460-bp *Hin*dIII–*Bam*HI fragment encoding $\Delta_{127-184}$TIMP and the equivalent 630-bp fragment encoding wild-type TIMP were subcloned between the hCMV promoter and the SV40 early polyadenylation signal in pEE12 (H. Caskey, unpublished work). This mammalian cell expression vector is a derivative of pEE6hCMV (Stephens & Cockett, 1989) containing a functional glutamine synthetase cDNA from pSV2GS under the control of the SV40 early promoter (Bebbington & Hentschel, 1987), which allows the selection of stable plasmid-bearing cell lines through growth in the absence of glutamine (C. R. Bebbington, unpublished work). Both plasmids were transfected into NSO mouse myeloma cells (ECACC catalog no. 85110503) by electroporation. *Sal*I-linearized plasmid DNA (40 μg) was added to 10^7 NSO cells that had been washed and resuspended in 1 mL of ice-cold PBS in an electroporation cuvette (Biorad 165-2088). The cells were incubated on ice for 5 min, followed by two pulses of 1500 V at 3 μF with a Bio-Rad gene pulser. After a further 5 min on ice, the cells were taken up in growth medium and diluted over several 96-well plates (75 μL/well) and incubated at 37 °C in 5% CO_2 for 24 h. Glutamine-free selection medium was added (75 μL/well), and the cells were incubated for a further 2–3 weeks until colonies were apparent. Conditioned media from selected colonies grown in mass culture were analyzed on substrate gels and in activity assays. The most productive cell lines were used to produce serum-free conditioned media from which the recombinant wild-type and $\Delta_{127-184}$TIMP were purified.

Purification of Recombinant $\Delta_{127-184}$TIMP-1 and TIMP-1. Culture medium from mouse myeloma cells expressing either the truncated or full-length TIMP was adjusted to 0.01 M Tris-HCl, pH 7.5, and 500-mL portions were loaded onto monoclonal anti-TIMP IgG (MAC 015) Sepharose columns (Cooksley et al., 1990; Williamson et al., 1990). The matrix was washed with 0.025 M Tris-HCl buffer pH 7.5, containing 0.5 M NaCl, 0.05% (w/v) Brij-35, and 0.02% azide to background A_{280} readings. The column was further washed with 5 bed volumes of 0.1 M sodium acetate buffer, pH 4.7, containing 0.5 M NaCl, 0.05% (w/v) Brij-35, and 0.02% toluene. The inhibitor was eluted with 0.1 M glycine hydrochloride buffer, pH 2.8, containing NaCl, Brij, and preservative as above. Fractions were collected into 0.1 volume of 2 M Tris-HCl, pH 8.0, to raise the pH. Inhibitory fractions were pooled and concentrated by using an Amicon concentration cell with a YM5 membrane. Yields of about 15 mg of inhibitor were obtained from each batch of culture medium. An $A^{1\%}_{280.1cm} = 1.0$ was used for wild-type TIMP, which has been established by amino acid analysis. For the $\Delta_{127-184}$TIMP, the various forms were titrated against stromelysin-1 in the dinitrophenyl-Pro-Leu-Gly-Leu-Trp-Ala-D-ArgNH_2 peptide assay of Stack and Gray (1989), assuming a 1:1 stoichiometry, in comparison with wild-type TIMP. It was deduced that an average $A^{1\%}_{280.1cm} = 1.4$ could be used for all forms of the truncated inhibitor. Further fractionation of the heterogeneous $\Delta_{127-184}$TIMP (3–5-mg batches) was effected by using a Sephacryl S200 column (100 × 1.5 cm), equilibrated with 0.025 M Tris-HCl buffer, pH 7.5, containing 0.5 M NaCl, 0.05% Brij-35, and azide.

Inhibitor Assays. Inhibitory activity was routinely assayed by using stable preparations of rabbit collagenase in a ^{14}C-labeled diffuse fibril assay, as described previously (Murphy et al., 1981). One unit of inhibitory activity gives 50% inhibition of 2 units of enzyme. Inhibitor subjected to heat treatment or trypsin proteolysis was assayed by this method and compared to untreated proteins. Inhibitory activity against pure samples of active human collagenase were also assessed by using this assay, against stromelysin and PUMP with ^{14}C-labeled casein as substrate and against 95-kDa and 72-kDa gelatinases with ^{14}C-labeled gelatin as substrate (Galloway et al., 1983). One unit of enzyme is defined as the activity turning over 1 μg of substrate/min.

Gel Electrophoresis. Samples of inhibitor were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) on 13% gels by using the Laemmli system (Laemmli & Favre, 1973) under reducing conditions. Gels were either silver stained (Merril et al., 1981) or immunoblotted onto nitrocellulose and immunoreactive material visualized by using a polyclonal sheep anti-human TIMP-1 antibody (Hembry et al., 1985) followed by a peroxidase-labeled anti-IgG and 4-chloro-1-naphthol substrate (Towbin et al., 1979).

Deglycosylation. $\Delta_{127-184}$TIMP was denatured by using 0.5% SDS and 1% 2-mercaptoethanol at 37 °C for 30 min and then treated with N-glycopeptidase F (500 ng, 20 units, volume 20 μL) at 37 °C for 5 h as instructed by Boehringer. The deglycosylated inhibitor was analyzed by polyacrylamide gel electrophoresis.

Metalloproteinase–Inhibitor Complex Studies. These were carried out as described by Murphy et al. (1989). TIMP-1 and $\Delta_{127-184}$TIMP-1 were trace-^{125}I-labeled by using Bolton–Hunter reagent to a specific activity of about 2.0×10^3 cpm/ng. Complexes of 6–30 pmol of ^{125}I-labeled inhibitor with different metalloproteinases were formed in a volume of 100 μL at room temperature for 30 min prior to chromatography on Sephacryl S200 as described above. Elution patterns were followed using a Packard γ counter. Molecular mass values were calculated from the elution pattern of standard proteins including alcohol dehydrogenase (150 000 kDa), bovine serum albumin (68 000 kDa), ovalbumin (45 000 kDa), carbonic anhydrase (29 000 kDa), soya bean trypsin inhibitor (22 000 kDa), and cytochrome *c* (12 500 kDa).

The formation of complexes of both $\Delta_{127-184}$TIMP and full-length TIMP with pro 95-kDa gelatinase were assessed by the above gel-filtration method. This was not entirely satisfactory, since the dissociation constant for wild-type TIMP–pro 95-kDa gelatinase complexes is apparently much higher than those for TIMP–active MMP complexes (Ward et al., 1991) and only low levels of stable complex were detectable at the low concentrations of reactants used in gel filtration experiments. Alternatively, the ability of the MAC015 Sepharose to bind TIMP–MMP complexes was exploited to assess the ability of different forms of TIMP to form complexes with pro or active 95-kDa gelatinase. TIMPs were incubated with pro 95-kDa gelatinase or its active form with 120 nM inhibitor in an approximately 1-fold molar excess with respect to enzyme under the conditions as described above. Incubations were chromatographed on MAC015 Sepharose as described for inhibitor purification. The elution of gelatinase and inhibitor in both unbound fractions and material eluted at pH 2.8 was assessed by either ^{14}C-labeled gelatin assay of free enzyme (unbound fractions) or gelatin–polyacrylamide substrate gel analysis of complexed enzyme and inhibitor (all fractions). This latter method is not quantitative, particularly with respect to gelatinase activity, but it gives a qualitative analysis of the distribution of activities.

A third method of complex assessment was employed that was a modification of the TIMP-1 sandwich ELISA described by Cooksley et al. (1990). A polyclonal sheep anti-TIMP IgG (Hembry et al., 1985) was coated onto microtiter plates and used to capture TIMP and TIMP–MMP complexes. After washing, TIMP binding could be measured by using MAC015 as the revealing antibody. TIMP complexed to MMPs can be distinguished in this assay since the complexes bind less efficiently to the polyclonal IgG layers and therefore give a significantly reduced signal. The recombinant TIMPs, either alone or in complexes in the presence of a 4-fold molar excess of pro 95-kDa gelatinase, were assayed in the ELISA. Pro 95-kDa gelatinase gave no detectable signal in this assay.

Preparation of Matrix Metalloproteinases. Recombinant forms of collagenase and stromelysin-1 were expressed and purified as described previously (Murphy et al., 1987; Docherty & Murphy 1990; Koklitis et al., 1991). The 95-kDa gelatinase was purified from U937 cell culture medium and the 72-kDa gelatinase from human gingival fibroblast culture medium as described by Ward et al. (1991). Matrix metalloproteinases were activated by treatment with (4-aminophenyl)mercuric acetate (APMA): 95-kDa gelatinase with 2 mM APMA at 37 °C for 1 h, 72-kDa gelatinase with 2 mM APMA at 25 °C for 1 h and PUMP with 1.5 mM APMA at 37 °C for 1 h. Stromelysin-1 and -2 were more efficiently activated with 5 μg/mL trypsin at 25 °C for 30 min, followed by the addition of 10× soya bean trypsin inhibitor. Collagenase was activated as for stromelysin except that active stromelysin-1 at a molar ratio of 0.01:1 (stromelysin:collagenase) was included and incubations were at 37 °C.

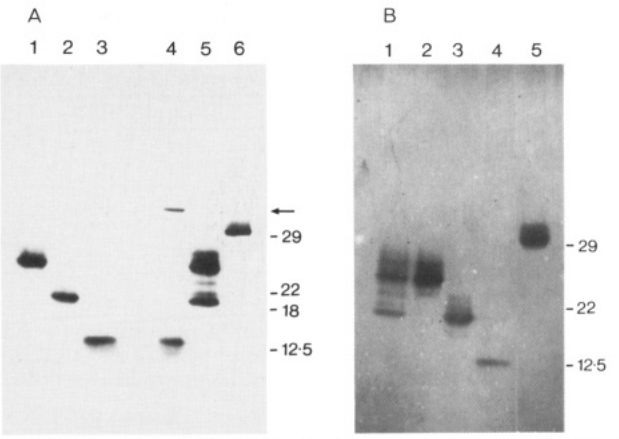

FIGURE 2: Electrophoretic analysis of $\Delta_{127-184}$TIMP and wild-type TIMP. Various forms of purified TIMPs were run on SDS–13% polyacrylamide gels (reducing conditions). (A) Silver-stained gel. $\Delta_{127-184}$TIMP was purified by affinity chromatography and gel filtration into three major forms of 24 kDa (lane 1), 19.5 kDa (lane 2), and 13 kDa (lane 3). If the mixed forms obtained from affinity chromatography (lane 5) were treated with N-glycopeptidase F (arrowed), all were converted to the 13-kDa species (lane 4). Wild-type TIMP-1 purified on the affinity column is shown in lane 6. (B) Affinity-purified mixed forms of $\Delta_{127-184}$TIMP (lane 1) and gel-purified 24-kDa (lane 2), 19.5-kDa (lane 3), and 13-kDa (lane 4) forms were recognized by a polyclonal sheep anti-TIMP-1 antibody on immunoblotting, as was wild-type TIMP (lane 5). The mobility of molecular mass standards (kilodaltons) is indicated.

Results and Discussion

Isolation of $\Delta_{127-184}$TIMP and Electrophoretic Characterization. The disulfide assignment in TIMP-1 indicates a two-domain structure in which three N-terminal loops are separated from the disulfide-bonded loops of the C-terminal domain by the glutamic acid residues E_{125} and E_{126} (Williamson et al., 1990; Figure 1 (bottom).

We expressed $\Delta_{127-184}$TIMP-1 containing the three N-terminal loops of TIMP-1 in mouse myeloma cells. Levels of greater than 30 mg/L of culture medium were obtained and purified on a monoclonal anti-TIMP-1 IgG (MAC015) Sepharose column. The truncated inhibitor bound as efficiently to this matrix as did wild-type TIMP and was eluted as two major bands of molecular mass 24 and 19.5 kDa and a minor band of 13 kDa (Figure 2A). All three bands were shown by Western blotting to react with a polyclonal antibody specific to TIMP-1 (Figure 2B). Treatment of the preparation with N-glycopeptidase F converted the two upper forms as well as a number of faint intermediate bands to 13 kDa (Figure 2A, lane 4). We conclude that the multiple bands of $\Delta_{127-184}$TIMP result from glycosylation heterogeneity. Rather less extensive heterogeneity is observed in both natural wild-type TIMP and the recombinant form made by NSO myeloma cells (Murphy & Werb, 1985; Figure 2A, lane 6). Like full-length TIMP, the truncated inhibitor sequence still possesses two glycosylation sites. Assuming the same amount of carbohydrate attachment, the theoretical molecular mass of $\Delta_{127-184}$TIMP would be about 23.5 kDa, that is, of a similar size to the 24-kDa species shown in Figure 2A, lanes 1 and 5. It may be that the intermediate 19.5-kDa species represent a form of the inhibitor that is glycosylated at only one of the two potential sites.

Comparison of the Inhibitory Activity of Wild Type and $\Delta_{127-184}$TIMP. The three major forms of $\Delta_{127-184}$TIMP could

Table I: Specific Activity of Full-Length and Truncated TIMPs on Activated Human Matrix Metalloproteinases[a]

	units of inhibitory activity/μg of inhibitor				
		$\Delta_{127-184}$ TIMP-1			
enzyme	wild-type TIMP-1	mixed forms	24 kDa	19.5 kDa	13 kDa
collagenase	4.0	3.6	3.5	3.8	4.1
stromelysin-1	2.5	4.9	6.7	6.2	7.6
95-kDa gelatinase	29.8	37.7	53.1	48.0	58.0
72-kDa gelatinase	51.7	14.4	18.3	18.1	18.6
PUMP	1.5	1.2			

[a] The inhibitors were titrated against the enzymes in the appropriate assays described under Experimental Procedures, and the values were calculated from the linear portion of their respective inhibition curves.

be separated by gel filtration (Figure 2), eluting with molecular masses of 29, 20, and 16 kDa, respectively. Analyses of each form of $\Delta_{127-184}$TIMP as well as the mixed forms eluted from the affinity matrix showed that all the preparations could inhibit activated stromelysin-1 and 95-kDa gelatinase with higher specific activities than TIMP-1 (Table I). Compared to wild-type TIMP, $\Delta_{127-184}$TIMP had very similar activity against human collagenase and PUMP but significantly reduced activity against 72-kDa gelatinase (Table I). Small differences between the wild-type and $\Delta_{127-184}$TIMP specific activities recorded here may be accounted for by differences in protein estimation and molecular mass. Nevertheless, it could be concluded that the major sites responsible for MMP inhibition reside in the truncated structure, although a contribution by the C-terminal domain cannot be discounted and will be the subject of further investigation. These results are in contrast to those of Coulombe and Skup (1988), who expressed mouse $\Delta_{100-181}$TIMP in an in vitro system and obtained no inhibitory activity. This may be due to the inability of a two-loop structure with an odd number of cysteine residues to fold correctly.

Stability of $\Delta_{127-184}$TIMP. We tested the stability of $\Delta_{127-184}$TIMP at a temperature of 90 °C for 2 h, when it retained 80% of its activity, whereas intact recombinant TIMP-1 retained 55% of its activity. This is in keeping with the reported thermal stability of natural TIMP (Murphy et al., 1981; Stricklin & Welgus, 1983). However, all forms of the truncated TIMP were extremely susceptible to trypsin proteolysis, losing 84% of their activity (2.5:1 w/w; 1 μg/mL trypsin at 37 °C) in 2 h, relative to a 17% loss for intact TIMP-1. We concluded that the $\Delta_{127-184}$TIMP was stably folded but that trypsin susceptible sites were more accessible to proteinase attack.

Formation of Stable Complexes between $\Delta_{127-184}$TIMP and Metalloproteinases. Using ^{125}I-labeled 24-kDa $\Delta_{127-184}$TIMP, we showed that stable complexes with active forms of 95-kDa gelatinase of molecular mass 81 kDa and with active stromelysin-1 of molecular mass 58 kDa were detectable down to 50 nM on gel filtration, as observed for intact TIMP-1 (Murphy et al., 1989a,b). Similar results were obtained for 19.5-kDa TIMP, which formed a stromelysin-1 complex of 51.5 kDa. We also demonstrated that all forms of the truncated TIMP formed SDS-stable complexes with the 52- and 44-kDa active forms of human stromelysin-2, as does full-length TIMP (Figure 3). The formation of such stable complexes with TIMP-1 is a property unique to stromelysin-2 among the human matrix metalloproteinases (G. Murphy, unpublished work).

The ability of $\Delta_{127-184}$TIMP to inhibit and form complexes with the active forms of all the MMPs tested here suggests that binding and inhibitory properties both reside in the N-terminal 1–126 amino acids. In addition to the interaction of TIMPs with active MMPs, it has been reported that complexes may occur between TIMP and the pro form of 95-kDa gelatinase (Wilhelm et al., 1989; Ward et al., 1991). Therefore, we examined whether this property also resides within the N-terminal part of TIMP.

FIGURE 3: Formation of stromelysin-2–TIMP complexes. The ability of the various TIMPs to form stromelysin-2 complexes was demonstrated on nonreducing gels (13%). Prostromelysin-2 (arrow) and two major active species generated by treatment with (4-aminophenyl)mercuric acetate (dotted arrows, lane 1) were incubated with each TIMP form. Wild-type TIMP (lane 3) formed complexes with the activated stromelysin-2 (lane 2), as did the $\Delta_{127-184}$TIMP, 24 kDa (lane 4); inhibitor alone (lane 5) and the 19.5-kDa TIMP (lane 6); inhibitor alone (lane 7) and the 13-kDa TIMP (lane 8); inhibitor alone (lane 9). The mobility of molecular mass standards (kilodaltons) is indicated.

Full-length TIMP–pro 95-kDa gelatinase complexes were shown to bind to a MAC015 anti-TIMP matrix and could be eluted at acid pH. Pro 95-kDa gelatinase alone did not bind significantly to this matrix; in the presence of wild-type TIMP, 92% of the activity was retained on the matrix, as assessed by ^{14}C gelatin assays. The qualitative distribution of gelatinase and TIMP in the column fractions is shown in Figure 4A, lanes 4–6. In contrast, $\Delta_{127-184}$TIMP did not retard the passage of pro 95-kDa gelatinase through the anti-TIMP Sepharose, 87% of the activity being detected in the drop-through fraction (Figure 4A, lanes 1–3). It was, however, demonstrable that 75% of the same amount of active 95-kDa gelatinase was retained by the matrix in the presence of $\Delta_{127-184}$TIMP (Figure 4A, lanes 10–12).

The apparent inability of truncated TIMP to bind to pro 95-kDa gelatinase was confirmed by an ELISA method. A reduced signal for full-length TIMP over a concentration range of 2–18 ng/mL occurred in the presence of excess pro 95-kDa gelatinase (Figure 4B). The ELISA detected free $\Delta_{127-184}$TIMP far more weakly than the full-length TIMP, but a linear response over a 2–70 ng/mL range was obtained (Figure 4B). In the presence of activated stromelysin-1 a reduced $\Delta_{127-184}$TIMP signal was obtained over the same concentration range indicating binding of stromelysin to the TIMP. However, in the presence of an excess of pro 95-kDa gelatinase no significant modification of the $\Delta_{127-184}$TIMP signal was detected in the assay (Figure 4B), suggesting that no binding of enzyme to the truncated inhibitor had occurred. No stable ^{125}I-labeled $\Delta_{127-184}$TIMP–pro 95-kDa gelatinase complex could be demonstrated by gel filtration, but even full-length TIMP complexes were rather unstable to these conditions (Ward et al., 1991).

In conclusion, we have expressed both a full-length and a truncated ($\Delta_{127-184}$) form of TIMP in mammalian cells and

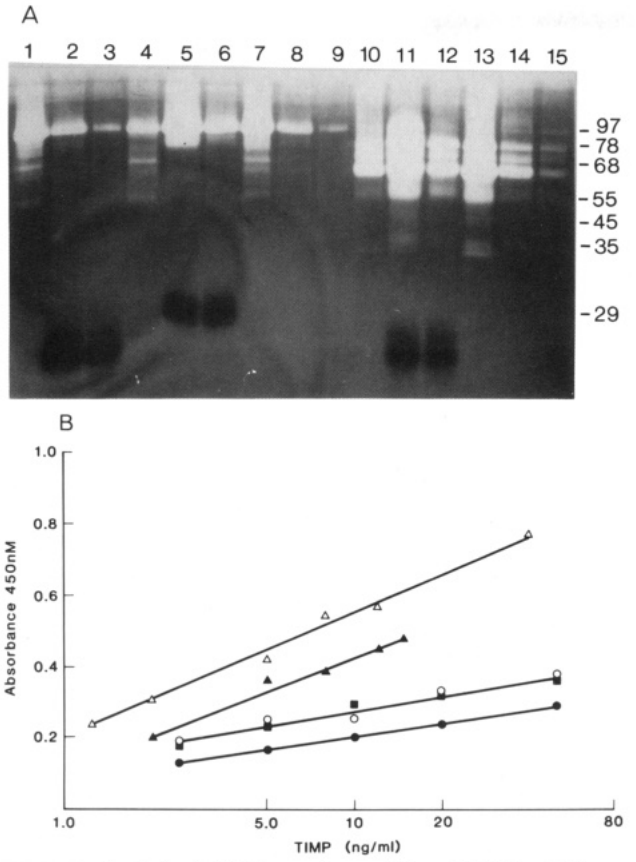

FIGURE 4: Analysis of TIMP complexes with pro 95-kDa gelatinase. (A) Gelatin–SDS–polyacrylamide gel (13%) analysis of the behavior of 95-kDa gelatinase and TIMPs on a monoclonal anti-TIMP affinity matrix. Pro 95-kDa gelatinase was incubated with a molar excess of 24-kDa $\Delta_{127-184}$TIMP and chromatographed on the affinity matrix; the bulk of the gelatinase activity (87%) did not bind to the column (lane 1), whereas the inhibitor bound and was eluted at pH 2.8 (lanes 2 and 3). In the case of pro 95-kDa gelatinase and a molar excess of wild-type TIMP, only 8% of the enzyme did not bind to the column (lane 4) and the majority eluted with the inhibitor at pH 2.8 (lanes 5 and 6). The elution pattern of pro 95-kDa gelatinase alone is shown in lane 7 (unbound material) and lanes 8 and 9 (pH 2.8 eluate). If the active form of 95-kDa gelatinase was incubated with 24-kDa $\Delta_{127-184}$TIMP, only 25% of the enzyme failed to bind to the column (lane 10), the majority eluting at pH 2.8 with the inhibitor (lanes 11 and 12). The elution pattern of active 95-kDa gelatinase alone, unbound (lane 13) and pH 2.8 eluate (lanes 14 and 15), are shown. The inhibitors are visualised as darker bands on the gel by brief incubation in crude collagenase to clear the gelatin background. (B) The formation of complexes of gelatinase with TIMPs was demonstrated by using an ELISA assay. Wild-type TIMP gives a linear response over a range from 1 to 70 ng/mL (△), which is reduced in the presence of a 4-fold molar excess of pro 95-kDa gelatinase (▲). The detection of $\Delta_{127-184}$TIMP is weaker but remains linear (○). In the presence of a molar excess of active stromelysin-1 (●), the signal is reduced. The presence of a 4-fold molar excess of pro 95-kDa gelatinase (■) failed to reduce the signal.

have shown that the purified proteins appear to be correctly folded and glycosylated. Comparison of their properties suggest that the major sites of interaction between TIMP and active MMPs, leading to enzyme inhibition and stable complex formation, reside in the N-terminal loops as depicted in Figure 1 (bottom). Further dissection of the structure–function relationships of TIMPs should be facilitated by these observations.

ACKNOWLEDGMENTS

We thank Robin Ward and Saroj Angal for the provision of enzymes, Frances Willenbrock and Jimi O'Connell for active-site titration analyses, and John Reynolds and Robert Freedman for stimulating discussions.

Registry No. TIMP, 86102-31-0; metalloproteinase, 81669-70-7; progelatinase, 119345-31-2; gelatinase, 9040-48-6; stromelysin, 79955-99-0.

REFERENCES

Bebbington, C. R., & Hentschel, C. C. G. (1987) in *DNA cloning* (Glover, D., Ed.) Vol. III, pp 163–188, Academic Press, New York.

Boone, T. C., Johnson, M. J., DeClerck, Y. A., & Langley, K. E. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2800–2804.

Cawston, T. E., Murphy, G., Mercer, G., Galloway, W. A., Hazelman, B. L., & Reynolds, J. J. (1983) *Biochem J. 211*, 313–318.

Cooksley, S., Hipkiss, J. B., Tickle, S. P., Holmes-levers, E., Docherty, A. J. P., Murphy, G., & Lawson, A. D. G. (1990) *Matrix 10*, 285–291.

Coulombe, B., & Skup, D. (1988) *J. Biol. Chem. 263*, 1439–1443.

Docherty, A. J. P., Lyons, A., Smith, B. J., Wright, E. M., Stephens, P. E., Harris, T. J. R., Murphy, G., & Reynolds, J. J. (1985) *Nature 318*, 66–69.

Galloway, W. A., Murphy, G., Sandy, J. D., Gavrilovic, J., Cawston, T. E., & Reynolds, J. J. (1983) *Biochem. J. 209*, 741–752.

Goldberg, G. I., Marmer, B. L., Grant, G. A., Eisen, A. Z., Wilhelm, S., & He, C. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8207–8211.

Hembry, R. M., Murphy, G., & Reynolds, J. J. (1985) *J. Cell Sci. 73*, 105–119.

Koklitis, P., Murphy, G., Sutton, C., & Angal, S. (1991) *Biochem. J. 276*, 217–221.

Laemmli, U. K., & Favre, M. (1973) *J. Mol. Biol. 80*, 575–599.

Melton, D. A., Kreig, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., & Green, M. R. (1984) *Nucleic Acids Res. 12*, 7035–7056.

Merril, C. R., Goldman, D., Sedman, S. A., & Ebert, M. H. (1981) *Science 211*, 1437–1438.

Mullis, K. B., & Faloona, F. A. (1987) *Methods Enzymol. 155*, 335–350.

Murphy, G., & Werb, Z. (1985) *Biochim. Biophys. Acta 839*, 214–218.

Murphy, G., Cawston, T. E., & Reynolds, J. J. (1981) *Biochem. J. 195*, 167–170.

Murphy, G., Cockett, M. I. C., Stephens, P. E., Smith, B. J., & Docherty, A. J. P. (1987) *Biochem. J. 248*, 265–268.

Murphy, G., Koklitis, P., & Carne, A. F. (1989) *Biochem. J. 261*, 1021–1034.

Murphy, G., Cockett, M. I., Ward, R. V., & Docherty, A. J. P. (1991) *Biochem. J. 277*, 277–279.

Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.

Stack, M. S., & Gray, R. D. (1989) *J. Biol. Chem. 264*, 4277–4281.

Stephens, P. E., & Cockett, M. I. (1989) *Nucleic Acids Res. 17*, 7110.

Stetler-Stevenson, W. G., Krutzsch, H. C., & Liotta, L. A. (1989) *J. Biol. Chem. 264*, 17374–1378.

Stricklin, G. P., & Welgus, H. G. (1983) *J. Biol. Chem. 258*, 12252–12258.

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.

Vogelstein, B., & Gillespie, D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 615–619.

Ward, R. V., Hembry, R. M., Reynolds, J. J., & Murphy, G.

(1991) *Biochem. J.* (in press).
Welgus, H. G., Jeffrey, J. J., Eisen, A. Z., Roswit, W. T., & Stricklin, G. P. (1985) *Collagen Rel. Res. 5*, 167-179.
Wilhelm, S. M., Collier, T. E., Marmer, B. L., Eisen, A. Z., Grant, G. A., & Goldberg, G. I. (1989) *J. Biol. Chem. 264*, 17213-17221.
Williamson, R. A., Marston, F. A. O., Angal, S., Koklitis, P., Panico, M., Morris, H. R., Carne, A. F., Simth, B. J., Harris, T. J. R., & Freedman, R. B. (1990) *Biochem. J. 268*, 267-274.

Articles

A Pancreatic Exocrine Cell Factor and AP4 Bind Overlapping Sites in the Amylase 2A Enhancer†

E. Fodor,‡ S. L. Weinrich,‡ A. Meister,‡ N. Mermod,§,‖ and W. J. Rutter*,‡
Department of Biochemistry and Biophysics, Hormone Research Institute, University of California, San Francisco, California 94143, and Department of Biochemistry, University of California, Berkeley, California 94720
Received February 21, 1991; Revised Manuscript Received June 10, 1991

ABSTRACT: A factor found in pancreatic exocrine cell lines and pancreatic nuclei binds selectively to the α-amylase 2A transcriptional enhancer. Pancreatic exocrine cell extracts protect asymmetrically an unusually large, 35 base pair region from DNase I digestion in vitro, suggesting the involvement of a multimeric DNA binding complex. We show that this region of the enhancer contains a major affinity recognition sequence for the HeLa transcription factor AP4. A 4 base pair mutation in the enhancer sequence shown previously to abolish activity in vivo [Boulet, A. M., Erwin, C. R., & Rutter, W. J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3599-3603] abolishes AP4 binding in vitro and weakens but does not eliminate the binding of adjacent enhancer factors. Further, sequences similar to the AP4 binding site are found within a consensus sequence of most pancreatic exocrine genes (Boulet et al., 1986). We have identified three AP4 binding sites in the pancreatic elastase gene: one occurs in the consensus sequence of the enhancer. Thus, protein(s) with the binding selectivity of AP4 may play a role in the expression of the pancreatic exocrine gene family.

Acinar cells of the exocrine pancreas produce and secrete hydrolytic enzymes which are involved in intestinal digestion. It is believed that the differentiated phenotype of this and other cells results from stringent control of activation and repression of gene sets by nuclear proteins which interact with DNA target sites in tissue-specific promoter and enhancer elements (Dynan & Tjian, 1985; Ptashne, 1988). Previous studies from this laboratory (Boulet et al., 1986) and others (Ornitz et al., 1985) have shown that high levels of transcription of many acinar cell-specific genes are determined by enhancer elements located in their 5'-flanking region.

The genes encoding the pancreatic hydrolytic enzymes contain a conserved 20 base pair sequence 100-200 base pairs from their mRNA CAP site (the pancreatic consensus sequence) (Boulet et al., 1986; Ornitz et al., 1985). This element appears essential for the activity of the amylase, chymotrypsin, elastase, and trypsin gene enhancers (Boulet et al., 1986; Hammer et al., 1987). The amylase 2A promoter–enhancer (+1 to −200 nucleotides from the mRNA CAP site) is necessary and sufficient to effect transcription of linked genes in cells of pancreatic exocrine origin but not in other cell types (Walker et al., 1983). In trangenic animals, this element has also been shown to be a determinant for the pattern of developmental expression of the amylase and elastase genes (Hammer et al., 1987; Osborn et al., 1988).

In the present work, we characterize by DNase I footprinting and electrophoretic mobility shift assays the binding in vitro of the amylase enhancer to nuclear factors present in amylase-expressing and -nonexpressing cell lines. The binding profile of the amylase enhancer is different from that of a mutant enhancer containing a 4 base pair transition mutation within the pancreatic consensus sequence that abolishes activity in vivo. We observed that the DNA sequence encompassing the region of the mutation is similar to the distal region of the mammalian virus SV40 late promoter that includes the binding site for transcription factor AP4 (Mermod et al., 1988). Experiments reveal that the enhancer binds purified AP4 with high affinity and that the 4 bp mutation eliminates binding activity. The pancreatic elastase 5' region contains multiple AP4 binding sites. One of these sites is present within the pancreatic consensus element of this gene (Ornitz et al., 1985).

Materials and Methods

DNA Probes. pAmy, containing the rat pancreatic α-amylase 2A promoter–enhancer (+30 to −350), was obtained from a partial digest of pAmy.CAT (Boulet et al., 1986) and inserted into the *Xho*I site of pUC9 by standard methods (Maniatis et al., 1982). The 57 base pair (−108 to −165) amylase minimal enhancer and enhancer mutant IV that contains a 4 base pair mutation (CAGT at −121 to −124) have been described (Boulet et al., 1986). The rat pancreatic elastase promoter–enhancer was obtained from R. MacDonald (Kruse et al., 1988) and the +8 to −205 *Sal–Bam* fragment subcloned into the polylinker of pUC19. A *Pvu*II–*Sph*I SV40

† N.M. was supported by a Swiss NSF postdoctoral fellowship. This work was supported by NIH Grant DK-21344.
‡ University of California, San Francisco.
§ University of California, Berkeley.
‖ Present address: Institut de Biologie Animale, Universite de Lausanne, 1015 Lausanne, Switzerland.

0006-2960/91/0430-8102$02.50/0