Articles

Pump-1 cDNA Codes for a Protein with Characteristics Similar to Those of Classical Collagenase Family Members†,‡

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ABSTRACT: Pump-1 cDNA has recently been isolated by screening a human tumor cDNA library with a transin (rat stromelysin) probe under low-stringency hybridization conditions. The cDNA codes for a potential protein with significant sequence similarity to the metalloproteinases collagenase and stromelysin, but which lacks the hemopexin-like domain characteristic of these enzymes. Expression of pump-1 cDNA in cos cells using an expression vector leads to secretion of a protein of M_r 28 000 with latent, organomercurial-activatable proteinase activity. Cos cells transfected with a partial pump-1 cDNA in the vector pPROTA secrete a fusion protein between the IgG-binding domains of staphylococcal protein A and pump-1. The fusion protein binds to IgG–Sepharose, and the bound fusion protein undergoes apparent autocleavage in the presence of 4-aminophenylmercuric acetate with elution of active pump-1 species of M_r 21 000 and 19 000. Active pump-1 degrades casein, gelatins of types I, III, IV, and V, and fibronectin and can activate collagenase. Active pump-1 is inhibited by EDTA, 1,10-phenanthroline, and the tissue inhibitor of metalloproteinases. These results show that, despite the absence of a hemopexin-like domain, pump-1 is a latent secreted metalloproteinase. Postpartum rat uteri contain elevated levels of rat pump-1 mRNA. On the basis of this observation, its size, and its substrate specificity, we suggest that pump-1 might correspond to a previously described uterine metalloproteinase, matrix metalloproteinase 7.

Metalloproteinases are believed to be active in many situations requiring connective tissue remodeling, for example, wound healing, bone resorption, or uterine or breast involution (Mullins & Rohrlich, 1983). They may also play a role in tumor invasion and metastasis and in other pathological conditions such as rheumatoid arthritis (Mullins & Rohrlich, 1983; Tryggvason et al., 1987). The characterization of these enzymes is thus of some importance. One of the best-studied metalloproteinases is the interstitial collagen-degrading enzyme collagenase (Murphy & Reynolds, 1985). Stromelysin [also known as MMP-3 (Okada et al., 1986), transin (Matrisian et al., 1986b), or collagenase activator (Fini et al., 1987)] is a collagenase-related metalloproteinase whose substrates include proteoglycans, fibronectin, gelatin, type IV collagen, and laminin (Okada et al., 1986; Galloway et al., 1983; Chin et al., 1985; Wilhelm et al., 1987). The amino acid sequences of collagenase and stromelysin are approximately 55% similar (Wilhelm et al., 1987; Whitham et al., 1986; Goldberg et al., 1986; Breathnach et al., 1987; Fini et al., 1987). Both enzymes have a C-terminal moiety which shows significant sequence similarity to the serum heme-binding protein hemopexin (Fini et al., 1987; Matrisian et al., 1986a).

We have recently described the isolation of cDNAs which potentially code for two new members of the collagenase family, stromelysin-2 and pump-1 (Muller et al., 1988). The putative protein pump-1 (267 amino acids) is much shorter than collagenase, stromelysin, or stromelysin-2 (each around 470 amino acids). The predicted amino acid sequence of pump-1 is 44% and 49% similar to those of the N-terminal domains of collagenase and stromelysin, respectively (Muller et al., 1988). An equivalent of the C-terminal, hemopexin-like domain of these enzymes is not present in pump-1 (Muller et al., 1988).

As pump-1 is a putative protein whose existence was deduced from the sequence of a cDNA clone, no data concerning the possible enzymatic activities of pump-1 were available. Our only way to produce pump-1 was from the corresponding cDNA. We therefore decided to use recombinant DNA technology to investigate whether pump-1 is a metalloproteinase with properties characteristic of a collagenase family member, despite the absence of a hemopexin-like domain, and whether pump-1 may correspond to any known metalloproteinase.

Experimental Procedures

Materials

Materials were purchased from the following sources: casein and Tween 20 were from Sigma. IgG–Sepharose and Sepharose 4B were from Pharmacia. 4-Aminophenylmercuric acetate (APMA)[1] and 1,10-phenanthroline were from Aldrich. L-[^{35}S]Methionine (~1000 Ci/mmol) was from Amersham.

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‡ The nucleic acid sequence in this paper has been submitted to the EMBL data bank under the name HSPUMP1.

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[1] Abbreviations: APMA, 4-aminophenylmercuric acetate; IgG, immunoglobulin G; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; TIMP, tissue inhibitor of metalloproteinases; TC buffer, 0.02 M Tris, pH 7.5, containing 5 mM $CaCl_2$; TCT buffer, TC buffer containing 0.05% Tween 20; kb, kilobase pair(s).

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Type I gelatin was prepared by denaturation of rat skin collagen (Galloway et al., 1983). TIMP was purified from human skin fibroblasts and procollagenase and stromelysin from human gingival fibroblasts (Whitham et al., 1986). Fibronectin was purified from bovine plasma (Vuento & Vaheri, 1979) and was kindly provided by C. Cockburn. All other materials were of reagent grade.

Methods

Plasmid Constructions. The vector pKCR3 (Matrisian et al., 1986b) contains sequentially the SV40 early gene promoter and origin of replication, a pair of splice sites from a rabbit β-globin gene, a unique *Eco*RI site for introduction of cDNAs, and a polyadenylation site from a rabbit β-globin gene. To create pPump, a 1.1-kb *Eco*RI fragment of pump-1 cDNA carrying the complete protein coding sequence (Muller et al., 1988) was introduced into the *Eco*RI site of pKCR3. The vector pPROTA (Sanchez-Lopez et al., 1988) is based on pKCR3 and contains the SV40 early gene promoter and origin of replication, a pair of splice sites from a rabbit β-globin gene, a sequence coding for the first 32 amino acids of transin (providing a signal peptide) fused to sequences coding for amino acids 23–270 of staphylococcal protein A (Uhlen et al., 1984) from pRIT5 (Nilsson et al., 1985), a unique *Eco*RI site for introducing cDNAs, and a globin polyadenylation site. pPAPP was prepared by inserting a 1.0-kb *Eco*RI fragment coding for amino acids 11–267 of pump-1 (Muller et al., 1988) (together with 3′-untranslated sequences) into the *Eco*RI site of pPROTA. This fragment was prepared with oligonucleotide-directed mutagenesis by fusing the sequence 5′-GAATTCCAGCCTGGCC-3′ onto nucleotide 58 of pump-1 cDNA using standard techniques (Grundström et al., 1985; Kumar et al., 1986). pTRC (Sanchez-Lopez et al., 1988) is a pKCR3 derivative containing a transin (rat stromelysin) cDNA fragment (Matrisian et al., 1985b) from which sequences coding for amino acids 33–262 have been deleted. It is designed to express the hemopexin-like domain (Matrisian et al., 1986a) of transin. pPA-TRC (Sanchez-Lopez et al., 1988) contains, in the vector pPROTA, an *Eco*RI fragment of transin cDNA encoding amino acids 12–32 followed by amino acids 263–475. pPA-TRC is designed to express a fusion protein between protein A and the hemopexin-like domain (Matrisian et al., 1986a) of transin.

Production of Proteins by Cos Cells. Cos cells (Gluzman, 1981) were transfected with recombinant plasmids by using the calcium phosphate procedure (Wigler et al., 1979). Cells were exposed to DNA for 24 h, then washed in serum-free medium, and incubated in medium with 5% fetal calf serum for 24 h. Cells were then washed twice with methionine-free Dulbecco's medium and incubated in this medium for 1 h to deplete intracellular methionine levels; 50 μCi/mL L-[^{35}S]-methionine ($\sim$1000 Ci/mmol, Amersham) was then added. Alternatively, cells were washed and incubated in normal Dulbecco's medium; 40 h later, the conditioned medium was recovered and centrifuged briefly to remove debris. L-[^{35}S]-Methionine-labeled samples were analyzed directly by SDS–polyacrylamide gel electrophoresis [SDS–PAGE (Laemmli, 1970)] or incubated with IgG–Sepharose (Pharmacia) as described below. Ammonium sulfate was added to unlabeled media (typically 2 mL of conditioned medium from an experiment involving transfection of 3×10^5 cos cells in a 6-cm-diameter petri dish) to a final saturation of 40%, and after 24 h at 4 °C, the precipitate was collected by centrifugation (77000*g* for 1 h at 4 °C). The precipitate was dissolved in 40 μL of 0.02 M Tris, pH 7.5, containing 5 mM $CaCl_2$ (TC buffer) before dialysis against TC buffer at 4 °C. Dialysates (20 μL) were used in digestion assays, or for experiments with IgG–Sepharose.

IgG–Sepharose Chromatography. IgG–Sepharose (Pharmacia) was prepared as recommended by the manufacturer before washing in 0.05 M Tris, pH 7.5, followed by TC buffer containing 0.05% Tween 20 (TCT buffer). Aliquots (0.5 mL) of ^{35}S-labeled media were brought to 0.05% Tween 20 and added to $\sim$25-μL packed volume of IgG–Sepharose. The mixture was left on ice for 1 h with occasional mixing. Resins were washed by sequential centrifugation and resuspension with 0.05 M Tris, pH 7.5 (1 mL), PBS containing 0.05% Tween 20 (7 × 1 mL), 0.05 M Tris, pH 7.5 (1 mL), and TCT buffer (2 × 1 mL) and then drained. Elution was carried out by adding 40 μL of TCT buffer, 2.5 μL of 2 M Tris, pH 7.5, and 1 μL of 50 mM APMA followed by incubation at 37 °C for 1 h. The eluate and eluted resins with residual bound proteins were separated by centrifugation. In some experiments, APMA was omitted from the elution buffer. Proteins in the eluates or remaining bound to the resin were analyzed by SDS–PAGE and fluorography.

Samples of unlabeled cos cell conditioned media containing protein A–fusion proteins were precipitated with ammonium sulfate and dialyzed as described above, adjusted to 0.05% Tween 20, and incubated with IgG–Sepharose ($\sim$25-μL packed volume) on ice for 2 h with occasional mixing. The resin was separated from the nonbound, "flow-through" fraction by centrifugation. The resin was extensively washed and eluted as described above. In some experiments, Sepharose 4B was used in place of IgG–Sepharose. "Flow-through" and eluate fractions were used in proteinase assays as described below.

To quantify amounts of pump-1 present, samples of eluates were analysed by SDS–PAGE followed by silver staining. The amount of pump-1 present was estimated by comparison of the pump-1 band intensity to the band intensities of known amounts of ovalbumin, carbonic anhydrase, and trypsin inhibitor. The pump-1 used here was at a concentration of 25 μg/mL. Proteolytic activity of pump-1 against ^{14}C-acetylated casein was determined as described (Galloway et al., 1983). The pump-1 used here was at 35 units/mL (specific activity 1400 units/mg) and the stromelysin at 70 μg/mL and 35 units/mL (specific activity 500 units/mg).

Substrate Cleavage by Pump-1. Digestions of the different substrates were performed as follows: Twenty microliters of fractions from IgG–Sepharose or Sepharose 4B chromatography was incubated with 2.5 μg of casein for 16 h at 37 °C in TC buffer (50-μL final volume). Four microliters (140 milliunits) of IgG–Sepharose chromatography purified pump-1 was incubated for 4 and 20 h with 0.5 μg of types III or IV collagen or 1.5 μg of type V collagen at 35 °C, or with 0.8 μg of fibronectin or 0.5 μg of type IV gelatin at 37 °C in TC buffer (30-μL final volume). Digests were performed in parallel with an equivalent volume of pPA-TRC control material or with 4 μL (140 milliunits) of stromelysin. One microliter (35 milliunits) of IgG–Sepharose chromatography purified pump-1 was incubated with 0.5 μg of type I or III gelatin or 1.5 μg of type V gelatin at 37 °C in TC buffer (30-μL final volume) for 4 and 20 h; equivalent amounts of the pPA-TRC control material or 1 μL (35 milliunits) of stromelysin were assayed in parallel.

Where appropriate, digests included 1 mM APMA. In some experiments, inhibitors were added to the reaction. Reactions were stopped by boiling for 3 min in gel sample buffer (Laemmli, 1970). Digestion products were analyzed by SDS–PAGE (Laemmli, 1970) followed by staining with

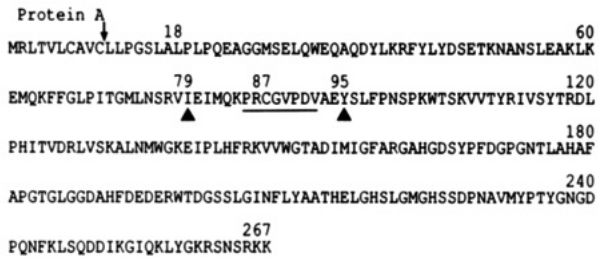

FIGURE 1: Predicted amino acid sequence of pump-1. On the basis of alignment (Muller et al., 1988) of the pump-1 sequence with those of stromelysin and collagenase, the following sequence features are proposed. Leu-18, N-terminus of secreted pump-1. Ile-79 and Tyr-95 (arrowheads), N-termini of APMA-treated pump-1. Cys-87, putative target of APMA in a conserved sequence block of eight amino acids (underlined). The structure of the protein A–pump-1 fusion protein is indicated (fusion of protein A sequences to Leu-11).

silver (Switzer et al., 1979) or Coomassie Brilliant Blue R-250.

Activation of procollagenase was carried out as described elsewhere (Murphy et al., 1987). Briefly, varying amounts (up to 525 milliunits) of active pump-1 eluted from IgG–Sepharose (or equivalent amounts of eluates from a parallel experiment using pPA-TRC or a similar amount of stromelysin in terms of units) were incubated at 37 °C for 2 h in the presence of procollagenase in a 30-μL final volume; collagenase activity was then assayed as described (Murphy et al., 1987) using ^{14}C-labeled type I collagen at 35 °C for 16 h in a 300-μL volume.

Rat Uterus RNA. Rat uteri were excised surgically and immediately frozen in liquid nitrogen. RNA was extracted from frozen powdered tissue as described elsewhere (Muller et al., 1988). Northern analysis of RNA samples using pump-1 (Muller et al., 1988) or glycolytic enzyme cDNA probes (Matrisian et al., 1985a) was as described in Muller et al. (1988). In particular, "high"-stringency conditions were used [50% (v/v) formamide] to avoid cross-hybridization of the pump-1 cDNA to transin–stromelysin or collagenase mRNAs.

Results

The significant sequence similarity shared by pump-1, collagenase, and stromelysin (Muller et al., 1988) suggests that available data concerning collagenase and stromelysin may be extrapolated to pump-1. It should be borne in mind that such extrapolations are based solely on the presence of conserved amino acid sequence features. They may nevertheless assist understanding of the experimental designs detailed below. To begin with, we would predict, on the basis of the amino acid sequence alignments presented elsewhere (Muller et al., 1988), that the N-terminus of secreted pump-1 will be Leu-18 (Figure 1). The predicted molecular weight of secreted pump-1 would then be 27 938.

Collagenase and stromelysin are secreted, latent, metalloproteinases which can be activated (Murphy & Reynolds, 1985) by treatment with organomercurials such as 4-aminophenylmercuric acetate (APMA). APMA may function by interacting with a thiol group [probably that of a Cys residue within the conserved sequence PRCGVPDV (Sanchez-Lopez et al., 1988)], provoking a conformation change leading to activation (Wilhelm et al., 1987; Whitham et al., 1986; Murphy et al., 1987; Vater et al., 1983, 1986; Stricklin et al., 1983; Grant et al., 1987). Activation can occur before a detectable decrease in size of the proenzyme is seen. However, activation is often accompanied by autocleavages on either side of the sequence PRCGVPDV (Wilhelm et al., 1987; Whitham et al., 1986; Grant et al., 1987). This sequence is also present in pump-1 (Figure 1). Extrapolation of data concerning

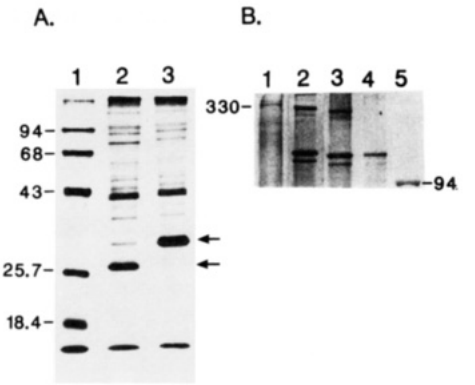

FIGURE 2: Proteolytic activity of secreted pump-1. (A) ^{35}S-Labeled proteins secreted by cos cells transfected with pPump (lane 2) or pTRC (lane 3) were analyzed by SDS–PAGE (10% gel) and fluorography. The M_r 28 000 and 30 000 proteins are indicated by arrows. Lane 1, molecular weight standards; $M_r \times 10^{-3}$ is shown. (B) Proteins secreted by cos cells transfected with pPump (lanes 3 and 4) were incubated with type I gelatin in the presence (lane 4) or absence (lane 3) of APMA for 8 h. Samples were analyzed by SDS–PAGE (7.5% gel) and silver staining. Lane 2, type I gelatin exposed to buffer alone. Lanes 1 and 5, molecular weight standards; $M_r \times 10^{-3}$ is shown.

APMA-activated collagenase (Grant et al., 1987) to pump-1 suggests that the N-termini of APMA-treated pump-1 will be Ile-79 and Tyr-95. The predicted molecular weights of APMA-treated pump-1 would then be 20 900 and 19 133.

Expression of Pump-1 in Cos Cells. We decided to express pump-1 in eukaryotic cells from its cDNA. The recombinant pPump was obtained by placing the pump-1 cDNA downstream from the SV40 early gene promoter in the expression vector pKCR3 (Matrisian et al., 1986b). As a control plasmid, we chose pTRC (Sanchez-Lopez et al., 1988), a pKCR3 derivative designed to direct synthesis of a secreted protein of M_r 30 000 corresponding to the C-terminal, hemopexin-like domain of transin (rat stromelysin). This fragment of transin does not exert any proteinase activity (Sanchez-Lopez et al., 1988). Transfection of cos cells with pPump or pTRC led to the secretion of proteins of M_r 28 000 and 30 000, respectively (see arrows in Figure 2A). Nontransfected cos cells do not secrete elevated levels of either an M_r 28 000 or an M_r 30 000 protein (data not shown). We identify the protein of M_r 28 000 as pump-1 and conclude that pump-1 is indeed a secreted protein. Conditioned medium from cos cells transfected with pPump contained a latent, APMA-activatable proteinase activity detectable by using type I gelatin (Figure 2B) or casein (data not shown) as substrates. As expected, conditioned medium from cos cells transfected with pTRC did not contain detectable levels of this activity (data not shown).

Chromatography of Protein A–Pump-1 Fusion Proteins. The results described above suggest that pump-1 is a secreted, APMA-activatable proteinase. However, alternative explanations of the data cannot be ruled out. Expression of pump-1 might induce secretion or activation of a cos cell proteinase, for example. One way of distinguishing between these possibilities would be to tag pump-1 with the IgG-binding domains of staphylococcal protein A and to use IgG–Sepharose chromatography to purify the fusion protein. This is possible when the vector pPROTA is used.

The vector pPROTA (Sanchez-Lopez et al., 1988) is designed to direct synthesis of secretable fusion proteins between the IgG-binding domains of staphylococcal protein A and a protein whose coding sequences are inserted behind the protein A sequences in the appropriate reading frame, using the unique *Eco*RI site of the vector. Oligonucleotide-directed mutagenesis was used to prepare an *Eco*RI fragment coding for amino acids

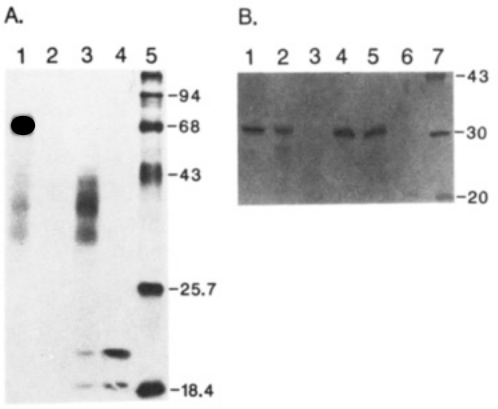

FIGURE 3: Purification and proteinase activity of active pump-1. (A) ^{35}S-Labeled proteins secreted by cos cells transfected with pPAPP were incubated with IgG–Sepharose. The resin was incubated with buffer containing APMA to induce autocleavage of the bound fusion protein (lanes 3 and 4) or with buffer alone (lanes 1 and 2). Proteins released from the resin (eluates) by these treatments were recovered in the supernatants after separation from the resin by a brief centrifugation. Eluates (lanes 2 and 4) and proteins remaining bound to IgG–Sepharose (lanes 1 and 3) were analyzed by SDS–PAGE (12% gel) and fluorography. Lane 5, molecular weight standards; $M_r \times 10^{-3}$ is shown. (B) Proteins secreted by cos cells transfected with pPAPP were exposed to Sepharose 4B (lanes 2–4) or IgG–Sepharose (lanes 5 and 6). Unbound material (lanes 2, 3, and 5) was incubated with casein in the presence (lanes 3 and 5) or absence (lane 2) of APMA. Bound material was eluted with APMA, and the eluates (lanes 4 and 6) were incubated with casein. Lane 1, casein exposed to buffer alone. Digests were analyzed by SDS–PAGE (12% gel) and staining with Coomassie Brilliant Blue R-250. Lane 7, molecular weight standards; $M_r \times 10^{-3}$ is shown.

11–267 of pump-1. This fragment was inserted into pPROTA. The resulting recombinant pPAPP should direct synthesis of a secretable fusion protein of the structure shown schematically in Figure 1. This fusion protein is essentially pump-1 with a long N-terminal extension. We would predict that it should bind to IgG–Sepharose via the protein A sequences. If the N-terminal extension does not interfere, the fusion protein should undergo APMA-induced cleavage at the sites appropriate for pump-1 (see Figure 1), generating species of M_r 21 000 and 19 000 consisting of pump-1 sequences alone.

Cos cells transfected with pPAPP secrete a protein of M_r 70 000 which binds to IgG–Sepharose (Figure 3A, lane 1). Treatment of the bound fusion protein with APMA leads to its disappearance from the resin (Figure 3A, lane 3) and elution of proteins of M_r 21 000 and 19 000 (Figure 3A, lane 4). We conclude that protein A–pump-1 fusion proteins will bind to IgG–Sepharose and that the bound fusion protein undergoes cleavage in the presence of APMA to generate putative active forms of pump-1.

From the above results, it is clear that if any proteinase activity secreted by cos cells transfected with pPAPP is due to the pump-1 sequences, this activity should (1) bind to IgG–Sepharose and (2) be eluted by APMA. Conditioned medium from cos cells transfected with pPAPP was incubated with either IgG–Sepharose or Sepharose 4B, and the unbound, "flow-through" fraction was collected. Resins were extensively washed before elution with APMA. The flow-through fractions and eluates were tested for their capacity to degrade casein (Figure 3B). The pPAPP Sepharose 4B flow-through fraction digested casein in the presence of APMA (lane 3). In the absence of APMA, no digestion occurred (lane 2). The APMA eluate of the Sepharose 4B did not digest casein (lane 4). The opposite behavior was observed when IgG–Sepharose was used: the APMA-activated flow-through fraction did not digest casein (lane 5), while the APMA eluate did (lane 6). Similar results were obtained when fibronectin or type I gelatin were used as substrates (data not shown). We conclude that "active" pump-1 can indeed act as a proteinase.

Substrates of Pump-1. We have used active pump-1 isolated by IgG–Sepharose chromatography from medium conditioned by cos cells transfected with pPAPP to investigate its substrate range. As already discussed, active pump-1 will digest casein. Using a standard casein degradation assay (Galloway et al., 1983), we have determined the specific activity of active pump-1 to be 1400 units/mg (1 unit degrades 1 μg of casein per minute at 37 °C). The corresponding figures for rabbit and human stromelysin are 2400 and 360–500 units/mg, respectively (Galloway et al., 1983; Murphy et al., 1987; this paper).

On the basis of its amino acid sequence, we might predict that pump-1 would more closely resemble stromelysin than collagenase in its substrate range. We therefore decided to compare the proteinase activity of pump-1 to that of human stromelysin. We chose to undertake a direct comparison by exposing given amounts of extracellular matrix components in parallel to the same number of units of pump-1 or stromelysin (this corresponds to about 3-fold less pump-1 than stromelysin on a weight-to-weight basis). As a control, conditioned medium from cos cells transfected with pPA-TRC [a plasmid designed to express a fusion protein between staphylococcal protein A and the C-terminal moiety of transin (Sanchez-Lopez et al., 1988)] was processed and assayed in parallel with the pPAPP samples. This latter control material should not have any proteinase activity. The results are shown in Figures 4–7. It may be stressed that the control preparation (see above) showed no detectable proteinase activity against any of the substrates tested here.

Active pump-1 readily degrades fibronectin (Figure 4A). The fibronectin digestion patterns obtained with pump-1 and stromelysin are very similar, although pump-1 is rather more effective. Pump-1 has little or no detectable activity against native collagens of types I (data not shown), III (Figure 4B), IV (Figure 4C), or V (Figure 4D). Stromelysin will, under the conditions used, degrade type III and type IV collagen to some extent. Pump-1 digests gelatins prepared from collagens of types III, IV, and V, giving results very similar to those obtained with stromelysin (Figure 5). Pump-1 will also digest gelatin prepared from collagen of type I (Figure 6). It may be noted that, as reported for stromelysin (Okada et al., 1986), pump-1 preferentially degrades the α2 chain of gelatin (Figure 6).

Although pump-1 will not digest native collagens, it might nevertheless play a role in collagen degradation. As has been shown previously, stromelysin can activate procollagenase (Fini et al., 1987; Murphy et al., 1987; Vater et al., 1983). In particular, treatment of human procollagenase simultaneously with APMA and prostromelysin increases the collagenase activity elicited by up to 12-fold compared to treatment with APMA alone (Murphy et al., 1987). Slightly lower stimulation was observed when already activated stromelysin was used (Murphy et al., 1987). Under similar experimental conditions, treatment of human procollagenase with active pump-1 increases the collagenase activity obtained up to 5-fold that obtained with APMA alone (Figure 7). Use of higher amounts of pump-1 did not lead to a further increase of collagenase activity (data not shown). In an experiment carried out in parallel using stromelysin, a maximum stimulation of 14-fold was obtained.

The activity of pump-1 on the substrate gelatin is blocked in the presence of EDTA, 1,10-phenanthroline, or TIMP

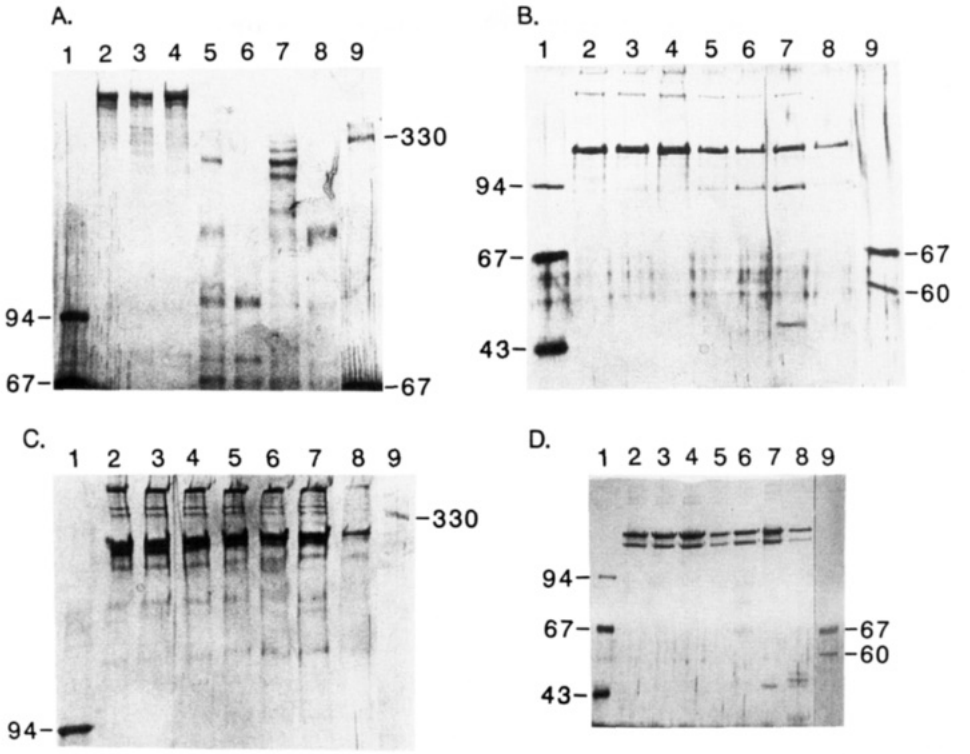

FIGURE 4: Comparative analysis of the proteolytic activity of pump-1 and stromelysin against fibronectin and collagen types III, IV, and V. Proteins secreted by cos cells transfected with pPAPP (lanes 5 and 6) or pPA-TRC (lanes 2 and 3) were exposed to IgG–Sepharose; bound material was eluted with APMA and incubated with fibronectin (A), type III collagen (B), type IV collagen (C), or type V collagen (D) for 4 h (lanes 2, 5, and 7) or 20 h (lanes 3, 6, and 8). Stromelysin was assayed in parallel (lanes 7 and 8). Digests were analyzed as follows: (A) SDS–PAGE (5% gel) under nonreducing conditions and silver staining. (B–D) SDS–PAGE (panels B and D, 7% gel; panel C, 5% gel) and silver staining. For panels A–D, lane 4 = substrate exposed to buffer alone; lanes 1 and 9 = molecular weight standards; $M_r \times 10^{-3}$ is shown.

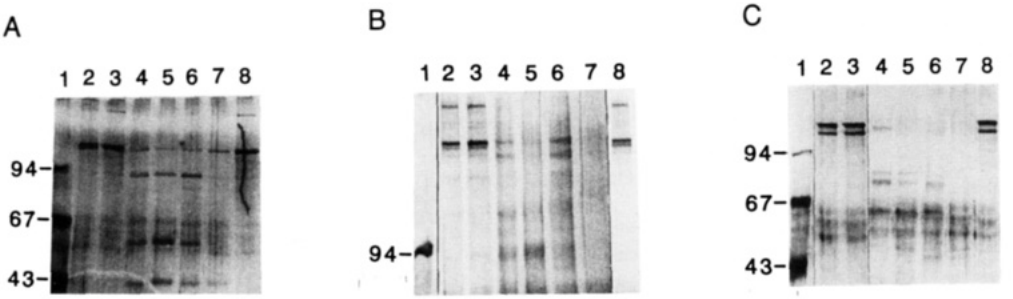

FIGURE 5: Comparative analysis of the proteolytic activity of pump-1 and stromelysin against type III, IV, and V gelatins. Proteins secreted by cos cells transfected with pPAPP (lanes 4 and 5) or pPATRC (lanes 2 and 3) were exposed to IgG–Sepharose; bound material was eluted with APMA and incubated with type III gelatin (A), type IV gelatin (B), or type V gelatin (C) for 4 h (lanes 2, 4, and 6) or 20 h (lanes 3, 5, and 7). Stromelysin was assayed in parallel (lanes 6 and 7). Digests were analyzed by SDS–PAGE (panels A and C, 7% gel; and B, 5% gel) and silver staining. For panels A–C, lane 1 = molecular weight standards; $M_r \times 10^{-3}$ is shown; lane 8 = substrate exposed to buffer alone.

(Figure 6), indicating that pump-1 is, as predicted, a metalloproteinase.

Pump-1 RNA in Involuting Rat Uterus. We conclude from the above data that pump-1 is a stromelysin-like metalloproteinase of the collagenase family. Does pump-1 correspond to any already characterized metalloproteinase? Most metalloproteinases described in the literature have molecular weights significantly higher than that of pump-1. However, Banda and Werb (1981) have described a metalloproteinase of M_r 22 000, termed macrophage elastase. This protein is secreted by the macrophage cell line WEHI-3 (Z. Werb, personal communication). We have attempted to detect a WEHI-3 mRNA capable of hybridizing to the pump-1 cDNA without success (data not shown) and tentatively conclude that pump-1 is not macrophage elastase.

The postpartum rat uterus is a site of very rapid connective tissue resorption [for a review, see Mullins and Rohrlich (1983)]. Within 4–6 days, the term uterus is reduced in weight by around 80% and regains its prepregnancy size, the most dramatic losses in weight occurring in the first 2-days postpartum. As well as a uterine collagenase (Woessner, 1962; Tyree et al., 1980; Halme et al., 1980; Roswit et al., 1983), a neutral metalloproteinase whose molecular weight, estimated by gel filtration, was 24 000 has been partially purified from this source (Sellers & Woessner, 1980). It seemed to us that this metalloproteinase might be uterine pump-1, and so we decided to search for pump-1 mRNA in involuting rat uteri. Total RNA was extracted from the uteri of rats ~5-days prepartum, and 1- or 5-days postpartum. The RNA was analyzed by Northern blotting using nick-translated pump-1 (Muller et al., 1988) cDNA as probe. The pump-1 cDNA probe detected a 1.2-kb RNA in uterine samples (Figure 8) which comigrated with pump-1 RNA from human tumor samples (data not shown). The sizes of transin–stromelysin

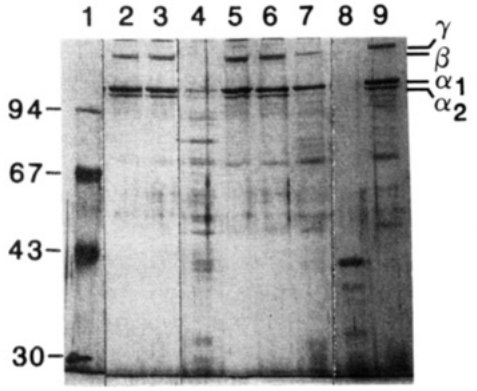

FIGURE 6: Inhibitors of pump-1. Proteins secreted by cos cells transfected with pPAPP (lanes 4–7) or pPA-TRC (lane 2) were exposed to IgG–Sepharose. Bound material was eluted with APMA and incubated for 20 h with type I gelatin. Stromelysin was assayed in parallel (lanes 8 and 9). Inhibitors were added as follows: 5 mM EDTA (lane 5), 5 mM 1,10-phenanthroline (lane 6), or 0.26 unit of tissue inhibitor of metalloproteinases (lanes 7 and 9). Samples were analyzed by SDS–PAGE (8% gel) and silver staining. The α_1 and α_2 chains of gelatin as well as the dimers (β) and trimers (γ) thereof are identified. Lane 3 = gelatin exposed to buffer alone. Lane 1 = molecular weight standards; $M_r \times 10^{-3}$ is shown.

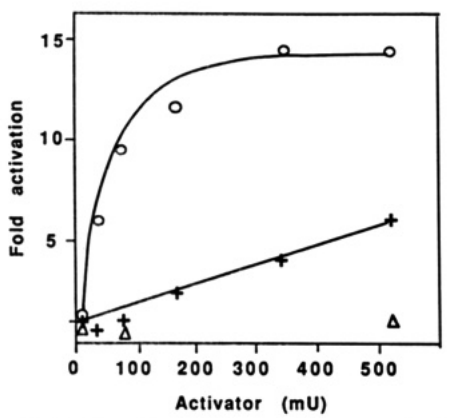

FIGURE 7: Activation of procollagenase by pump-1. Samples of procollagenase were incubated with various amounts of active pump-1 (represented by the APMA eluate of an IgG–Sepharose column exposed to proteins secreted by cos cells transfected with pPAPP), or equivalent amounts of eluate from an identical experiment using pPA-TRC or equivalent amounts of stromelysin, for 2 h at 37 °C. Collagenase activity was then assayed using ^{14}C-labeled type I collagen. Results are expressed as the *x*-fold activation observed relative to the activity elicited with APMA alone. (+) Pump-1 samples; (Δ) PA-TRC samples; (O) stromelysin samples.

and collagenase mRNAs are greater than 1.8 kb. Furthermore, under the hybridization conditions used, the pump-1 cDNA will not cross-hybridize with transin or collagenase mRNAs, conditions of low stringency being necessary to observe such cross-hybridization (Muller et al., 1988). The 1.2-kb RNA cannot therefore be collagenase or transin RNA. Blots were reprobed with cDNAs corresponding to two glycolytic enzymes, lactate dehydrogenase and enolase. Blots were scanned densitometrically, and the ratio of the pump-1 signal to that of the enolase signal was calculated for the various samples. Relative expressions were derived by comparing the ratio for a given sample to that of the 5-day preparturient sample. As shown in Figure 8, elevated levels of pump-1 RNA can be detected in uteri 1-day postpartum (relative expression 10.8) but have fallen to low levels by 5-days postpartum (relative expression 0.6). The temporal expression of pump-1 mRNA correlates well with the process of involution, with low levels prepartum or 5-days postpartum and high levels 1-day postpartum.

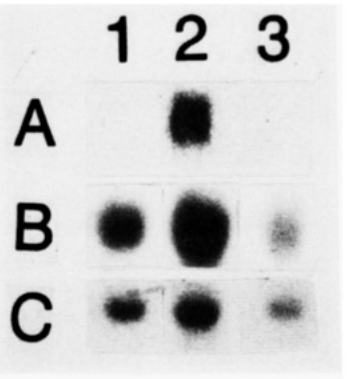

FIGURE 8: Detection of pump-1 mRNA in involuting rat uteri. Total RNA was extracted from rat uteri at the following stages: animals 5-days prepartum (lane 1) or 1-day (lane 2) or 5-days (lane 3) postpartum. RNA samples (10 μg) were subjected to gel electrophoresis; transfer to Hybond-N membranes and hybridization with nick-translated pump-1 (A), enolase (B), or lactate dehydrogenase (C) probes.

Discussion

We have described elsewhere the isolation of a cDNA corresponding to pump-1, a putative metalloproteinase of the collagenase family (Muller et al., 1988). Pump-1 lacks the hemopexin-like domain characteristic of this family of enzymes. We show here that pump-1 is nonetheless a bona fide member of the collagenase family, sharing characteristics of stromelysin and collagenase such as latency, activation by organomercurials, or inhibition by TIMP or metal chelators. Pump-1 is clearly more closely related to stromelysin than to collagenase in terms of substrate specificity (it digests readily casein, for example). However, pump-1 is not identical with stromelysin in terms of proteinase activity: while, like stromelysin, pump-1 will digest casein, fibronectin, and gelatins of types I, III, IV, and V, it will not digest collagens of type III or IV, and is a less efficient collagenase activator than stromelysin. Nonetheless, it is worthwhile pointing our that had pump-1 been purified by classical means it would have been difficult to prove that it was not one of the multiple low molecular weight forms of stromelysin (Chin et al., 1985). This sort of problem clearly does not arise when using material expressed from a specific, known cDNA. We have, as a consequence, been able to demonstrate that low molecular weight matrix metalloproteinases are not necessarily degradation products of larger enzymes.

It appears that at least three different human genes code for metalloproteinases with a stromelysin-like specificity: genes for stromelysin, stromelysin-2, and pump-1 (Muller et al., 1988; R. Nicholson, G. Murphy, and R. Breathnach, unpublished observations). The reason for this multiplicity of genes may be linked to the observation that in the rat embryo fibroblast system the two stromelysin-like genes, termed transin and transin-2 (Breathnach et al., 1987), are controlled differently. For example, the transin gene but not the transin-2 gene is induced in quiescent fibroblasts by epidermal growth factor. Both genes can be activated by ras oncogenes in fibroblasts. The different enzymes may thus have similar roles but be expressed under different, defined circumstances.

Comparison of pump-1 with stromelysin suggests that the hemopexin-like domain of the latter (Fini et al., 1987; Matrisian et al., 1986a) (and which is absent from pump-1) is not necessary to maintain it in an inactive conformation, to bind TIMP, or for enzyme activity against purified extracellular matrix components such as fibronectin or gelatin. A truncated form of APMA-activated rabbit stromelysin

(proteoglycanase of M_r 24 000) was originally purified from rabbit bone (Galloway et al., 1983). From N-terminal amino acid sequence data (Whitham et al., 1986), it was deduced to have lost the C-terminal hemopexin-like domain but still had the ability to form stable TIMP complexes and degrade matrix macromolecules. Similarly, the M_r 28 000 form of human stromelysin (Okada et al., 1986) is known to be the N-terminal portion of the M_r 45 000 enzyme and retains almost identical properties (G. Murphy and P. Koklitis, unpublished observations). The role, if any, played by the hemopexin-like domain remains unclear [see also Sanchez-Lopez et al. (1988)], though it may perhaps be of importance in fixing the enzyme at its site of action in vivo, where the structure of the extracellular matrix is more complex. Alternatively, the hemopexin-like domain may play a role in disposal of enzyme–inhibitor complexes, if these are phagocytosed as hypothesized previously (Murphy & Reynolds, 1985) [hemopexin itself is involved in disposal of circulating heme (Takahashi et al., 1985)].

We have been able to detect pump-1 RNA in involuting rat uterus, where its temporal expression correlates well with the process of involution. It is possible that pump-1 corresponds to a low molecular weight metalloproteinase partially characterized previously (Sellers & Woessner, 1980) from this source. Indeed, this enzyme has recently been purified and shown to be a latent metalloproteinase of M_r 28 000 with an APMA-activated counterpart of M_r 19 000 (Woessner & Taplin, 1988). The active enzyme digests casein, fibronectin, and gelatins of types I, III, IV, and V but not the corresponding native collagens. The enzyme level rises 5-fold from 1-day prepartum to 1-day postpartum and rapidly declines by 5-days postpartum (Sellers & Woessner, 1980). It is clear that the properties of pump-1 and the uterine metalloproteinase (termed matrix metalloproteinase 7) are quite similar, and we suggest that the pump-1 cDNA codes for human matrix metalloproteinase 7. In any case, the association of pump-1 with a system where extensive tissue degradation is known to take place is consistent with the proteinase activity of pump-1. Pump-1 can function both as a collagenase activator and by degrading directly other noncollagen components of the connective tissue.

Further studies of the control of expression of the pump-1 gene during involution would be assisted by knowing the cell type which is expressing the mRNA. It has been suggested that the elevated levels of metalloproteinases observed in postpartum uteri may be produced by infiltrating cells (Sellers & Woessner, 1980; Woessner, 1980). The same phenomenon could of course explain expression of pump-1 RNA in human tumors (Muller et al., 1988). The use of in situ hybridization techniques with pump-1 cDNA and appropriate cell markers should allow us to address this question.

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Characterization of Genes Encoding Rat Tonin and a Kallikrein-like Serine Protease[†,‡]

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ABSTRACT: Tissue kallikreins are a group of serine proteases which may function as peptide hormone processing enzymes. Two rat kallikrein genomic clones (RSKG-5 and RSKG-50) were sequenced and characterized. The rat tonin gene and a kallikrein-like gene were found in clones RSKG-5 and RSKG-50, respectively. The tonin gene is 4146 base pairs in length, with both the variant CCAAA and TTTAAA boxes in the 5′-end region and an AATAAA polyadenylation signal at the 3′ end of the gene. It has five exons which are separated by four introns. Sequence analysis of 3.7-kb 5′ upstream and 7.5-kb 3′ downstream of the tonin gene failed to reveal a second kallikrein gene. Sequence comparisons of the RSKG-5 exons with tonin cDNA revealed that only one base in the 3′-noncoding region was different from that in the previously reported rat tonin cDNA. Characteristic TC- and TG-repeated sequences were also found in the first and second introns of the tonin gene. The tonin gene encodes a preprotonin of 259 amino acids (aa). The active enzyme consists of 235 aa and is preceded by a deduced signal peptide of 17 aa and a profragment of 7 aa. Northern blot analysis indicates that RSKG-5 is expressed in a sex-dependent manner in rat submandibular gland, with a higher level expressed in males. The RSKG-50 gene was truncated at an *Eco*RI site in the second intron, excluding its 5′ end. Compared to the coding sequence of pancreatic kallikrein, 12 nucleotides have been deleted in exon 3 of the RSKG-50 gene. The nucleotide sequences of the third, fourth, and fifth exons of the RSKG-50 gene encode a polypeptide of 188 aa residues. The translated peptide is 80% homologous to rat pancreatic kallikrein and 75% homologous to rat tonin in the corresponding regions. Key residues in the RSKG-50 gene product indicate a serine protease with kallikrein-like cleavage specificity at basic amino acids.

Tissue kallikrein belongs to a multigene family coding for a subgroup of serine proteases which are involved in the processing of bioactive peptides (Schachter, 1980; Mason et al., 1983). These enzymes including pancreatic kallikrein, tonin, arginine esterase A, γ subunit of nerve growth factor, and epidermal growth factor binding protein (Bothwell et al., 1979; Chao, 1983; Chao et al., 1984; Swift et al., 1982; Mason et al., 1983; Ashley & MacDonald, 1985a,b; Gerald et al., 1986a) show a high degree of amino acid and nucleotide sequence homology. The structural and functional similarities of the kallikreins have hindered the identification and definition of their physiological roles. Therefore, molecular biology approaches have been implemented to analyze kallikrein gene structure, organization, function, and expression in mammalian systems (Mason et al., 1983; Gerald et al., 1986a,b).

Tonin (EC 3.4.99.–), a member of the rat tissue kallikrein family, produces angiotensin II from angiotensinogen, angiotensin I, or the tetradecapeptide renin substrate (Boucher et al., 1972). Angiotensin II is the most potent vasoconstrictive peptide known. It maintains arterial pressure in peripheral arterioles, modulates renin release in the kidney, and stimulates secretion of aldosterone in the adrenal cortex. Tonin has been shown to cleave proopiomelanocortin to produce adrenocorticotropin hormone (ACTH)[1] and to yield opiate-like peptides from β-lipotropic hormone (β-LPH) (Seidah et al., 1979a,b) and to degrade substance P (Chretien et al., 1980). Moreover, tonin has been reported to have kininogenase activity and can produce bradykinin from both high and low molecular weight kininogens (Ikada & Arakawa, 1984). In addition, tonin was shown to have the capability to activate renin from prorenin (Gutkowska et al., 1982). In these proteolytic activities, tonin prefers to cleave the substrate selectively at Phe– or Arg–peptide bonds (Seidah et al., 1979a,b; Chretien et al., 1980), giving tonin both trypsin- and chy-

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[1] Abbreviations: ACTH, adrenocorticotropin hormone; β-LPH, β-lipotropic hormone; aa, amino acid(s).

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