Sheep mast cell proteinase-1, a serine proteinase with both tryptase- and chymase-like properties, is inhibited by plasma proteinase inhibitors and is mitogenic for bovine pulmonary artery fibroblasts

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Sheep mast cell proteinase-1 (sMCP-1), a serine proteinase with dual chymase/tryptase activity, is expressed in gastrointestinal mast cells, and released systemically and on to the mucosal surface during gastrointestinal nematode infection. The potential for native plasma proteinase inhibitors to control sMCP-1 activity was investigated. Sheep α_1-proteinase inhibitor (α_1PI) inhibited sMCP-1 slowly, with second-order association rate constant (k_{ass}) 1.1×10^3 $M^{-1} \cdot s^{-1}$, whereas sheep contrapsin inhibited trypsin (k_{ass} 2.2×10^6 $M^{-1} \cdot s^{-1}$) but not sMCP-1. Western-blot analysis and gel filtration showed that when added to serum or plasma, sMCP-1 was partitioned between α_1PI and α_2-macroglobulin. The possibility that significant cleavage of plasma proteins could occur before sMCP-1 was inhibited was investigated using gel filtration and SDS/PAGE after adding sMCP-1 to plasma. Cleavage of ovine fibrinogen occurred in the presence of excess α_1PI and α_2-macroglobulin, the α-chain being cleaved C-terminally and the β-chain at the putative Lys-27. In addition, sMCP-1 was found to be mitogenic for bovine pulmonary artery fibroblasts, but was not mitogenic in the presence of soya-bean trypsin inhibitor. In terms of fibrinogen cleavage and fibroblast stimulation, sMCP-1 shows functional similarities to mast cell tryptase.

INTRODUCTION

Sheep mast cell proteinase-1 (sMCP-1) is a member of the mast cell chymases, a group of serine proteinases with broadly chymotrypsin-like character. Other chymases have been described in human, rat, mouse and canine mast cells [1–4]. sMCP-1 is expressed in ovine gastrointestinal mucosal mast cells (MMCs) [5], and is unusual in that it has been found to cleave some substrates with trypsin-like specificity [6].

The release of MMC chymases into the circulation and into the gut lumen occurs in rodents and sheep during nematode infection or after the induction of systemic anaphylaxis [7]. One of the most likely functions of MMC chymases is to increase epithelial permeability, since mouse mast cell proteinase-1 (mMCP-1) and sMCP-1 are detected in the lumen of the gut during nematode infections [8,9]. Furthermore, using an *ex vivo* perfusion system, the introduction of rat mast cell proteinase-2 (rMCP-2) into the mesenteric artery of rats caused increased jejunal mucosal permeability within minutes [10]. Increased mucosal permeability is thought to facilitate the translocation of anti-worm antibodies into the gut lumen [7].

Our finding that sMCP-1 has trypsin-like properties, in addition to chymase activity, suggests that it may have some functions in common with tryptase. Human tryptase is a tetrameric serine proteinase, which is stabilized by strong electrostatic interactions with proteoglycans [11]. Some of the known functions of tryptase are fibrinogen degradation [12], prostromelysin activation [13] and mitogenic activity for fibroblasts [14], the latter activity not being observed with chymase [14]. Human tryptase is not inhibited by plasma serine proteinase inhibitors (serpins), nor by α_2-macroglobulin (α_2M), and remains active after prolonged incubation in plasma [15]. This is in contrast with our previous studies on the MMC chymases rMCP-2 and mMCP-1, which suggest that α_1-proteinase inhibitor (α_1PI) is highly effective in inhibiting both proteinases [16,17].

It was therefore of interest to determine whether sMCP-1, which, like rMCP-2 and mMCP-1, is abundantly expressed in gastrointestinal MMCs [7], might be regulated in the same way, or like tryptase, be unaffected. In addition, sMCP-1, like tryptase [14], is shown to be mitogenic for pulmonary fibroblasts.

MATERIALS AND METHODS

Materials

SMCP-1 [5], equine neutrophil elastase [18] and rMCP-2 [19] were prepared as described previously. Bovine pancreatic trypsin was supplied by Boehringer-Mannheim. HPLC-grade solvents were obtained from Rathburn Chemicals Ltd., Walkerburn, Scotland, U.K. All other solvents and Coomassie Brilliant Blue R250 were obtained from BDH Laboratory Supplies Ltd. Unless otherwise stated, all chromatography columns and stationary phases were supplied by Pharmacia Biotech Ltd. All other reagents were purchased from Sigma Chemical Co.

Electrophoresis and N-terminal sequencing of proteins

SDS/PAGE was performed using the Mini-Protean II format (Bio-Rad), following the manufacturer's protocols. Samples for sequencing were separated by SDS/PAGE, using 10% acrylamide Tris/Tricine gels [20], then transferred to Immobilon-P membrane (Millipore) by semi-dry blotting with 10 mM 3-

Abbreviations used: sMCP-1, sheep mast cell proteinase-1; rMCP-2, rat mast cell proteinase-2; mMCP-1, mouse mast cell proteinase-1; MMC, mucosal mast cell; PBS/Brij, PBS containing 0.1% Brij 35; Suc, succinyl; STI, soya-bean trypsin inhibitor; α_1PI, α_1-proteinase inhibitor; α_1AC, α_1-antichymotrypsin; α_2M, α_2-macroglobulin; serpin, serine proteinase inhibitor.

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cyclohexylamino-1-propanesulphonic acid in 10% methanol buffer at 75 mA/minigel for 1 h. Blots were stained with Coomassie Brilliant Blue R250 and bands of interest were sequenced by B. Dunbar, University of Aberdeen Protein Sequencing Facility, Aberdeen, Scotland, U.K., and P. Barker, Microchemical Facility, Babraham Institute, Cambridge, U.K.

Inhibitory effect of serum on sMCP-1, rMCP-2 and chymotrypsin

To dilutions (neat to 1:20) of sheep serum in PBS containing 0.1% Brij 35 (BPS/Brij; 40 μl) were added 10 μl of sMCP-1 (10.75 μM), rMCP-2 (18.8 μM) or chymotrypsin (18.0 μM). The mixtures were incubated at 25 °C for 24 min and residual activity with Suc-Ala-Ala-Pro-Phe-nitroanilide substrate was determined.

Purification of proteinase inhibitors from sheep serum

The following protocol was used to purify sheep α_1PI [21] and the serpin previously termed α_1PI [22] but now recognized as sheep contrapsin [23]. Sheep α_2M was also purified.

Ovine serum (9 ml) was fractionated on Blue Sepharose 6 Fast Flow (430 ml; 50 mm × 220 mm; Pharmacia), equilibrated in 50 mM Tris/HCl, pH 7.5. The small first peak (high-M_r-exclusion peak) contained α_2M, and the larger later peak contained the serpins of interest; the majority of ovine serum albumin remained bound to the column. $(NH_4)_2SO_4$ (solid) was added to the exclusion peak at 4 °C to 50% saturation. The precipitate recovered by centrifugation was buffer-exchanged into 20 mM Tris/HCl, pH 7.5, and further purified by anion-exchange chromatography on Mono Q in 20 mM Tris/HCl, pH 7.5, with a 0–240 mM NaCl gradient. Fractions were monitored by reducing SDS/PAGE, and those containing a single band at approximate M_r 180000 were pooled as pure α_2M.

The main peak from Blue Sepharose chromatography of ovine serum was treated with solid $(NH_4)_2SO_4$ at 4 °C to give 50% saturation, and the supernatant after centrifugation was raised to 80% saturation by further addition of $(NH_4)_2SO_4$. The solids obtained by centrifugation of the 50–80% preparation were redissolved, then desalted into 20 mM Tris/HCl, pH 7.5. Anion-exchange chromatography (Mono Q) was then performed on the preparation in 20 mM Tris/HCl, pH 7.5, with a 0–240 mM NaCl gradient. Fractions were monitored for trypsin-inhibitory capacity by incubating 50 μl of trypsin solution (30 μg/ml in 50 mM Tris/HCl, pH 7.5) with 10 μl of each sample for 5 min in microtitre plate wells, then adding 10 μl of substrate (10 mM *N*-Cbz-Arg-nitroanilide in DMSO). Inhibitory fractions were pooled and diluted 1:2 with 20 mM Tris/HCl, pH 8.5, for further chromatography on Mono Q at pH 8.5, with a 0–320 mM NaCl gradient. Fractions were monitored by reducing SDS/PAGE, and those containing a protein of apparent M_r 55000 were combined and rechromatographed until a homogeneous α_1PI preparation was obtained. Partial purification of a second serpin (sheep contrapsin; apparent M_r 123000 by SDS/PAGE) was also achieved during the pH 8.5 separation, being eluted before α_1PI.

Inhibitor kinetics: determination of association constant

sMCP-1 was active-site-titrated by the method of Jameson et al. [24] for chymotrypsin-like enzymes. The Spi1 variant of equine α_1PI was purified from horse plasma known to be homozygous for the I haplotype of equine α_1PI by the method of Pemberton et al. [25]. Equine elastase was active-site-titrated at pH 2.2 with Cbz-Ala-nitrophenyl ester, by the method of Kezdy and Kaiser [26], and used to titrate equine α_1PI. This inhibitor was used in titrations to determine the active-site concentrations of bovine pancreatic trypsin, bovine pancreatic chymotrypsin and rMCP-2. The molarities of sheep α_1PI and sheep contrapsin were determined by titration against standardized bovine pancreatic trypsin stock.

For each determination, the proteinase and inhibitor were diluted in PBS/Brij to approximately equal concentration. To determine the association rate constant, typically 20 μl aliquots of inhibitor solution were added to 20 μl of proteinase in a semi-micro polystyrene cuvette (BDH) and incubated for different lengths of time at 25 °C. After incubation, 140 μl of PBS/Brij was added, followed by 20 μl of the appropriate 4-nitroanilide substrate solution (10 mM in DMSO: Suc-Ala-Ala-Pro-Phe-nitroanilide for chymotrypsin, rMCP-2 and sMCP-1; MeOSuc-Ala-Ala-Pro-Val-nitroanilide for equine elastase; Cbz-Arg-nitroanilide for trypsin). The cuvette was agitated to mix well, then the rate of change of A_{405} was measured over 1–2 min (Beckman DU 650 spectrophotometer). The experimental data were fitted to the theoretical model as described by Boudier and Bieth [27].

Analysis of sMCP-1–α_1PI complex for SDS/PAGE

sMCP-1 (2 μl of 0.5 mg/ml in PBS/Brij) was added to sheep α_1PI (20 μl of 0.18 mg/ml in 20 mM Tris/HCl, pH 8.5) and incubated at 20 °C for 30 min. Non-reducing sample buffer (i.e. lacking 2-mercaptoethanol) (8 μl) was added and the mixture heated at 95 °C for 3 min to denature the enzyme. Reducing SDS sample buffer (30 μl) was then added and the sample was heated for a further 3 min at 95 °C, followed by analysis by SDS/PAGE.

Rabbit-anti-sMCP-1 polyclonal antibody

This was raised and affinity-purified as described previously [5], and its specificity confirmed by Western blotting and immunohistochemistry as described [5].

Western-blot analysis of sMCP-1 in the presence of serum, α_1PI and α_2M

sMCP-1 (3 μl of 0.5 mg/ml in PBS/Brij) was incubated variously with sheep serum (1 μl), sheep α_1PI (21 μl of 0.22 mg/ml in PBS) or sheep α_2M (25 μl of 0.28 mg/ml in 20 mM Tris/HCl, pH 7.5) for 30 min at 25 °C. After incubation, 10% SDS was added to the mixtures (and to controls lacking sMCP-1), to a total volume of 50 μl, which were then heated at 95 °C for 3 min. Samples were treated for SDS/PAGE with an equal volume of reducing sample buffer, and after electrophoresis of 10 μl aliquots, were electroblotted on to Immobilon-P membrane as described above. The blot was probed with affinity-purified rabbit anti-sMCP-1 IgG (see above; 4 μg/ml), followed by alkaline phosphatase-conjugated monoclonal mouse anti-rabbit IgG (Sigma; diluted 1 in 20000). Finally, the blot was developed with 5-bromo-4-chloro-3-indolyl phosphate/NitroBlue Tetrazolium colour development substrate (Promega).

Analysis of plasma treated with sMCP-1

Sheep plasma (100 μl) was diluted with PBS (90 μl), then 10 μl of PBS or sMCP-1 (0.8 mg/ml) was added, and the mixture incubated at 20 °C for 15 min. Since the plasma concentration of sheep α_1PI is approx. 1.6 mg/ml [21], the incubation mixture contained an estimated 10-fold molar excess of α_1PI over sMCP-1. The incubation mixture was then injected on to a gel-filtration column (Superose 12), eluted with PBS containing 3.8% trisodium citrate at 1 ml/min, and fractions of 1 ml collected. All fractions were analysed by SDS/PAGE and for residual pro-

teinase activity. The fraction containing degraded fibrinogen in the plasma + sMCP-1 incubation was separated by SDS/PAGE and blotted on to Immobilon-P membrane. Resolved fibrinogen bands were cut out for N-terminal amino acid sequencing.

Bovine pulmonary artery fibroblast culture

Fibroblast cultures were isolated from pulmonary arteries of adult cows using a primary explant procedure [28]. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin and maintained at 37 °C in a humidified atmosphere of 95% air/5% CO_2. Fibroblasts were utilized for experiments at passage levels 4–10 and rendered quiescent in serum-free Dulbecco's modified Eagle's medium for 48 h before treatment.

Measurement of DNA synthesis

Quiescent fibroblasts in triplicate wells of 24-well plates were incubated with agonists for 24 h. [*methyl*-^{3}H]Thymidine was added for the final 4 h of the incubation to give a final concentration of 0.1 μCi/ml. After 24 h, cells were washed twice with PBS, three times with 5% trichloroacetic acid and twice with ethanol. The trichloroacetate-insoluble material was solubilized in 0.3 M NaOH, and the level of incorporation of [^{3}H]-thymidine determined by liquid-scintillation counting.

RESULTS

Titration of chymases with sheep serum

In order to determine whether sMCP-1, like the rodent MMC chymases, is inhibited by plasma serpins [16,17], the inhibitory effects of sheep serum on sMCP-1, rMCP-2 and chymotrypsin were compared. The addition of serum at increasing concentrations to the rat mast cell chymase rMCP-2 resulted in complete inhibition of the activity of this enzyme towards the nitroanilide substrate used. However, in the cases of chymotrypsin and sMCP-1, the residual amidolytic activity could not be reduced to less than approx. 10 and 20% respectively (Figure 1).

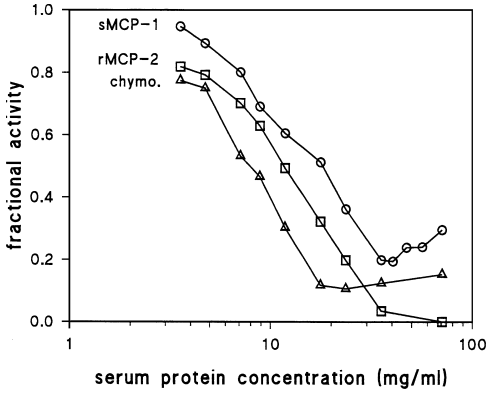

Figure 1 Inhibition of sMCP-1, rMCP-2 and chymotrypsin by addition of sheep serum

Dilutions of serum were added to sMCP-1 (10.75 μM), rMCP-2 (18.8 μM) or chymotrypsin (chymo.; 18.0 μM). After incubation at 25 °C for 24 min, residual hydrolytic activity was measured spectrophotometrically after the addition of Suc-Ala-Ala-Pro-Phe-4-nitroanilide. Fractional activity is plotted against serum protein concentration in each incubation.

Purification of sheep serum proteinase inhibitor

To characterize the native inhibitors of sMCP-1, three of the important proteinase-inhibitory components of ovine serum were purified. The α_2M-like protein was shown by SDS/PAGE (Figure 2) to have a subunit M_r of approx. 177000, and had substantial N-terminal sequence homology (> 50%) with human α_2M (Figure 3). Both α_1PI and contrapsin [21,22] were purified. The serpin with the lower M_r (55000 by SDS/PAGE; Figures 2 and 4) was analogous to the α_1PI described in other species in that it inhibited trypsin, chymotrypsin and leucocyte elastase (Table 1), and had an N-terminal sequence identical with that reported by Mistry et al. [21] (Figure 3). The other serpin (apparent M_r = 123000 by SDS/PAGE; Figure 2) was larger than reported by Sinha et al. [22] (M_r 62000), but had the same inhibitory specificity for trypsin and an identical N-terminal sequence over the first 10 residues (Figure 3). This protein, initially described by Sinha et al. [22], was more recently identified as sheep contrapsin by comparison with goat contrapsin (M_r 60000) [23], which readily forms dimers as an artifact of SDS/PAGE analysis.

Inhibition kinetics

The purified sheep α_1PI and the equine α_1PI elastase inhibitor (Spi1) were similar in their second-order association rate constants with a range of serine proteinases. The order of k_{ass} was the same in both: neutrophil elastase > chymotrypsin > rMCP-2 > trypsin > sMCP-1 (Table 1). Importantly, both were rather slow inhibitors of sMCP-1. Contrapsin did not inhibit sMCP-1 or chymotrypsin (Table 1), but was a very efficient inhibitor of trypsin ($k_{ass} = 2.2 \times 10^6$ M$^{-1} \cdot$ s^{-1}), as has been described for goat contrapsin ($k_{ass} = 1.9 \times 10^6$ M$^{-1} \cdot$ s^{-1}) [23].

SDS/PAGE analysis of sMCP-1–α_1PI complex-formation

Incubation of sMCP-1 with α_1PI resulted in the formation of a complex band at approximate M_r 80000 by SDS/PAGE (Figure 4). Minimal degradation of complex and/or residual α_1PI occurred, as observed with rMCP-2-rat serpin incubations [16], and in contrast with human chymase and rat mast cell proteinase-1, which both substantially hydrolysed α_1PI [16,30].

Western-blot analysis of sMCP-1 in the presence of inhibitors

In order to determine which inhibitors complexed with sMCP-1 in ovine serum, the chymase was added as described in the Materials and methods section to serum, to purified α_1PI and to purified α_2M. The blots (Figure 5) showed three sMCP-1-containing complex bands. One band of approximate M_r 80000 was found when sMCP-1 was incubated with serum and also with purified α_1PI. Another two bands, of approximate M_r 155000 and > 205000, were seen in incubations of sMCP-1 with serum and with purified α_2M (Figure 5). The same results were observed when plasma was used in place of serum (results not shown).

Plasma protein degradation by sMCP-1

Because sMCP-1 was inhibited slowly by α_1PI (k_{ass} 1.1×10^3 M$^{-1} \cdot$ s^{-1}), it was possible that, when released systemically or into the tissues, sMCP-1 might exert significant proteolytic activity. Therefore sMCP-1 was added to plasma at a dilution

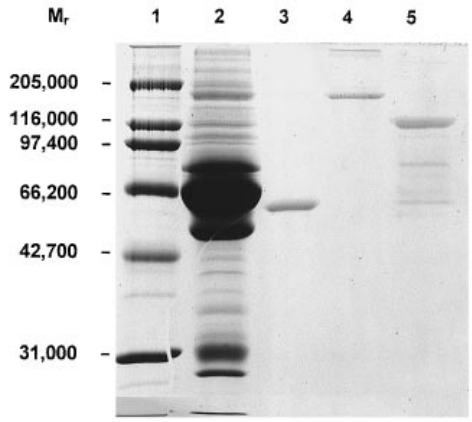

Figure 2 SDS/polyacrylamide gel showing inhibitors purified from sheep serum

Lane 1, M_r standard (Sigma); lane 2, sheep serum (0.25 μl); lane 3, sheep α_1PI (0.5 μg); lane 4, sheep α_2M (0.5 μg); lane 5, sheep contrapsin (1.0 μg). The gel was stained with 0.25% Coomassie Brilliant Blue R250.

	1 10 20
sheep α_1PI	G V L Q G H A V Q E T D D T S H Q E A A
sheep α_1PI (Mistry *et al.*)	G V L Q G H A V Q E T D D T S H Q E A A
sheep contrapsin	L P E N V V V K D Q
sheep serpin (Sinha *et al.*)	L P E N V V V K D Q D R R A S V D D L A
human α_2M	S V S G K P Q Y M V L V P S L L H
sheep α_2M	– V S x E P Q Y M V L V P x x L x

Figure 3 N-Terminal amino acid sequences of inhibitors isolated from sheep serum

The α_1PI- and contrapsin-like inhibitors are compared with the sheep serpin sequences reported by Mistry et al. [21] and Sinha et al. [22] respectively. The partial sheep α_2M N-terminal sequence is aligned with that deduced for the human protein [29]. ×, Residues that could not be assigned; —, spaces inserted to improve sequence alignment.

ensuring inhibitor excess (see the Materials and methods section), which was confirmed after Superose 12 fractionation of the plasma, where residual chymotrypsin-like activity was associated only with the excluded peak containing α_2M (results not shown). The only major change detected by SDS/PAGE of all the Superose 12 fractions was in the fraction containing fibrinogen. In the fibrinogen subunit pattern (Figure 6), the intensity of the α-chain band was much reduced. A band remained in the position of the β-chain (approximate M_r 59000), but amino acid sequencing showed that this contained the N-termini of both α- and β-chains, indicating C-terminal cleavage of the α-chain. The band that appeared at approximate M_r 56000 had an N-terminal sequence (Figure 7) corresponding to a conserved region of the β-chain, indicating cleavage of a peptide of approx. 27 residues from the N-terminus of the β-chain. The γ-chain was uncleaved at the N-terminus, and was found to be superimposed on N-terminal sequence for the α-chain, indicating further C-terminal cleavage of fibrinogen α-chain.

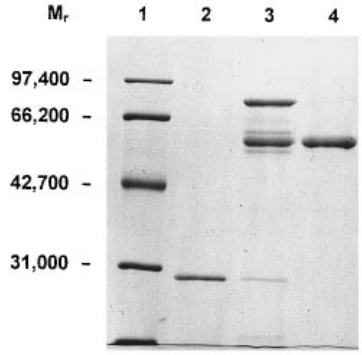

Figure 4 SDS/PAGE analysis of sMCP-1–α_1PI complex

SDS/10% polyacrylamide gels were used. Lane 1, M_r (Bio-Rad); lane 2, sMCP-1 (0.25 μg); lane 3, sMCP-1 (0.25 μg) + sheep α_1PI (0.88 μg); lane 4, sheep α_1PI (0.88 μg). Details of sample preparation are given in the Materials and methods section.

Table 1 Second-order association rate constants of α_1PIs with a range of serine proteinases

Association rate constants (k_{ass}) were determined as described in the Materials and methods section. N.I., negligible inhibition (estimated $k_{ass} < 10^2\ M^{-1} \cdot s^{-1}$); N.D., not done.

	k_{ass} ($M^{-1} \cdot s^{-1}$)		
Proteinase	Sheep α_1PI	Horse α_1PI (Spi1)	Sheep contrapsin
sMCP-1	1.1×10^3	7.5×10^2	N.I.
rMCP-2	1.2×10^6	$2.2 \times 10^{5*}$	N.D.
Bovine pancreatic chymotrypsin	1.3×10^6	1.7×10^6	N.I.
Bovine pancreatic trypsin	5.0×10^4	9.2×10^4	2.2×10^6
Equine neutrophil elastase 2A	1.7×10^7	$1.9 \times 10^{7*}$	N.D.

* Data taken from [25].

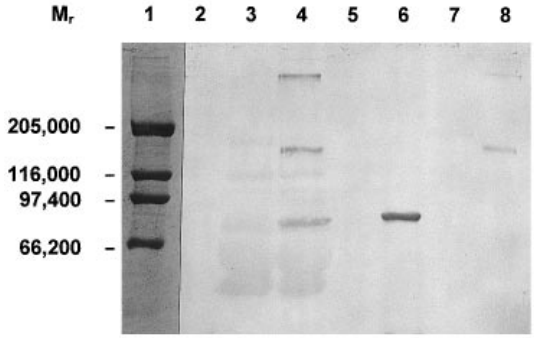

Figure 5 Western-blot analysis of sMCP-1 in the presence of inhibitors

The following samples were separated by SDS/PAGE (7.5%); lane 1, M_r standards (Sigma); lane 2, sMCP-1 (0.15 μg); lane 3, sheep serum (0.1 μl); lane 4, sheep serum + sMCP-1 (0.1 μl + 0.15 μg respectively); lane, 5, sheep α_1PI (0.46 μg); lane 6, sheep α_1PI + sMCP-1 (0.46 μg + 0.15 μg respectively); lane 7, sheep α_2M (0.70 μg); lane 8, sheep α_2M + sMCP-1 (0.70 μg + 0.15 μg respectively). Incubations with sMCP-1 were carried out at 25 °C for 30 min before SDS/PAGE separation and anti-sMCP-1 Western blotting, as described in the Materials and methods section. Under these conditions, free sMCP-1 ran off the gel, but complexed sMCP-1 was readily detected in lanes 4, 6 and 8 by Western blotting.

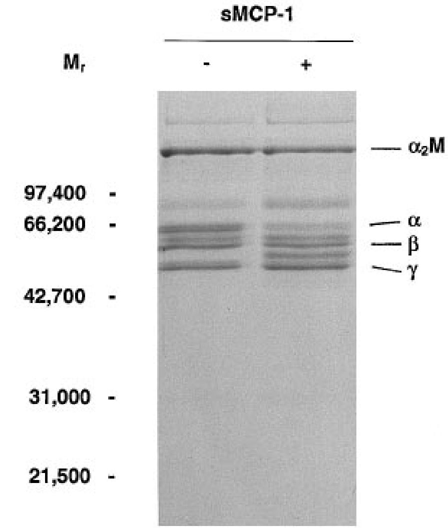

Figure 6 SDS/polyacrylamide gel of fibrinogen-containing fractions from plasma with and without preincubation with sMCP-1

Samples (2 μl) of excluded fractions from Superose 12 gel filtration of plasma incubated with and without sMCP-1 were analysed on a 10% gel. The lane marked 'sMCP-1 — ' was from untreated plasma. The lane marked 'sMCP-1 + ' was from plasma (100 μl) treated with sMCP-1 (10 μl of 0.8 mg/ml).

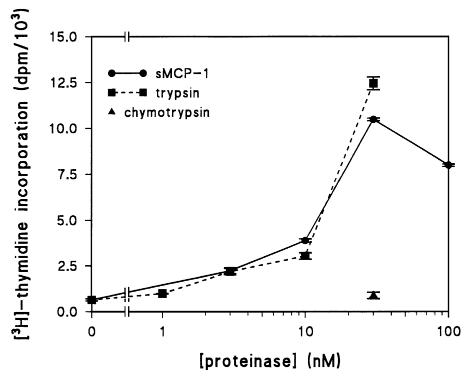

Figure 8 Concentration-dependence of sMCP-1 (●)- and trypsin (■)-stimulated DNA synthesis in bovine pulmonary artery fibroblasts

A chymotrypsin control (30 nM) is indicated (▲). Each point is the mean ± S.E.M. of triplicate measurements for one typical experiment.

DNA synthesis in pulmonary artery fibroblasts

sMCP-1 stimulated the incorporation of [^{3}H]-thymidine in a dose-dependent manner (Figure 8). This experiment was repeated on three occasions with similar results. Trypsin also stimulated incorporation, optimally at approx. 10–30 nM. At higher concentrations, trypsin caused detachment of fibroblasts from the plate. In order to determine whether the fibroblast stimulation required catalytically active enzyme, the effect of soya-bean trypsin inhibitor (STI) on proteinase-induced fibroblast DNA synthesis was investigated. Trypsin and sMCP-1 caused 12- and 7-fold increases respectively ($P < 0.05$) in [^{3}H]thymidine incorporation over control, and pretreatment of the proteinases with STI abrogated this effect (Figure 9). In this experiment, chymotrypsin caused a slight increase in [^{3}H]thymidine incorporation over control, but pretreatment of chymotrypsin with STI did not reduce the level of [^{3}H]thymidine uptake, indicating that this was due to an effect other than chymotryptic activity. The cells were also stimulated by 10% newborn calf serum (results not shown), but not by soya-bean trypsin inhibitor and buffer-only controls.

DISCUSSION

Chymases derived from intestinal MMCs in rodents are thought to be important mediators of gut permeability and, at the same time, are released systemically into the bloodstream, where they are effectively inhibited by serpins [16,17]. We have therefore purified α_1PI, contrapsin and α_2M from sheep serum in order to determine their interaction with sMCP-1. Two different serpins have been described as α_1PI in sheep [21,22]. One of these [21] is an inhibitor of M_r 56000 with substantial N-terminal amino acid sequence homology with and similar specificity to other mammalian α_1PI (i.e. inhibition of leucocyte elastase and trypsin). The other inhibitor, of M_r 62000, is an efficient inhibitor of trypsin but not elastase, and has a lower N-terminal sequence homology with human α_1PI [22]. This inhibitor was subsequently recognized as the sheep homologue of goat contrapsin by Potempa et al. [23]. The present study confirmed the presence of both inhibitors in sheep serum.

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                      1         2         3         4
                1         0         0      ⇓  0         0
bovine β-chain  Q F P T D Y D E G Q D D R P K V G L G A R G H R P Y D K K K E E A P S L R P V P

sheep β-chain     G Y L D Y D E V D D N R A K L P L D A R x x x x x x x ? ? E E A P S L R P

human β-chain                 Q G V N D N E E G F F S A R G H R P L D K K R E E A P S L R P A P
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Figure 7 N-Terminal sequence analysis of sheep fibrinogen β-chain cleavage

Bovine [31], sheep and human [32] fibrinogen β-chain sequences are aligned at the conserved region, C-terminal to the thrombin-cleavage site (residue 21 in bovine β-chain). The unknown residues marked × are inserted to join the known sheep fibrinopeptide B sequence [33] with the N-terminal sequence observed here for sMCP-1-cleaved sheep fibrinogen β-chain (residues marked ? were obscured by an impurity during sequencing). Bovine fibrinogen β-chain was cleaved by sMCP-1 at residue 28 (⇓) [6].

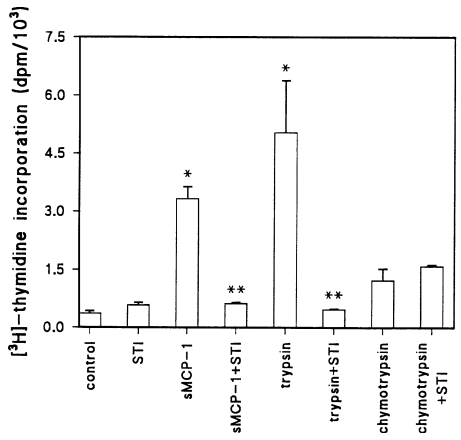

Figure 9 Effect of proteinases on [^{3}H]thymidine incorporation by bovine pulmonary artery fibroblasts

Mean values ± S.E.M. for triplicate determinations are shown. *Significant difference ($P < 0.05$) in proteinase-treated fibroblasts compared with buffer-only controls. **Significant difference ($P < 0.05$) in cells treated with STI-inhibited proteinases compared with uninhibited proteinases. sMCP-1, trypsin and chymotrypsin were used at final concentrations of 100 nM, 10 nM and 10 nM respectively with or without preincubation with STI at a concentration of 3 mg/ml for 5 min at 20 °C.

In common with human mast cell chymase [30], sMCP-1 reacted slowly ($k_{ass} = 1.1 \times 10^3\ M^{-1} \cdot s^{-1}$) with sheep α_1PI, although there was no evidence for substantial cleavage of α_1PI, as was observed with human mast cell chymase [30]. Sheep contrapsin was a very efficient inhibitor of trypsin, but it showed no appreciable rate of complex-formation with chymotrypsin or sMCP-1. The contrapsins of ruminants and rodents are a subset of serpins with closer sequence similarity to α_1-antichymotrypsin (α_1AC) than to α_1PI [23,34,35], and are good inhibitors of trypsin, but not chymotrypsin and elastase. Interestingly, goat and bovine plasma contain other structurally related serpins, termed goat elastasin [23] and bovine elastase inhibitor [34] respectively. These are very effective inhibitors of elastase and chymotrypsin, both possessing Leu-Ser at P1-P1′ in the reactive-site loop, in common with α_1AC. No sheep homologues of elastasin or α_1AC were observed during this study, but it is of note that sMCP-1 is not inhibited by human α_1AC [6], in contrast with human chymase [36].

When titrated with sheep serum, the amidolytic activity of rMCP-2 decreased to undetectable levels, as a result of the efficient inhibition by sheep α_1PI. However, the activity of sMCP-1 towards the nitroanilide substrate could not be reduced to zero by the addition of serum, even when the α_1PI concentration was in great excess, because of inhibition by the pan-specific proteinase inhibitor, α_2M. The active site of the proteinase is not affected when trapped within and covalently linked to α_2M [37], permitting the observed cleavage of low-M_r substrates.

Further evidence for sMCP-1 complexing with both α_1PI and α_2M was obtained by Western blotting of sMCP-1 to which serum, α_1PI and α_2M had variously been added. The absence of any other significant bands containing sMCP-1 immunoreactivity suggests that α_1PI and α_2M are the important inhibitors of sMCP-1 present in serum, and that sMCP-1 is partitioned between both inhibitors. A similar partition of human chymase between α_2M and serpins has been observed by Schechter et al. [30] with human serum, where the serpins included both α_1PI and α_1AC.

The addition of sMCP-1 to plasma caused degradation of fibrinogen (Figure 6), despite the presence of excess α_1PI and α_2M. Fibrinogen α-chain was degraded by cleavage of a fragment of more than M_r 5000 from the C-terminus. Hydrolysis of the β-chain was also observed, at a position corresponding to Lys-28 of bovine fibrinogen (Figure 7). This observation is supported by our finding that sMCP-1 cleaved purified bovine fibrinogen β-chain at this position [6]. Such a cleavage removes the GHR sequence (residues 22–24 of bovine β-chain), which is normally exposed when fibrinogen is cleaved by thrombin, and is important for polymerization to form fibrin. The γ-chain was uncleaved at the N-terminus, and underwent no obvious shift in M_r by SDS/PAGE.

Mast cell chymases are not reported to stimulate cell growth, so it is particularly interesting that sMCP-1, at concentrations as low as 3 nM, stimulates DNA synthesis in bovine pulmonary artery fibroblasts. Tryptase, but not chymase, from dog mast cells is mitogenic for pulmonary fibroblasts [14], which suggests that sMCP-1 may be mitogenic for bovine pulmonary artery fibroblasts by virtue of its trypsin-like properties, particularly since chymotrypsin was not mitogenic for these cells.

In summary, it has been demonstrated that the activity of sMCP-1 is controlled primarily by the serum inhibitors α_1PI and α_2M. The rate of inhibition is relatively slow, which may allow proteinase released into intercellular space sufficient opportunity to exert physiological effects by acting on a target substrate rather than being inhibited. Our *in vitro* studies strongly suggest that, when released from the cell, sMCP-1 will cleave fibrinogen with trypsin-like specificity before it can be inhibited. The observed fibrinogen cleavages by sMCP-1 are likely to affect the nature of fibrin formed near sites of mast cell activation. Equally, during this window of activity, sMCP-1 may act like tryptase in its interaction with cell-surface substrates, for example, causing fibroblast proliferation. Thus sMCP-1, in addition to having dual chymase/tryptase substrate specificity, also has physiological properties in common with both chymase and tryptase.

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