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Human and Rat Malignant-Tumor-Associated mRNAs Encode Stromelysin-like Metalloproteinases†

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ABSTRACT: Rat transin and human stromelysin 2 mRNAs, which have been associated with malignant tumors, code for potential proteins with significant sequence homology to the metalloproteinases collagenase and stromelysin. We have used an expression system that allows easy purification of these proteins after transfection of COS cells with a vector containing the corresponding cDNA. This system has allowed us to prepare transin and stromelysin 2 as active proteinases that are inhibited by inhibitors of metalloproteinases. Further analysis of these enzymes indicates that they degrade several components of the extracellular matrix including collagen types III, IV, and V and fibronectin, as well as gelatins formed from several denatured collagen types. In addition, both transin and stromelysin 2 are capable of activating procollagenase in vitro. Thus, in malignant tumors these proteinases may act, both directly and indirectly, to degrade the extracellular matrix and permit tumor invasion of neighboring tissues.

The extracellular matrix (ECM)[1] plays many important roles in the establishment and structural integrity of tissues. Although turnover of the ECM components is normally very low in the adult, an increase in this rate is a necessary event during many normal biological processes including cell migration during development, wound healing, angiogenesis, or postpartum uterine involution. Uncontrolled ECM degradation can have severe consequences, however, as in pathological conditions such as rheumatoid arthritis or tumor invasion and metastasis [for reviews see Mullins and Rohrlich (1983), Yamada (1983), Murphy and Reynolds (1985), and Tryggvason et al. (1987)].

The degradation of the ECM is accomplished through the action of several proteolytic enzymes, including members of the metalloproteinase family (Tryggvason et al., 1987). Several members of this metalloproteinase family have been characterized, including the following: collagenase, which

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[1] Abbreviations: ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DTT, dithiothreitol; TIMP, tissue inhibitor of metalloproteinases; SV40, simian virus 40; IgG, immunoglobulin G; APMA, (4-aminophenyl)mercuric acetate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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degrades the three interstitial collagens (Werb & Reynolds, 1975; Murphy et al., 1982); stromelysin, which degrades fibronectin and gelatin and has limited activity against type IV collagen as well as other ECM components (Galloway et al., 1983; Chin et al., 1985; Okada et al., 1986); gelatinase, which degrades gelatin and native types IV and V collagen (Murphy et al., 1982; Hibbs et al., 1985); and a collagenase specific for degradation of type IV collagen (Salo et al., 1983). The association of such enzymes with invasive tumor-associated ECM degradation has become a well-established concept [see Liotta (1986) and Tryggvason et al. (1987) and references cited therein].

Our group has previously reported the cloning of two cDNAs, rat transin cDNA (Matrisian et al., 1985) and human stromelysin 2 cDNA (Muller et al., 1988), which resemble the cDNAs of stromelysin and collagenase in nucleotide sequence (Whitham et al., 1986; Breathnach et al., 1987; Muller et al., 1988). Transin mRNA levels have been shown to be greatly increased following oncogenic transformation of rat embryo fibroblast cell lines (Matrisian et al., 1985, 1986a; Breathnach et al., 1987), and to be more abundant in invasive mouse skin squamous cell carcinomas than in benign papillomas (Matrisian et al., 1986b). The predicted amino acid sequence of transin suggests that it may be the rat equivalent of human stromelysin (Whitham et al., 1986; Breathnach et al., 1987; Wilhelm et al., 1987), while the predicted amino acid sequence of stromelysin 2 indicates that it encodes a proteinase distinct from stromelysin (Muller et al., 1988). Given the correlation between malignancy and the degradation of the ECM, it is important to confirm that the proteins encoded by the transin and stromelysin 2 cDNAs are metalloproteinases and to determine which components of the ECM are degraded by these malignancy-associated enzymes.

We have previously described a novel scheme to purify transin from medium conditioned by COS cells that were transfected with a vector designed to direct synthesis of a secretable fusion protein between transin and the IgG-binding domains of staphylococcal protein A (Sanchez-Lopez et al., 1988). Here we have used this system to produce active recombinant rat transin and human stromelysin 2 for comparison of their proteolytic activities in vitro to those of two well-characterized metalloproteinases, human collagenase and stromelysin.

Experimental Procedures

Plasmid Constructions. We have described previously construction of the vector pPROTA (Sanchez-Lopez et al., 1988). This plasmid contains the SV40 early gene promoter and origin of replication, splice sites from a rabbit β-globin gene, sequences coding for amino acids 1–32 of transin (providing a signal peptide), sequences coding for amino acids 23–270 of protein A (Uhlén et al., 1984) followed by a unique *Eco*RI site, and a polyadenylation signal from a rabbit β-globin gene.

The cDNA sequences encoding transin (Matrisian et al., 1985) or the C-terminal domain of transin have been inserted into the *Eco*RI site of pPROTA, as has been described elsewhere (Sanchez-Lopez et al., 1988), and the recombinant plasmids have been termed pPA-TR and pPA-TRC, respectively. pPA-TR contains transin cDNA sequences encoding amino acids 12–475 following the protein A encoding DNA sequences. pPA-TRC contains transin cDNA sequences encoding amino acids 263–475 following the protein A encoding DNA sequences.

An *Eco*RI cleavage site was created at nucleotide position 56 of the cDNA for human stromelysin 2 (Muller et al., 1988) by oligonucleotide-directed deletion mutagenesis (Grundstrom et al., 1985; Kumar et al., 1986) using a specific oligonucleotide, 5′-TCTCCCGGGAATTCCCTGCCAGTCT-GCT-3′, and the vector M13tg130 (Kieny et al., 1983). *Eco*RI digestion of this mutant plasmid created a 1570-bp DNA fragment (containing stromelysin 2 encoding cDNA sequences from the position of amino acid 12 to beyond the translational termination codon) that was inserted into pPROTA and the recombinant plasmid termed pPA-S2. This plasmid thus contains cDNA sequences encoding the first 32 amino acids of transin, followed by DNA sequences encoding amino acids 23–270 of protein A and cDNA sequences encoding amino acids 12–476 of stromelysin 2.

To create such a fusion plasmid containing human collagenase cDNA sequences (a gift of P. Herrlich, Kernforschungzentrum Karlsruhe), we converted the unique *Eco*RI cleavage site of pPROTA to an *Xba*I cleavage site using an oligonucleotide adaptor, 5′-AATTTTCTAGAA-3′. Insertion of a 1435-bp DNA fragment, created by *Xba*I digestion, from human collagenase (Whitman et al., 1986) into the new *Xba*I cleavage site of pPROTA resulted in a fusion plasmid termed pPA-C. This plasmid contains cDNA sequences encoding amino acids 24–469 of collagenase following the protein A encoding DNA sequences.

Other molecular biological techniques used have been described in detail by Maniatis et al. (1982).

Preparation of Recombinant Enzymes. Recombinant plasmids containing fused protein A and rat transin, human stromelysin 2, or collagenase cDNA sequences were transfected into COS cells (Gluzman, 1981) by using the calcium phosphate procedure (Wigler et al., 1979). DNA (10 μg/3 $\times$ 10^5 COS cells) was left on cells for 15 h before cells were washed and allowed to recover for 24 h in fresh Dulbecco medium containing 5% fetal calf serum. Cells were then washed and incubated in serum-free Dulbecco medium for 48 h. The medium was harvested and cleared by centrifugation, and proteins were precipitated at 4 °C in 80% ammonium sulfate (ultrapure, EDTA free from Bethesda Research Labs).

The precipitated proteins were collected by centrifugation, resuspended in TC (20 mM Tris, pH 7.5, 10 mM $CaCl_2$) buffer, and dialyzed against TC buffer at 4 °C. The fusion proteins were purified from this concentrate by using the IgG–Sepharose chromatography method that we have described previously (Sanchez-Lopez et al., 1988). The dialysates containing the fusion proteins were incubated with IgG–Sepharose for 2 h at 4 °C before the matrix was washed throughly. The active enzymes were eluted from the matrix by rocking for 4 h at 37 °C in TC buffer containing 0.05% Tween 20 and 1 mM APMA.

[^{35}S]Methionine-labeled proteins were prepared as above except that transfected cells were incubated in methionine-free Dulbecco medium for 1 h before addition of 100 μCi of [^{35}S]methionine ($\sim$1000 Ci/mmol). Labeled medium was incubated directly with IgG–Sepharose, and the ^{35}S-labeled proteins eluting from the IgG–Sepharose after APMA treatment were analyzed by SDS–PAGE (12%) and visualized by fluorography.

Recombinant human stromelysin was purified by using standard techniques described elsewhere (Galloway et al., 1983) from medium conditioned by C127 cells (Lowy et al., 1978) transfected with a vector containing cDNA sequences encoding human stromelysin under control of a mouse metallothionein I promoter as described elsewhere (Murphy et al., 1987).

Proteolytic Activity Quantification. The proteolytic activities of transin, stromelysin, and stromelysin 2 were quantified by degrading ^{14}C-acetylated casein in TC buffer at 37 °C for 2 h as described by Galloway et al. (1983). One unit of activity is defined as the amount of enzyme required to degrade 1 μg of casein/min at 37 °C. The proteolytic activity of collagenase was quantified by using a ^{14}C-acetylated collagen (I) diffuse fibril assay (Cawston & Barrett, 1979). Type I rat skin collagen (100 μg, 0.04 μCi/mg) was digested in TC buffer at 35 °C for 4 h. One unit of collagenase is defined as the amount of enzyme required to degrade 1 μg of type I collagen/min at 35 °C.

Inhibition Studies. The effect of inhibitors on proteolytic activity of transin and stromelysin 2 was determined by using casein as substrate. Twenty milliunits (mU) of enzyme was incubated at room temperature with or without inhibitor for 30 min before addition of 1 μg of substrate and incubation for 4 h at 37 °C. Digestion was assayed by SDS–PAGE (12%) and staining with silver nitrate (Merril et al., 1984). The concentrations of the inhibitors tested were 2 mM 1,10-phenanthroline (dissolved in ethanol), 2 mM EDTA, 2 mM EGTA, 1 mM DTT, and 0.1 unit of human tissue metalloproteinase inhibitor (TIMP). A unit of TIMP is defined as the amount of inhibitor required to inhibit 2 units of collagenase by 50% (Murphy et al., 1985).

Digestion of ECM Components. Proteolytic activities against ECM components were assayed in digestions of various purified ECM component proteins as follows: 1 μg of rat tail type I collagen was incubated with 30 mU of enzyme in TC buffer for 18 h at 32 °C; 1 μg of bovine collagen type III (a gift of M. Barnes, Strangeways Research Laboratory) was incubated with 20 mU of enzyme in TC buffer for 6 h at 32 °C; 0.5 μg of mouse EHS collagen type IV was incubated with 50 mU of enzyme in TC buffer for 6 h at 37 °C; 1 μg of bovine type V collagen (a gift of M. Barnes) was incubated with 20 mU of enzyme in TC buffer for 6 h at 35 °C; 1 μg of bovine plasma fibronectin (a gift of C. Cockburn, Strangeways Research Laboratory) was incubated with 20 mU of enzyme in TC buffer for 6 h at 37 °C. Alternatively, the collagens were heated at 45 °C for 10 min to form gelatin before incubation with enzyme for 6 h at 37 °C. For Figure 5 the EHS type IV collagen (0.5 μg) was incubated at 37 °C for 6 h with from 0 to 500 mU of stromelysin (350 units/mg). Digestions were stopped by boiling for 5 min in Laemmli sample buffer containing 5% β-mercaptoethanol. Digestion products were separated by SDS–PAGE (7.5% for type I or III collagen, 6% for type IV or V collagen, or 5% for fibronectin) and observed by staining with silver nitrate.

Procollagenase Activation. Recombinant procollagenase (Murphy et al., 1987) was mixed with various quantities of transin or stromelysin 2 and incubated at 37 °C for 2 h. Collagenase activity was assayed by using the ^{14}C-acetylated collagen diffuse fibril assay described above. The level of activation is expressed as a fold increase over the value obtained with procollagenase incubated with TC buffer. The amount of digestion was determined by measuring the soluble radioactivity after centrifugation and subtraction of background radioactivity obtained in mock digests containing no enzyme.

Results

Purification of Recombinant Metalloproteinases. The amino acid sequences predicted from the cDNAs for transin and stromelysin 2 suggest that, like stromelysin and collagenase, these proteins are secreted as latent proenzymes that will undergo a series of molecular weight changes during activation (Vater et al., 1983, 1986; Stricklin et al., 1983; Okada et al., 1986; Murphy et al., 1987; Grant et al., 1987). These changes are shown schematically, for transin, in Figure 1A. The primary translation product (Figure 1Aa) is shortened to the latent enzyme form (Figure 1Ab) by removal of a secretory signal peptide during the secretion process. The activation process involves removal of a propeptide to give the major active enzyme form (Figure 1Ac) by a self-cleavage reaction that can be induced in vitro by exposure of the latent enzyme to organomercurials such as APMA. We have previously described a procedure that exploited these characteristics to aid the purification of recombinant transin (Sanchez-Lopez et al., 1988). The vector pPROTA (Figure 1B) is designed to direct synthesis of secretable fusion proteins containing the IgG-binding domains of staphylococcal protein A fused to a protein (such as transin) encoded by cDNA sequences inserted into the vector at the unique *Eco*RI site. As we have shown elsewhere (Sanchez-Lopez et al., 1988), insertion of cDNA sequences encoding transin (amino acids 12–475) at the *Eco*RI site of pPROTA and transfection of COS cells with the recombinant plasmid result in the synthesis by the COS cells of a fusion protein (Figure 1Ca) comprised of amino acids 1–32 of transin (acting as the secretion signal) followed by amino acids 23–270 of protein A (containing the IgG-binding domain) and amino acids 12–475 of transin. This protein is secreted from the cell (Figure 1Cb) and can be recovered from the conditioned medium by incubation with IgG–Sepharose, thereby allowing separation from other proteins in the conditioned medium. Treatment of the IgG–Sepharose-bound fusion protein with APMA results in activation of the enzyme, cleavage of the propeptide, and elution of a protein corresponding to active transin (Figure 1Cc).

We have now inserted cDNA sequences encoding human stromelysin 2 or collagenase into pPROTA (as described under Experimental Procedures) to allow production in COS cells of fusion proteins comprised of the first 32 amino acids of transin, the IgG-binding domains of protein A, and either amino acids 12–476 of stromelysin 2 or amino acids 24–469 of collagenase. These fusion proteins should bind to IgG–Sepharose and be eluted by treatment with APMA in the same manner as was observed with transin.

Figure 2 shows that APMA treatment of all these IgG–Sepharose-bound fusion proteins results in the elution of proteins corresponding to either transin, stromelysin 2, or collagenase. The eluate from the rat transin fusion protein contains two major proteins with apparent molecular weights of 48 000 and 29 000, while the eluate from the human stromelysin 2 fusion protein contains two major proteins of 47 000 and 28 000. These are similar to the sizes (48 000 and 28 000) reported for active human stromelysin (Murphy et al., 1987). The eluate of the collagenase fusion protein contains a triplet of bands between 39 000 and 43 000, as well as smaller bands around 24 000, similar to the species sizes (42 000–44 000 and 28 000) reported previously for activated collagenase (Vater et al., 1983; Grant et al., 1987). Smaller bands visible with all three eluted enzymes are likely remnants of the cleaved C-terminal domain of the proteins.

The proteins eluted by APMA, after binding of the fusion proteins to IgG–Sepharose, were used in the experiments described below and are referred to simply as transin, stromelysin 2, or collagenase.

To generate a negative control for proteolytic activity assays, we have inserted cDNA sequences encoding amino acids 263–475 (see Figure 1) of transin, referred to here as the C-domain, into the *Eco*RI site of pPROTA. This plasmid,

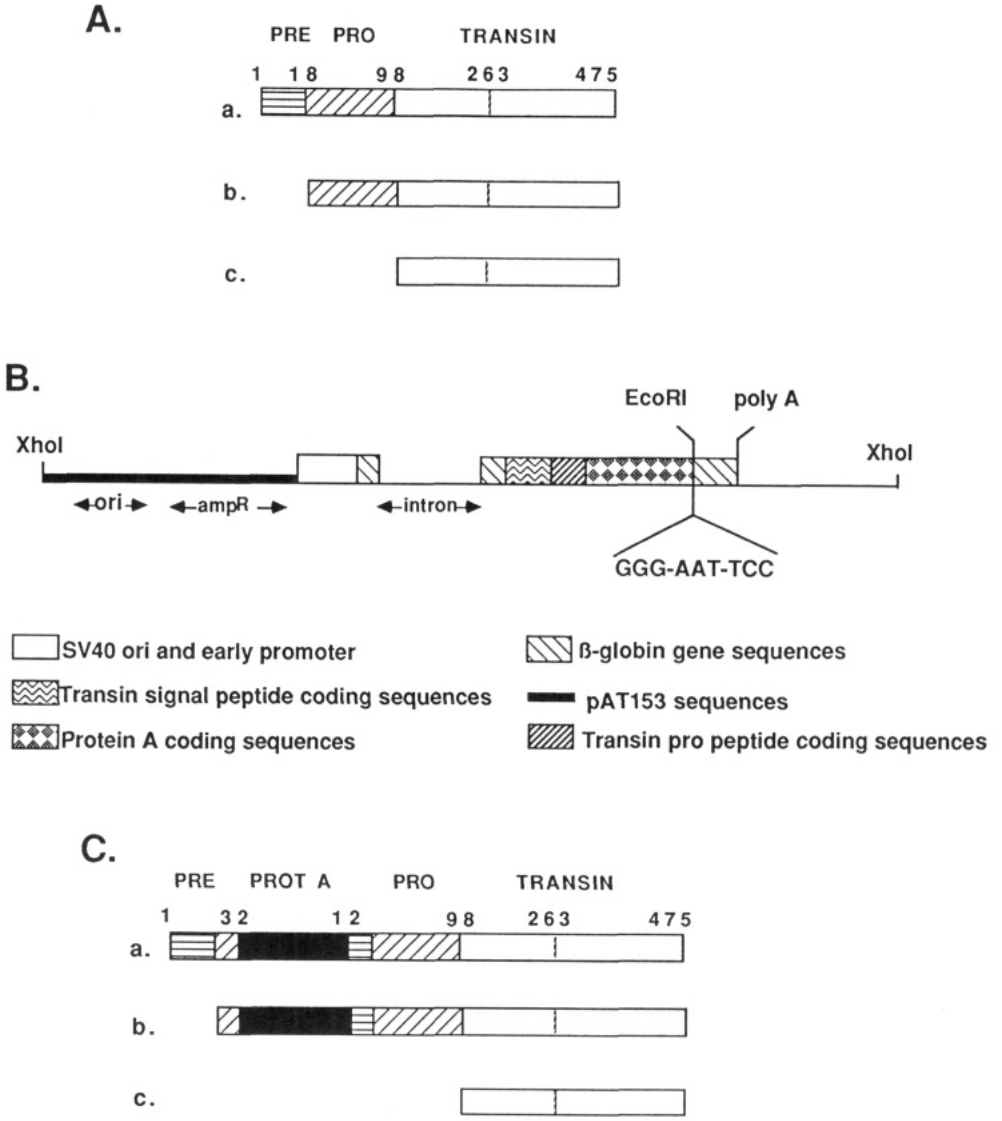

FIGURE 1: (A) Schematic representation of the transin protein structure: (a) primary translation product; (b) secreted form; (c) after activation. Numbers refer to transin amino acid sequence (Breathnach et al., 1987). The transin sequences are separated into pre (boxes with horizontal lines), pro (boxes with slanted lines), and active (open boxes) regions. The active region is divided by a line indicating the position of amino acid 263, which represents the start of the C-domain as described in the text. (B) Schematic representation of the vector pPROTA. The vector is shown linearized by *Xho*I digestion. The pAT153 origin of replication (ori) and β-lactamase gene (amp^r) are shown, as is the location of a β-globin gene polyadenylation site (poly A). The unique *Eco*RI site of pPROTA is shown, as is the phase of the protein A coding sequences surrounding it. (C) Schematic representation of the protein A–transin fusion protein structure: (a) primary translation product; (b) secreted form; (c) after activation. Numbers and box shadings are as in panel A above, with the addition of a solid box representing protein A sequences.

pPA-TRC, directs synthesis of a fusion protein that binds to IgG–Sepharose. Treatment of this bound fusion protein with APMA does not result in release of the fusion protein from the IgG–Sepharose (Sanchez-Lopez et al., 1988) and the eluate does not contain any detectable proteolytic activity. Volumes of this eluate, corresponding to the volumes of active enzyme used, were used as the negative control in all experiments.

Inhibition of Proteinase Activity. Degradation of casein (Figure 3) by transin or stromelysin 2 was inhibited by chelating agents such as EDTA, EGTA, and 1,10-phenanthroline, by a thiol compound (DTT), and by a specific tissue inhibitor of metalloproteinases (TIMP). These data support the classification of transin and stromelysin 2 as metalloproteinases.

Analysis of Proteolytic Activities against ECM Components. Previous studies of metalloproteinases have indicated that stromelysin has activity against several protein components of the ECM, but not type I collagen (Galloway et al., 1983; Chin et al., 1985; Okada et al., 1986), while collagenase is known to have activity primarily against the interstitial collagens (Werb & Reynolds, 1975; Stricklin et al., 1978). Since transin and stromelysin 2 resemble both stromelysin and collagenase in amino acid sequence, we have used purified components of the ECM to compare the proteolytic activities of transin and stromelysin 2 (both purified from protein A fusion proteins) to those of collagenase (purified from a protein A fusion protein) and stromelysin (purified from C127 conditioned medium). Equivalent quantities of general proteolytic activity of transin, stromelysin or stromelysin 2, or collagenase activity were used in digests of the ECM component proteins.

In Figure 4 we compare the proteolytic activity of these four enzymes against four types of native collagen: type I (Figure 4A), type III (Figure 4B), type IV (Figure 4C), and type V (Figure 4D). Collagenase (C) was able to degrade the interstitial collagens, types I and III, but not collagen type IV or V. Stromelysin (S), stromelysin 2 (S2), and transin (T) showed no activity against type I collagen and low activity against type IV collagen. It should be noted that relative to previous reports of the ability of stromelysin to degrade type IV collagen (Chin et al., 1985; Okada et al., 1986) the en-

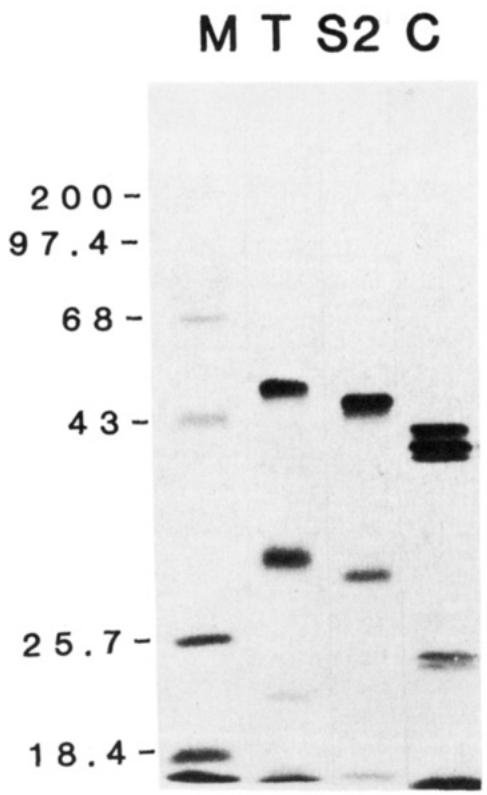

FIGURE 2: Fluorograph of the [^{35}S]methionine-labeled transin (T), stromelysin 2 (S2), and collagenase (C) polypeptides eluted from IgG–Sepharose after APMA treatment. Indicated at the left are the apparent molecular weights ($\times 10^{-3}$) of the ^{14}C-labeled molecular weight standards: myosin H chain (200 000); phosphorylase *b* (97 400); bovine serum albumin (68 000); ovalbumin (43 000); α-chymotrypsinogen (25 700); and β-lactoglobulin (18 400).

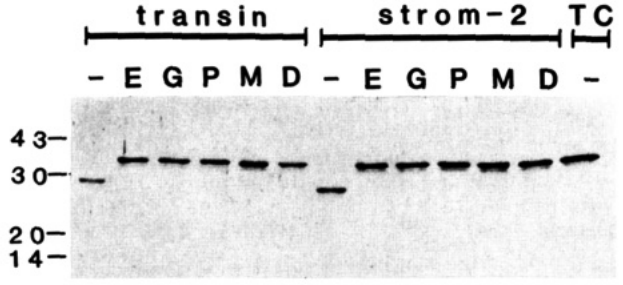

FIGURE 3: Inhibition of proteolytic activity by several metalloproteinase inhibitors. Casein was incubated with IgG–Sepharose-purified transin (first six lanes labeled –, E, G, P, M, and D), stromelysin 2 (second set of six lanes), or the control preparation (TC) either in the presence (lanes E, G, P, M, and D) or in the absence (–) of inhibitors. The inhibitors used were EDTA (E), EGTA (G), 1,10-phenanthroline (P), TIMP (M), and DTT (D). Indicated at left are the migratory positions of the molecular weight size markers: ovalbumin (43 000); carbonic anhydrase (30 000); trypsin inhibitor (20 000); and α-lactalbumin (14 400).

zyme–substrate ratios used here were much lower. Nevertheless, we detected low activity against the proα2 (IV) chain of type IV collagen [presumably present as the homotrimer $[\alpha 2(IV)]_3$] by stromelysin, transin, and to a lesser extent stromelysin 2. We are certain that at much higher enzyme–substrate ratios more degradation of the type IV collagen molecule would be observed since the stromelysin used in these studies will degrade type IV collagen extensively when used at much higher enzyme–substrate ratios (see Figure 5), yet it preferentially degrades the proα2 (IV) chain at low enzyme–substrate ratios. Unfortunately, the low activity in the protein A–fusion purified enzyme preparations did not allow us to obtain sufficiently concentrated enzymes to examine this possibility with transin or stromelysin 2.

When native collagens undergo an initial cleavage by collagenases in vivo, they relax their helical structures and form gelatin, thereby becoming susceptible to degradation by other enzymes. To mimic such a situation in vitro, we have heated the collagen at 45 °C to form gelatin prior to incubation with enzyme (Figure 6). Type I gelatin (Figure 6A) was only slightly degraded by collagenase (C) but was extensively degraded by stromelysin (S), stromelysin 2 (S2), and transin (T). It should be noted that, as previously reported for stromelysin (Okada et al., 1986), the α2 chain is degraded more rapidly than the α1 chain of type I gelatin. Type III gelatin (Figure 6B) was degraded in a similar manner by all four enzymes. Similar activity was also observed by all four enzymes against type V gelatin (Figure 6D). No activity was detected by collagenase, and very little by stromelysin 2, against type IV gelatin (Figure 6C). Type IV gelatin was, however, extensively degraded by stromelysin or transin.

Stromelysin, stromelysin 2, and transin all have the ability to degrade both chains of fibronectin, resulting in similar fragment patterns on the gel (Figure 7), suggesting that digestion occurs at specific locations on the fibronectin molecule.

Activation of Procollagenase. An endogenous collagenase activator protein has been described that closely resembles stromelysin in size and activity (Vater et al., 1983). More recently we have shown that stromelysin can activate procollagenase in vitro (Murphy et al., 1987). Therefore, we have now examined the possibility that transin and stromelysin 2 might also be able to activate procollagenase. Incubation of procollagenase with transin or stromelysin 2 prior to assay for activity against type I collagen (Figure 8) resulted in a significant increase (12.5-fold with transin, 8.5-fold with stromelysin 2) above the level of digestion observed with procollagenase in the absence of activator. This activating ability is certainly a quality of the enzyme rather than a result of residual APMA remaining in the enzyme preparation, since incubation of procollagenase with maximal volumes of the control transin C-domain preparation resulted in a much lower level of collagenase activity. If the activation due to residual APMA is subtracted from the levels of activity observed after incubation with transin or stromelysin 2, there remain 10-fold and 6-fold increases in collagenase activity that must be attributed to the presence of transin and stromelysin 2, respectively.

Discussion

Since the isolation of the transin cDNA from oncogenically transformed rat fibroblast cells (Matrisian et al., 1985), many groups, including our own, have noted the sequence similarity between transin and the ECM-degrading metalloproteinases collagenase and stromelysin (Goldberg et al., 1986; Matrisian et al., 1986; Whitham et al., 1986; Fini et al., 1987; Wilhelm et al., 1987; Frisch & Ruley, 1987). The rat transin cDNA has since been used to isolate a related human cDNA that has been termed stromelysin 2 (Muller et al., 1988). Comparison of amino acid sequences determined from the cDNAs predicts that transin is very probably the rat equivalent of stromelysin, while stromelysin 2 is a related, but distinct, protein. However, because transin and stromelysin 2 were isolated as cDNAs, it is necessary to confirm that the proteins encoded by these cDNAs have functional properties similar to those of the related, and well-characterized, metalloproteinases.

Given the clear association of transin, as well as the ECM-degrading metalloproteinases, with the transformed phenotype and malignancy, it is important to compare directly the ac-

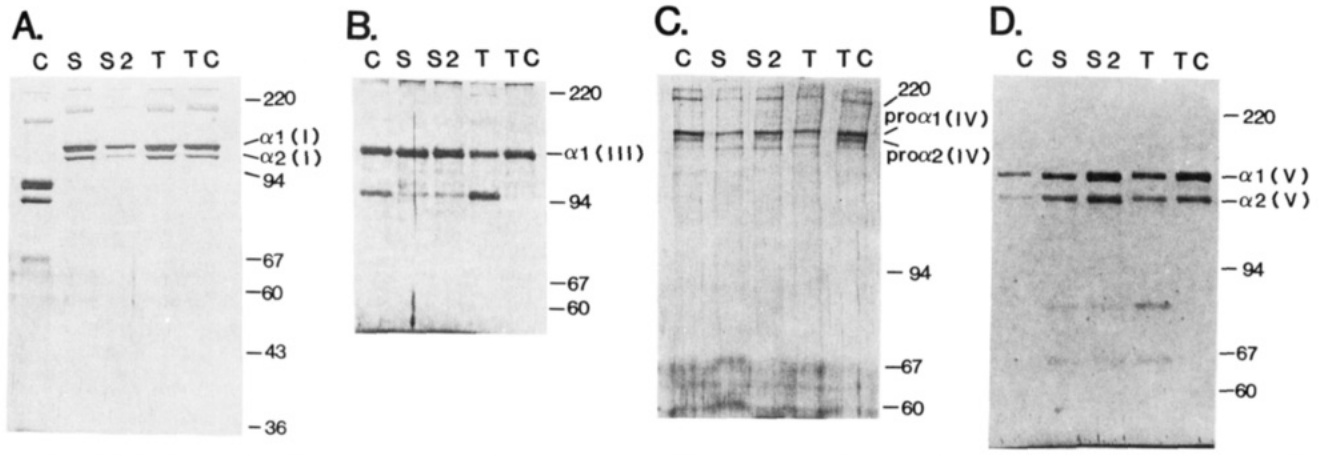

FIGURE 4: Electrophoretic banding pattern of degradation products resulting from incubation of native collagen with IgG–Sepharose-purified collagenase (C), stromelysin 2 (S2), or transin (T) or with purified recombinant stromelysin (S) or the control preparation (TC). Collagens used were type I (panel A), type III (panel B), type IV (panel C), and type V (panel D). The position of the α-chains of each collagen type is indicated. Also indicated are the migratory positions of the molecular weight markers: ferritin (220 000); phosphorylase *b* (94 000); albumin (67 000); catalase (60 000); ovalbumin (43 000); and lactate dehydrogenase (36 000).

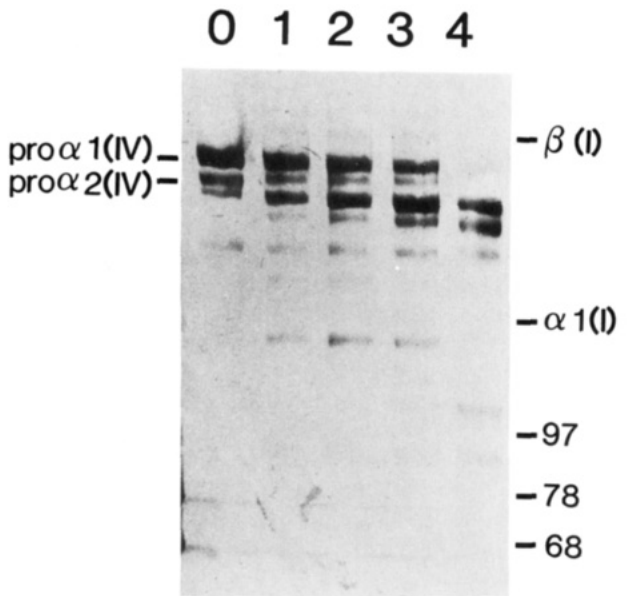

FIGURE 5: Electrophoretic banding pattern of type IV collagen after incubation with 0 (0), 25 (1), 50 (2), 250 (3), or 500 mU (4) of purified recombinant stromelysin. The position of each proα chain of type IV collagen is indicated. Also indicated are the migratory positions of the molecular weight markers: the β-chains of type I collagen; the α1-chain of type I collagen; phosphorylase *b* (97 000); transferrin (78 000); and serum albumin (86 000).

tivities of transin and stromelysin 2 to stromelysin and collagenase to see the relationship of transin to the human enzymes and to better understand the action and interaction of the metalloproteinases. We have therefore utilized a novel system for purification of recombinant enzymes, which we have used previously to produce transin for structure–function studies (Sanchez-Lopez et al., 1988), to produce purified transin, stromelysin 2, and collagenase. We compare here the in vitro activities of the enzymes produced in this manner to the activity observed by using recombinant stromelysin purified by standard procedures (Murphy et al., 1987).

Stromelysin has been reported as having two major active forms with apparent molecular weights of 45 000–48 000 and 28 000 (Chin et al., 1985; Okada et al., 1986; Murphy et al., 1987). The two major species of active stromelysin have been separated, and it was determined that the proteolytic activities were identical (Okada et al., 1986; P. Koklitis and G. Murphy, unpublished data). We have shown previously that these forms correspond in transin to a large active species (48 000) which can be separated into N-terminal and C-terminal domains. We have shown that the N-terminal species (28 000) can be created by engineering the removal of the C-terminal domain (amino acids 263–475). This N-terminal region of transin contains the same proteolytic activity against casein as we observed with the entire transin molecule and is inhibited by metal chelating agents as well as by TIMP (Sanchez-Lopez et al., 1988). The function of the C-terminal domain has not yet been determined, although the similarity in the amino acid sequence of this region with hemopexin suggests a possible role in protein–protein interactions (Breathnach et al., 1987). We now see that APMA activation of a protein A–stromelysin 2 fusion protein also results in two major polypeptide species with sizes similar to those produced with activated transin and stromelysin. The collagenase produced in our system resulted in two major and a minor polypeptide species with apparent molecular weights of 39 000–43 000. Multiple collagenase species in this size range have also been described by others (Stricklin et al., 1983; Grant et al., 1987) and vary at their N-termini depending on the method of activation. It is also possible, however, that the multiple species may occur as a result of aberrant cleavage during activation. However, it is clear from the substrate digestion assays that the collagenase produced by this method displays the same substrate specificity as has been described for natural collagenase.

Metalloproteinases are known to require Zn^{2+} and Ca^{2+} for activity, and thus the proteolytic activity is inhibited in the presence of metal chelating agents. DTT has also been shown to inhibit metalloproteinases, and Okada et al. (1986) have suggested that this inhibition may involve interaction with a zinc ion believed to be located at the active site. A protein (TIMP) has been purified and characterized that is a specific inhibitor of metalloproteinases (Murphy et al., 1981; Welgus & Stricklin, 1983; Murphy et al., 1985). The activities of both stromelysin 2 and transin proved to be sensitive to all of the above types of inhibitor, indicating that they are typical metalloproteinases.

Stromelysin has been shown to degrade many ECM components including fibronectin, type IV collagen, proteoglycans, and denatured collagen type I, but not native type I collagen. Collagenase is known to degrade native and denatured interstitial collagens (types I–III), but to have little activity against other components of the ECM. The data presented here (summarized in Table I) indicate that transin and stro-

Table I: Summary of Substrate Recognition by the Four Recombinant Metalloproteinases Assayed[a]

enzyme	collagen type I	collagen type III	collagen type IV	collagen type V	gelatin type I	gelatin type III	gelatin type IV	gelatin type V	fibronec
collagenase	+	+	–	–	(+)	+	–	+	–
stromelysin transin	–	+	+	+	+	+	+	+	+
stromelysin 2	–	+	+	+	+	+	(+)	+	+

[a] Lack of detectable activity by an enzyme against a substrate is indicated by a –, good activity by a +, and slight activity by a (+).

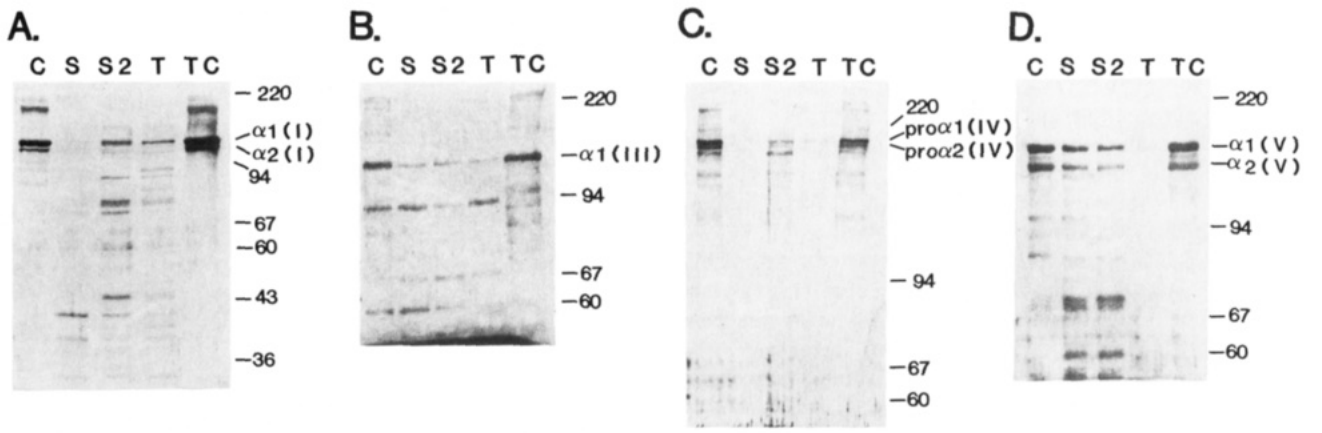

FIGURE 6: Electrophoretic banding pattern of degradation products resulting from incubation of denatured collagen (gelatin) with IgG–Sepharose-purified collagenase (C), stromelysin 2 (S2), or transin (T) or with purified recombinant stromelysin (S) or the control preparation (TC). Denatured collagens used were type I (panel A), type III (panel B), type IV (panel C), and type V (panel D). The position of the α-chains of each collagen type is indicated, as are the migratory positions of the molecular weight markers as described in Figure 4.

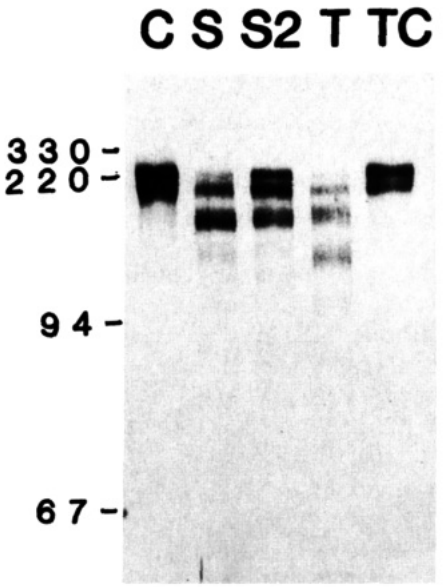

FIGURE 7: Electrophoretic banding pattern of fibronectin after incubation with IgG–Sepharose-purified collagenase (C), stromelysin 2 (S2), or transin (T) or with purified recombinant stromelysin (S) or the control preparation (TC). The migratory position of the molecular weight markers thyroglobulin (330000), ferritin (220000), phosphorylase *b* (94000), and albumin (67000) is indicated at *left*.

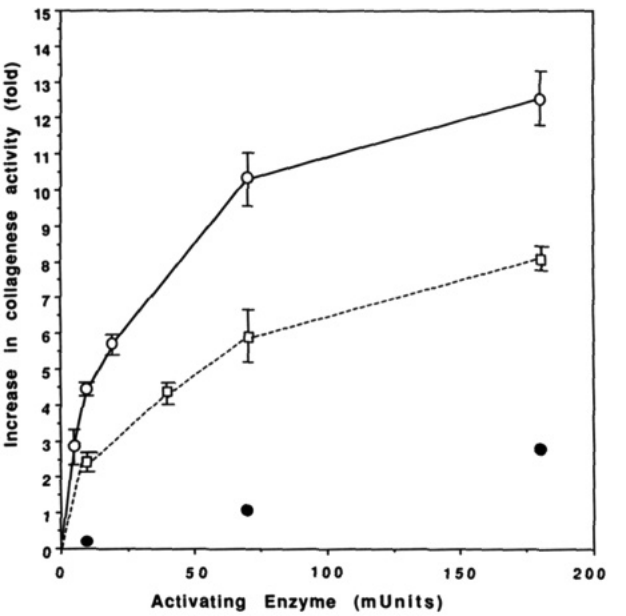

FIGURE 8: Activation of procollagenase. Open symbols represent the fold increase in collagenase activity against type I collagen after incubation of collagenase with various amounts (milliunits) of IgG–Sepharose-purified transin (circles) or stromelysin 2 (squares) as compared to activity observed after assay with inactive procollagenase. Solid circles represent the amount of activation observed when procollagenase was incubated with volumes of transin C-domain corresponding to the maximal volumes of activating enzyme used at that location.

melysin 2 degrade components of the ECM in a way more closely resembling that of stromelysin than collagenase. Stromelysin 2 and transin, like stromelysin, do not cut native type I collagen but do degrade types III–V collagen and fibronectin.

The ECM components degraded by stromelysin, stromelysin 2, and transin are representative of proteins located in all parts of the ECM. Thus, these enzymes should play an important role in degradation of the ECM during the normal and abnormal situations where ECM turnover is required. Stromelysin, stromelysin 2, and transin may also act in association with other enzymes, such as type I or type IV collagenases, to continue degradation of the proteins after an initial enzymatic attack has relaxed the tertiary structure of the proteins. That we have observed activity by stromelysin, stromelysin 2, and transin against four different types of denatured collagen supports this notion.

Since it is clear that the complex ECM is not degraded in a simple manner, but rather requires several enzymes to re-

move it sufficiently to allow cell migration (i.e., during tissue remodeling or tumor invasion), it has been proposed that the enzymes involved in ECM degradation may interact with each other directly in an activation cascade (Vater et al., 1983, 1986; Mignatti et al., 1986; Murphy et al., 1987). To this end, we have shown previously that human stromelysin can act as an activator of human procollagenase in vitro (Murphy et al., 1987). In that study, the collagenase activity was increased by up to 12-fold by treatment of procollagenase simulantaneously with APMA and prostromelysin. Somewhat less activation was detected when previously activated stromelysin was used. We show here that transin and stromelysin 2 can also act as activators of procollagenase in vitro.

The RNA levels for transin, stromelysin, and collagenase are increased in cells exposed to growth factors or tumor-promoting phorbol esters (Matrisian et al., 1986; Whitham et al., 1986; Brinckerhoff et al., 1986; Edwards et al., 1987), and stromelysin is often synthesized and secreted coordinately with collagenase (Sellers et al., 1978; Chin et al., 1985; Chua et al., 1985). Therefore, the observed ability of stromelysin, stromelysin 2, and transin to activate procollagenase in vitro suggests a similar in vivo role for these enzymes. Since type I collagen is the major component of the ECM, its degradation during the invasive process is clearly essential. The enzymes that can activate collagenase may thus play an important role in controlling the malignant phenotype.

We have shown in this paper that the protein encoded by the rat transin cDNA has enzymatic activity in vitro, against several ECM components, similar to that of the human metalloproteinase stromelysin. This supports the notion that transin is the rat equivalent of stromelysin. We have also shown that in human there is an additional enzyme, stromelysin 2, the activity of which closely parallels the activity spectrum of stromelysin. This indicates that there exists a subset of enzymes similar to stromelysin acting to degrade the ECM. The ability of this subset of enzymes to digest ECM components from all areas of the ECM suggests an important role for these proteinases in the many situations where the ECM must be removed. The ability of these enzymes to work in association with other ECM-degrading enzymes, by activation of proenzymes and/or by degrading many different denatured collagens, reinforces the importance of these enzymes in such situations. Finally, the association of rat stromelysin and human stromelysin 2 with the malignant phenotype strongly supports the notion that metalloproteinases play an important role in the progression of such pathological conditions.

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Registry No. Stromelysin 2, 118368-07-3; procollagenase, 39287-99-5.

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Expression of Two Kallikrein Gene Family Members in the Rat Prostate†

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ABSTRACT: We have characterized two kallikrein gene family members expressed in the prostate and submaxillary glands of rats. One mRNA (S3) is identical with the previously characterized submaxillary gland S3 mRNA that encodes an enzyme closely related to tonin. The second mRNA (P1) encodes a novel kallikrein-like enzyme that retains key amino acid residues responsible for the characteristic enzymatic cleavage specificity of kallikrein. Two P1-specific oligonucleotide probes derived from the P1 mRNA sequence were used to demonstrate the presence of P1 mRNA in the prostate and submaxillary glands and its absence in eight other rat tissues known to express one or more members of the kallikrein family. The P1-coding gene (rGK-8) was identified among genomic clones containing kallikrein family members by hybridization with a P1-specific oligonucleotide probe. The identification of the P1 gene was verified by nucleotide sequencing; the exon sequences of rGK-8 match the P1 mRNA sequence. The upstream region of rGK-8, where transcriptional regulatory elements likely reside, is very similar to that of other rat kallikrein family genes which are expressed in distinct tissue-specific patterns.

The kallikrein gene family encodes serine proteases that process biologically active peptides. This subfamily of serine proteases is characterized by limited substrate specificity and high sequence conservation when compared to other simple serine proteases. Tissue kallikrein is the best characterized enzyme of this family, which also includes tonin, the γ subunit of nerve growth factor (γ-NGF), epidermal growth factor binding proteins (EGF-BPs), prostate-specific antigen (PSA), and other less well-characterized proteases. Tissue kallikrein specifically cleaves the protein kininogen to release the potent vasodilatory peptide lysyl-bradykinin (Yamada & Erdos, 1982). Tonin cleaves angiotensinogen in vitro to release angiotensin II, a potent vasoconstrictor (Boucher et al., 1974). γ-NGF processes the precursor of nerve growth factor (Thomas et al., 1981), and the EGF-BPs process the precursor of epidermal growth factor (Lundgren et al., 1984; Drinkwater et al., 1987). Human PSA cleaves the high molecular weight seminal vesicle protein (Watt et al., 1986), causing liquification of the seminal fluid clot (Lilja et al., 1987). Thus, the biochemical role of the enzymes of the kallikrein family appears to be the selective cleavage of polypeptide precursors, principally prohormones [reviewed in Fuller and Funder (1986), Drinkwater et al. (1987), and MacDonald et al. (1988)].

In rodents, the kallikrein gene family comprises 12–24 members (Mason et al., 1983; Evans et al., 1987; Ashley & MacDonald, 1985a; Wines et al., 1989) disparately expressed in a variety of tissues. In the rat, kallikrein-like enzymes or their mRNAs have been detected in the submaxillary gland, sublingual and parotid glands, pancreas, prostate, kidney, spleen, pituitary gland, testis, brain, and liver (Tschesche et al., 1979; Swift et al., 1982; Ashley & MacDonald, 1985b; Pritchett & Roberts, 1987; Chao & Chao, 1987). For the mouse, from which all 24 kallikrein family genes have been characterized, only 1 gene encodes tissue (true) kallikrein (van Leeuwen et al., 1986); this is probably true for human, rat, and hamster as well. The other genes either encode proteins closely related to kallikrein or are pseudogenes (Evans et al.,

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