Identification of targeting proteinase for rat α_1 -macroglobulin *in vivo*

Mast-cell tryptase is a major component of the α_1 -macroglobulin–proteinase complex endocytosed into rat liver lysosomes

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The α_1 -macroglobulin–proteinase complex endocytosed into rat liver lysosomes was purified by a series of column chromatographic steps on concanavalin A–Sephadex, Sephacryl S-300, DEAE-cellulose and TSK gel DEAE-5PW columns. The complex contained no detectable α_2 -macroglobulin. Studies on the substrate specificity indicated that the complex had tryptase-like activities towards various synthetic substrates, but no elastase, chymotrypsin, cathepsin-B and cathepsin-L activities. The proteinase activity was completely inhibited by di-isopropyl fluorophosphate, leupeptin and antipain, indicating that the proteinase

bound to α_1 -macroglobulin is a serine proteinase. Two protein bands (62 and 59 kDa) of the complex were labelled with [3 H]di-isopropyl fluorophosphate and both bands cross-reacted with anti-(mast-cell tryptase)antibody. These results suggest that mast-cell tryptase is a major targeting proteinase for α_1 -macroglobulin *in vivo*. The main α -macroglobulin–proteinase complex in the adjuvant-treated rats was also the α_1 -macroglobulin–tryptase complex, even though the plasma level of α_2 -macroglobulin was elevated.

INTRODUCTION

α -Macroglobulins are broad-spectrum proteinase inhibitors that inactivate mammalian and even prokaryotic endopeptidases of all known classes such as serine-, cysteine-, aspartic- and metallo-type proteinases [1–4]. Rat plasma contains two α -macroglobulins [5]. α_1 -Macroglobulin is a tetramer of subunits, each consisting of a heavy chain (143 kDa) and a light chain (40 kDa) which are held together by disulphide bonds, whereas α_2 -macroglobulin is a tetramer of 159 kDa chains. Normal adult rat serum has a high α_1 -macroglobulin concentration of 2–4 mg/ml and a low α_2 -macroglobulin concentration of less than 50 μ g/ml [3,5].

The proteinase inhibitory mechanism of α -macroglobulins has been analysed in detail using human α_2 -macroglobulin and typical serine proteinases [6,7] such as trypsin, plasmin or thrombin, but other α -macroglobulins are also inhibitory [8]. Complex formation between proteinases and α -macroglobulin is initiated by specific proteolysis in the so-called ‘bait region’ of α -macroglobulin. In the rat α_1 -macroglobulin the bait region is located in the heavy chains. Cleavage of the bait region induces a set of conformational changes in α -macroglobulins leading to entrapment of the proteinase, activation of internal β -cysteinyl- γ -glutamyl thiol ester and exposure of the receptor recognition sites important for rapid clearance from the circulation. A large fraction of the proteinase is covalently bound, primarily through ϵ -lysyl (proteinase)– γ -glutamyl (α -macroglobulin) cross-links without blocking the proteinase active site. Although trapped proteinases retain enzymic activities, they can only cleave small molecules such as synthetic substrates, because of steric hindrance. The α -macroglobulin–proteinase complex is rapidly cleared from the circulation and from extracellular spaces by receptor-mediated endocytosis by various kinds of cells such as hepatocytes [9], fibroblasts [10] and macrophages [11]. Davidsen et al. [9] examined the plasma clearance of an 125 I-labelled α_2 -

macroglobulin–trypsin complex in the rat and reported that about 90% of the complex injected intravenously was incorporated into hepatocytes. After internalization, the α -macroglobulin–proteinase complex is transported into lysosomes and rapidly degraded [12,13].

In contrast with this detailed knowledge of the α -macroglobulins, the physiological function of α -macroglobulin is poorly understood. The fact that complete deficiency of α_2 -macroglobulin has never been identified in human indicates the importance of this plasma proteinase inhibitor. In view of their interaction with a broad range of endopeptidases and their presence in the extravascular space, α -macroglobulins have been proposed to function as inhibitors of foreign proteinases introduced by invading pathogens [1] or as a second-line regulatory element in extracellular proteolysis [14,15] such as coagulation, fibrinolysis and inflammation. On the other hand, α -macroglobulin can act as a carrier for various cytokines [16]. Because of the abundance of other proteinase inhibitors in plasma, it has been difficult to identify the specific physiological roles of α -macroglobulins and to determine target proteinases for α -macroglobulins *in vivo*. In order to clarify specific functions of α -macroglobulins, the proteinases complexed with α -macroglobulins *in vivo* must be isolated and characterized. Recently, Dahlman et al. purified an α_1 -macroglobulin–cathepsins (B, H and L) complex from rat skeletal muscle [17] using whole-tissue extract as the starting material for purification. The possibility that the complex is artificially produced during purification can not be excluded because α -macroglobulin can bind to proteinases from all four catalytic classes.

The α -macroglobulin–proteinase complex is internalized and concentrated into lysosomes by a specific receptor system [9,12,18]. If proteolytic degradation of the complex in lysosomes is inhibited, the complex is thought to accumulate in the lysosomes. In fact, some lysosomal proteins can be protected from degradation by cysteine proteinase inhibitor, resulting in an

Abbreviations used: APMSF, (*p*-amidino)phenylmethanesulphonyl fluoride; Box, *t*-butoxycarbonyl; Bz, benzoyl; Suc, succinyl; Z, benzyloxycarbonyl; E-64, *trans*-epoxysuccinyl-L-leucylamide-(4-guanidino)butane; DFP, di-isopropyl fluorophosphate; MCA, 4-methylcoumaryl 7-amide; ML, mitochondria-lysosome.

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elevation of their intracellular concentration [19]. Typical prolongation of turnover of unstable proteins by proteinase inhibitors is observed *in vivo* in galactosialidosis [20]. In affected patients, proteolytic degradation of β -galactosidase is accelerated due to a molecular defect in the protective protein which prevents rapid proteolytic degradation of the enzyme. Administration of a cysteine-proteinase inhibitor such as leupeptin restored the β -galactosidase activity in the fibroblasts of these patients. Previously, we showed that the α_2 -macroglobulin-thrombin complex, which was endocytosed from culture media, accumulates in the lysosome of cells cultured in the presence of a cysteine-proteinase inhibitor such as E-64 or leupeptin, as β -galactosidase does in fibroblasts of patients with galactosialidosis and established a method of purifying the endocytosed native complex from the cultured cells [12,13]. Recently, we found that the concentration of the α -macroglobulin-proteinase complex increased greatly in rats treated with leupeptin, as had been observed in cultured cells.

In this study, we administered leupeptin to rats, then purified the α_1 -macroglobulin-proteinase complex from the lysosomal fraction of their livers, as a first step in the elucidation of the physiological function of α -macroglobulin. Studies on the catalytic and immunological properties of the complex showed that the α_1 -macroglobulin-proteinase complex formed *in vivo* contained mast-cell tryptase as the major proteinase.

EXPERIMENTAL

Animals

Male Wistar rats weighing 300–450 g were injected twice with leupeptin or E-64 intraperitoneally, 6 h and 12 h before they were killed, at a dose of 2 mg per 100 g body weight. More than 95% of cathepsin-B and -L activities were inhibited by these treatments. Rats with inflammation (α_2 -macroglobulin-induced) were prepared by intraperitoneal injection with complete Freund's adjuvant (0.2 ml/100 g body weight), and used 3 weeks after the injection. The induction of α_2 -macroglobulin in plasma was confirmed by immunoblotting.

Enzyme assay

Proteinase activity was assayed using Boc-Phe-Ser-Arg-MCA (where BOC is *t*-butoxycarbonyl and MCA is 4-methylcoumaryl 7-amide) (Peptide Institute, Osaka, Japan) as described previously [12]. Cathepsin-B and -L activities were assayed using Z-Phe-Arg-MCA (where Z is benzyloxycarbonyl) at pH 5.5 by the method of Barrett and Kirschke [21]. Protein concentrations were determined by the method of Bradford [22] using a Bio-Rad protein assay reagent with BSA as a standard.

Immunological analysis

Rat α_1 - and α_2 -macroglobulins were purified by the methods of Lonberg-Holm et al. [5]. Antisera were raised by immunizing Japanese White rabbits with purified α_1 - and α_2 -macroglobulins. Both antibodies were purified by protein A-agarose affinity chromatography. Anti- α_1 -macroglobulin and anti- α_2 -macroglobulin antibodies were incubated with purified bovine α_2 -macroglobulin and rat α_1 -macroglobulin overnight at 4 °C respectively. The supernatants obtained by centrifugation were analysed by immunochemical means. The specificities of the two antibodies were confirmed by Western blotting. SDS/PAGE

[7.5% (w/v) acrylamide gel] was performed by the method of Laemmli [23]. The separated proteins were transferred to a nitrocellulose membrane [24], and detected using anti- α_1 -macroglobulin or anti- α_2 -macroglobulin antibodies, and goat anti-(rabbit IgG) antibody conjugated with alkaline phosphatase according to the instructions of the manufacturer (Bio-Rad). Immunoprecipitation of purified complex with anti- α_1 -macroglobulin or anti- α_2 -macroglobulin antibody was carried out as follows. Purified complex (3.5 μ g) was incubated with anti- α_1 - or anti- α_2 -macroglobulin antibodies (3.5 μ g) at 4 °C overnight. The reaction mixture was centrifuged and the supernatant was incubated with 20 μ l of protein A-agarose at 4 °C for 1 h. The mixture was centrifuged and the enzyme activity in the supernatant was assayed. Anti-(rat serum albumin) antibody was used as a control.

Anti-(rat cathepsin B), anti-(rat cathepsin L) and anti-(rat mast-cell tryptase) antibodies were kindly provided by Dr. T. Towatari and Dr. H. Kido (Division of Enzyme Chemistry, Institute for Enzyme Research, University of Tokushima, Japan). Anti-(human prothrombin) and anti-(human plasminogen) antibodies were purchased from Nordic Immunological Laboratories (Tiburg, The Netherlands) and Athens Research (Athens, GA, U.S.A.) respectively.

Purification of α_1 -macroglobulin-proteinase complex

The α_1 -macroglobulin-proteinase complex was purified from the liver lysosomal fraction of rats treated with leupeptin as described previously [12] with some modifications. All purification procedures were performed at 4 °C unless otherwise stated. Animals were decapitated and most of the blood in the liver was removed by perfusion with ice-cold saline. The liver was then minced and homogenized in 5 vol. of ice-cold 0.25 M sucrose containing 10 mM Tris/HCl, pH 7.0. The homogenate was centrifuged at 1200 g for 10 min at 4 °C, and the postnuclear supernatant was centrifuged at 17000 g for 20 min. The precipitated mitochondria-lysosome (ML) fraction was suspended in 10 mM sodium phosphate buffer, pH 6.0, and frozen at -30 °C. The ML fraction was thawed and homogenized in 10 vol. of 10 mM sodium phosphate buffer, pH 6.0, using a Polytron homogenizer (Kinematica, Littau, Switzerland). The homogenate was centrifuged at 17000 g for 15 min and the resulting supernatant was applied to a concanavalin A-Sepharose (Pharmacia, Uppsala, Sweden) column equilibrated with 20 mM sodium phosphate buffer, pH 7.0, containing 0.5 M NaCl. The column was washed extensively with the same buffer, then the enzyme was eluted with 20 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl and 10% (w/v) α -methylglucoside at 37 °C. The eluate was concentrated by ultrafiltration (Amicon, PM-10 membrane) and applied to a Sephacryl S-300 column (2.0 cm $\times$ 95 cm) equilibrated with 20 mM Tris/HCl buffer, pH 7.5, containing 0.15 M NaCl. The enzyme was eluted with the same buffer at a flow rate of 20 ml/h. Fractions containing activity were pooled, concentrated by ultrafiltration and dialysed against 20 mM Tris/HCl, pH 7.5. The dialysate was applied to a DEAE-cellulose column (1.0 cm $\times$ 4.0 cm) equilibrated with the dialysis buffer. The enzyme was eluted with 100 ml of a linear gradient of 0–0.25 M NaCl in 20 mM Tris/HCl, pH 7.5, at a flow rate of 10 ml/h. Fractions containing activity were concentrated by ultrafiltration, dialysed against 50 mM Tris/HCl buffer, pH 7.5, and applied to a TSK DEAE-5PW (Tosoh, Tokyo, Japan) column (0.75 cm $\times$ 0.75 cm) equilibrated with the dialysis buffer. The enzyme was eluted with a linear gradient of 0–0.5 M NaCl in the same buffer at a flow rate of 1 ml/min. The fractions with

Figure 1 Sephacryl S-300 gel filtration of trypsin-like proteinase activities in ML fractions of liver from control rats and those treated with leupeptin or E-64

Rats were treated, intraperitoneally with leupeptin or E-64 (2 mg/100 g body weight) twice, 6 h and 12 h before killing. Glycoprotein fractions were isolated from ML fractions by concanavalin A-Sepharose chromatography as described in the Experimental section. The glycoprotein fractions were applied to a Sephacryl S-300 column (2.0 cm $\times$ 95 cm) equilibrated with 20 mM Tris/HCl buffer, pH 7.5, containing 0.15 M NaCl at a flow rate of 20 ml/h and assayed for protein content (●). Proteinase activity (△) on Boc-Phe-Ser-Arg-MCA was measured using

activity were pooled, concentrated and stored at -30°C until required.

[^3H]DI-isopropyl fluorophosphate labelling and immunoprecipitation

The purified complex (5 μg of protein) was incubated in an ice bath for 45 min in the absence or presence of 5 mM (*p*-amidino-phenyl)methanesulphonyl fluoride (APMSF). Thereafter, [^3H]DFP (74 kBq, NEN, Boston, MA, U.S.A.) was added and the mixture was incubated in an ice bath for 30 min. Samples (3 μg of protein) were boiled in 2% (w/v) SDS in the presence of 2-mercaptoethanol and resolved by SDS/PAGE. The gel was treated with EN 3 HANCE (NEN) according to the instructions of the manufacturer, then visualized by fluorography at -80°C using Konica X-ray film.

The complex labelled with [^3H]DFP was immunoprecipitated as follows. The complex (5 μg) was incubated with anti- α_1 -macroglobulin antibody (5 μg) at 4°C for 2.5 h. Immunoprecipitate was collected by centrifugation after addition of 20 μl of IgG-sorb (The Enzyme Center, Boston, MA, U.S.A.). The pellet was washed three times with phosphate-buffered saline. SDS/PAGE and fluorography proceeded as described above.

RESULTS

Elevation of α_1 -macroglobulin-proteinase-complex activity by leupeptin

First, we examined whether leupeptin induced accumulation of the intracellular α -macroglobulin-proteinase complex *in vivo* as it does the α_2 -macroglobulin-thrombin complex in cultured cells. Leupeptin was administered twice, 6 h and 12 h before killing, and the concanavalin A-bound fraction isolated from the ML fraction of the liver was subjected to Sephacryl S-300 gel filtration. As shown in Figure 1, the hydrolytic activity towards a synthetic substrate (tube numbers 50–60) was 10-fold greater than that of the control. The molecular mass of the enzyme was estimated to be 700 kDa by gel filtration, and the elevated proteinase activity was completely immunoprecipitated with anti- α_1 -macroglobulin antibody. These results suggested that leupeptin also induced a significant accumulation of α_1 -macroglobulin-proteinase complex *in vivo*. The effect of E-64, an irreversible cysteine-proteinase inhibitor [25], on the activity of α_1 -macroglobulin-proteinase complex *in vivo* was also examined. As shown in Figure 1, E-64 had the same effect on the increase in activity of the α_1 -macroglobulin-proteinase complex. The possibility of artificial production of α_1 -macroglobulin-proteinase complex during purification was excluded. Production of α_1 -macroglobulin-proteinase complex was not prevented by adding methylamine, which inactivates rat α_1 -macroglobulin [26], to the buffers used for purification. These results indicate that the α_1 -macroglobulin-proteinase complex is formed *in vivo* and can be protected from degradation by inhibition of cysteine proteinase with leupeptin or E-64, resulting in an elevated intracellular concentration.

Purification of the α_1 -macroglobulin-proteinase complex

The α_1 -macroglobulin-proteinase complex that accumulated after leupeptin treatment was purified from the ML fraction by chromatography on concanavalin A-Sepharose, Sephacryl S-300 and DEAE-cellulose and by h.p.l.c. on TSK DEAE-5PW. A typical purification of α_1 -macroglobulin-proteinase complex

50 μl volumes of eluate. The molecular mass was estimated by comparison of the elution position with those of standard proteins; bovine α_2 -macroglobulin (720 kDa), ferritin (450 kDa), aldolase (150 kDa) and BSA (66.2 kDa).

Table 1 Purification summary of α_1 -macroglobulin–proteinase complex from the liver of rats treated with leupeptin

Abbreviation: ConA, concanavalin A.

Step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/mg per min)	Purity (-fold)	Yield (%)
ConA–Sephacryl	11.5	7.04	0.61	1.0	100
Sephacryl S-300	2.23	6.81	3.05	5.0	96.7
DEAE-cellulose	0.55	3.99	7.25	11.9	56.7
TSK-DEAE 5 PW	0.072	1.37	19.04	31.1	19.5

Figure 2 SDS/PAGE of the purified enzyme

Purified enzyme (0.5 μ g) was boiled in 2% (w/v) SDS in the presence of 10% (v/v) 2-mercaptoethanol and resolved by electrophoresis under denaturing conditions. Proteins were then detected by silver staining (a) or immunostaining with anti- α_1 -macroglobulin antibody (b). The standard proteins compared using silver staining were myosin heavy chain (200 kDa), β -galactosidase (116 kDa), phosphorylase b (97.4 kDa), BSA (66.2 kDa), ovalbumin (43 kDa) and carbonic anhydrase (31 kDa).

from rat liver treated with leupeptin is summarized in Table 1. About 70 μ g of purified complex was obtained from 50 g of liver. The final preparation gave a broad single band on PAGE under non-denaturing conditions (results not shown). On SDS/PAGE, two major bands (160 and 90 kDa) and faint bands (84, 70, 62, 59 and 38 kDa) were detected by silver staining (Figure 2a). This profile is very similar to that of α_1 -macroglobulin–cathepsin (B, H and L) complexes [17]. The bands of 160 and 38 kDa are considered to be native α_1 -macroglobulin heavy and light chain respectively. As shown in Figure 2, Western blots indicated that these protein bands (160, 90 and 38 kDa) cross-reacted with the anti-(rat α_1 -macroglobulin) antibody. Moreover, the trypsin-like activities towards Boc-Phe-Ser-Arg-MCA and Boc-Leu-Lys-Arg-MCA of the final preparation were both completely immunoprecipitated by the anti- α_1 -macroglobulin antibody, but not by the anti- α_2 -macroglobulin antibody (Table 2). These results

confirmed that the high-molecular-mass proteinase that was elevated by leupeptin is a complex of α_1 -macroglobulin with proteinase. α_2 -Macroglobulin is barely detectable in normal rat plasma, but its concentration is increased dramatically *in vivo* after injection of complete adjuvant [5]. Therefore, the proteinase was also purified from adjuvant-treated (inflammatory) rats in the same way and its properties were compared with those of the enzyme from normal rats to determine the difference between the targeting proteinases for α_1 - and α_2 -macroglobulin. During its purification, the complex from rats with inflammation showed the same elution profiles on various column chromatographies as that from normal rats, and the yield of the purified enzyme was also almost the same. The final preparation gave the same protein and immunostaining profiles on SDS/PAGE. The enzyme activity was completely immunoprecipitated with anti- α_1 -macroglobulin antibody, but not precipitated with anti- α_2 -macroglobulin antibody.

Optimum pH of proteinase activity of the complex

The effects of pH on the activity towards Boc-Phe-Ser-Arg-MCA were determined. The complexes purified from normal rats and those with inflammation both showed the highest activities at pH values of 9–10 as shown in Figure 3.

Substrate specificity

The activities of the enzyme on various synthetic substrates are shown in Table 3. The complex hydrolysed substrates that had a basic amino acid at the P1 position, which in general are good substrates for trypsin-like proteinases. Substrates with single or double amino acid residues, such as Bz-Arg-MCA or Z-Phe-Arg-MCA, were only slightly hydrolysed by the complex. Moreover between pH 4 and pH 10, the complex hardly hydrolyses Suc-Leu-Leu-Val-Tyr-MCA (where Suc is succinyl), which is a good substrate for chymotrypsin-like proteinases [27] or Suc-Ala-Pro-Ala-MCA, a good substrate for elastase [28]. Furthermore, no cathepsin B or L activity was detected on Z-Phe-Arg-MCA. Of the substrates tested, Boc-Phe-Ser-Arg-MCA, Boc-Val-Pro-Arg-MCA, Boc-Leu-Lys-Arg-MCA and Boc-Ile-Glu-Gly-Arg-MCA were good substrates. The substrate specificity of the complex was somewhat similar to that of rat mast-cell tryptase [29].

Susceptibility to inhibitors

The effects of various proteinase inhibitors on the enzyme activity were tested. As seen in Table 4, the trypsin-like activities of the purified enzymes from normal rats assayed with Boc-Phe-Ser-Arg-MCA were inhibited by DFP, leupeptin and antipain. As shown in Figure 4, 62 and 59 kDa bands were labelled with [3 H]DFP on SDS/polyacrylamide gel, and both bands were immunoprecipitated with anti- α_1 -macroglobulin antibody.

Table 2 Immunoprecipitation of α_1 -macroglobulin–proteinase complex with anti- α_1 -macroglobulin or anti- α_2 -macroglobulin antibodies

Immunoprecipitation was performed as described in the Experimental section.

Addition	Boc-Phe-Ser-Arg-MCA		Boc-Leu-Lys-Arg-MCA	
	Activity (pmol/ml per min)	Inhibition (%)	Activity (pmol/ml per min)	Inhibition (%)
Anti-(rat serum albumin)	285.5	0	211.1	0
Anti-(rat α_1 -macroglobulin)	3.4	98.8	2.4	98.9
Anti-(rat α_2 -macroglobulin)	290.0	0	257.7	0

Figure 3 Effects of pH on the activity of α_1 -macroglobulin–proteinase complex towards a synthetic substrate

Assays were performed under standard conditions except for the buffers. The buffers include 0.1 M acetate buffer for pH values of 5 and 6 (open triangles), 0.1 M Tris/HCl buffer for pH values of 7, 8 and 9 (closed circles), and 0.1 M glycine/NaOH buffer for pH values of 9, 10 and 11 (open circles). Values are shown as a percentage of the maximum activity.

Moreover, labelling with [3 H]DFP was completely abolished by APMSF which is a competitive inhibitor of DFP that binds to active serine residues. On the contrary E-64, pepstatin, chymostatin and bestatin had no effect on the enzyme activities. EDTA inhibited the activity slightly. Similar results were obtained with Boc-Leu-Lys-Arg-MCA as substrate (results not shown). These results show that the complex purified from normal rats contained trypsin-like serine proteinases. The substrate specificity and the proteinase inhibitor susceptibility of the complex from rats with inflammation were also examined. No significant differences in these properties between both complexes were observed.

Identification of the complexed proteinase

The complexes purified from normal rats and those with inflammation contained trypsin-like serine proteinases as mentioned above. We therefore investigated the type of trypsin-like protein-

Table 3 Substrate specificity of the purified α_1 -macroglobulin–proteinase complex

Enzyme activities were determined at 37 °C with various substrates at concentrations of 10 μ M at pH 10.

Substrate	Activity	
	Specific activity (nmol/mg per min)	Percentage of control level (%)
Boc-Gln-Arg-Arg-MCA	24.4	100
Boc-Phe-Ser-Arg-MCA	20.2	82.7
Boc-Val-Pro-Arg-MCA	17.1	70.0
Boc-Leu-Lys-Arg-MCA	13.0	53.2
Boc-Glu-Lys-Lys-MCA	2.56	10.5
Boc-Ile-Glu-Gly-Arg-MCA	8.52	34.9
Boc-Val-Leu-Lys-MCA	7.97	32.6
Z-Phe-Arg-MCA	2.29	9.3
Bz-Arg-MCA	0.24	0.98
Suc-Ala-Pro-Ala-MCA	0.03	0.12
Suc-Leu-Leu-Val-Try-MCA	1.07	4.38

Table 4 Effects of various inhibitors on the enzyme activity of the α_1 -macroglobulin–proteinase complex

Activities towards Boc-Phe-Ser-Arg-MCA were assayed at pH 10 in the presence of various inhibitors.

Inhibitor	Enzyme activity	
	Specific activity (nmol/mg per min)	Inhibition (%)
No addition	20.2	0
DFP (1 mM)	3.03	85
Leupeptin (1 μ g/ml)	0	100
Antipain (1 μ g/ml)	1.82	91
E-64 (1 μ g/ml)	20.2	0
Chymostatin (1 μ g/ml)	20.2	0
Pepstatin (1 μ g/ml)	17.8	12
EDTA (5 mM)	14.5	28
Bestatin (1 μ g/ml)	17.8	12

ases trapped by α_1 -macroglobulin in normal and inflammatory states. The results suggested that both complexes contain trypsin, thrombin, Factor X or plasmin. Boc-Phe-Ser-Arg-MCA, Boc-Val-Pro-Arg-MCA, Boc-Ile-Glu-Gly-Arg-MCA and Boc-Glu-Lys-Lys-MCA are good substrates for trypsin [29], α thrombin [3], Factor X [30] and plasmin [31] respectively. Enjyoji

Figure 4 [^3H]DFP labelling and immunoblot analysis of the purified enzyme

[^3H]DFP labelling and immunoprecipitation with anti- α_1 -macroglobulin antibody; samples of purified enzyme from normal (lanes 1 and 2) and adjuvant-treated (lanes 3 and 4) rats were labelled with [^3H]DFP after incubation with (lanes 1 and 3) and without (lanes 2 and 4) APMSF as described in the Experimental section. The purified enzymes from normal (lanes 5 and 6) and adjuvant-treated (lanes 7 and 8) rats labelled with [^3H]DFP were immunoprecipitated with anti- α_1 -macroglobulin antibody. Supernatant (lanes 5 and 7) and precipitate (lanes 6 and 8) after immunoprecipitation. Samples were resolved by SDS/PAGE and radioactive bands were visualized by fluorography. The [^{14}C]-labelled methylated standard proteins were myosin heavy chain (200 kDa), phosphorylase b (97.4 kDa) BSA (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa) and lysozyme (14.3 kDa). Immunoblot: samples of purified enzyme (10 μg) from normal (lanes 9, 11 and 13) and adjuvant-treated (lanes 10, 12 and 14) rats were resolved by SDS/PAGE. Proteins were transferred to nitrocellulose and incubated with anti-tryptase (lanes 9 and 10), anti-prothrombin (lanes 11 and 12) and anti-plasminogen (lanes 13 and 14) antibodies.

et al. reported that rat Factor X is resistant to α_2 -macroglobulin [32], therefore we attempted to identify the trypsin-like proteinases bound to α_1 -macroglobulin by immunoblotting with antibodies to mast-cell tryptase, prothrombin and plasminogen. As shown in Figure 4 (lanes 9 and 10), anti-tryptase antibody reacted in Western blots of the complexes. The protein bands in the blots binding the anti-tryptase IgG have a molecular mass of 62 and 59 kDa, which were also detected by silver staining (Figure 2) and [^3H]DFP labelling (Figure 4). The intensity of both protein bands was increased in rats with inflammation compared with normal rats. These molecular mass values are higher than those of free tryptase (37 and 35 kDa [33]). A protein band corresponding to free tryptase was barely detectable in immunoblots of the complexes, indicating that most of the tryptase was covalently linked to proteolytic fragments of α_1 -macroglobulin. No immunoreactive bands with antibodies to prothrombin and plasminogen were detected (Figure 4, lanes 11–14). Furthermore, the proteinase activities of the complexes from normal rats and those with inflammation towards Boc-Glu-Lys-Lys-MCA and Boc-Val-Leu-Lys-MCA (a good substrate for plasmin) were not inhibited by D-Val-Phe-Lys-chloromethyl ketone, a specific plasmin inhibitor (results not shown). Protein bands immunoreactive with antibodies to cathepsins B and L were undetectable in our preparation.

One distinguishing feature of tryptase is that its activity decreased with increasing NaCl concentration [34]. To confirm our results, the effects of NaCl of α_1 -macroglobulin–proteinase complex were examined. The activities toward Boc-Phe-Ser-Arg-MCA of the complex decreased to 53 % of the control in the presence of 1 M NaCl.

DISCUSSION

α -Macroglobulins are well-characterized proteinase inhibitors and their mechanisms of internalization into the cells have also been analysed in detail. However, the physiological function of

α -macroglobulins is poorly understood. Isolation of the α -macroglobulin–proteinase complex formed *in vivo* is necessary to determine which proteinases are targeted by α -macroglobulins. The α -macroglobulin–proteinase complex can be identified by the combination of a proteinase-activity assay and immunoprecipitation with antibody raised against α -macroglobulins, because proteinase complexed with α -macroglobulin is still active toward synthetic substrates. However, rapid degradation of the α -macroglobulin–proteinase complex in lysosomes is the most urgent problem needing to be solved to isolate the complex formed *in vivo*. Previously we showed that the α_2 -macroglobulin–thrombin complex, which was endocytosed from culture media, was highly accumulated in the lysosomes of baby hamster kidney (BHK) cells cultured in the presence of a cysteine-proteinase inhibitor such as leupeptin or E-64 [12].

In this study, we attempted to isolate the α -macroglobulin–proteinase complex formed *in vivo* from the livers of rats treated with the cysteine-proteinase inhibitor leupeptin, using the method established for cultured cells. The α -macroglobulin–proteinase complex present in the circulation is endocytosed by a specific receptor system mainly into hepatocytes, as shown by Davidsen et al. [9]. The complex was hardly detectable in control liver, but accumulated in liver after the administration of leupeptin or E-64. These results indicate that the purified complex is endocytosed into hepatocytes from the circulation and is not an artifact of purification.

Structural and immunological studies have indicated that the purified complex consists of α_1 -macroglobulin–proteinase. The proteinase complexed with α_1 -macroglobulin was identified by studies of the substrate specificity toward various synthetic substrates, susceptibility to inhibitors and the immunological reactivity to various antibodies against proteinases, indicating that mast-cell tryptase is a major proteinase in the complex. The optimal pH of the proteinase is too high compared with that of mast-cell tryptase, being pH 9–10. On the contrary the optimal pH of free mast-cell tryptase is 7–8 [34]. Similarly Dahlman and co-workers reported that the optimal pH of cathepsins B, H and L complexed with α_1 -macroglobulin was alkaline, although free cathepsins B, H, and L had pH optima of pH 5–6 [17]. The optimal pH of the proteinase might become more alkaline after complex formation with α -macroglobulin. Two bands (62 and 59 kDa) were specifically labelled with [^3H]DFP and both were cross-reactive with anti-tryptase IgG. Free tryptase (37 and 35 kDa) was barely detectable in our preparation. These results indicated that tryptase is covalently linked to the proteolytic fragment of α_1 -macroglobulin. Recently Uchino and co-workers purified a 25 kDa serine proteinase complexed with α_2 -macroglobulin from pig gastric mucosa, and reported that a serine proteinase is bound to α_2 -macroglobulin non-covalently [35]. The molecular mass and binding mode of this proteinase were quite different from those of our preparation.

Rat plasma contained two kinds of α -macroglobulin [3,5]. In normal rats, the concentration of α_1 - and α_2 -macroglobulin in serum are 2–4 mg/ml and less than 50 $\mu\text{g}/\text{ml}$ respectively. Rat α_2 -macroglobulin is an acute-phase reactant, and it is induced to high levels by adjuvant [5]. Therefore we also purified the α -macroglobulin–proteinase complex from adjuvant-treated rat liver in the same manner. The purified complex was composed of α_1 -macroglobulin and tryptase the same as the complex from normal rats. The concentration of α_2 -macroglobulin in plasma was increased after injection with adjuvant, but the concentration of α_1 -macroglobulin was still higher than that of α_2 -macroglobulin. Further analysis is necessary to investigate the roles of α_2 -macroglobulin in rat.

Recently Poller and co-workers reported on a patient with

chronic lung disease and a partial α_2 -macroglobulin deficiency, and suggested that, in lung, α_2 -macroglobulin plays a role in the regulation of proteinase activity that is not inhibited by α_1 -antitrypsin [36]. Our results agree with their suggestion, if rat α_1 - and human α_2 -macroglobulin have the same function *in vivo*. α_1 -Antitrypsin is a major proteinase inhibitor in plasma and its deficiency causes pulmonary emphysema [37]. α_1 -Antitrypsin is thought to regulate neutrophil elastase activity in lung. However, tryptase is not inhibited by α_1 -antitrypsin [34]. Tryptase is distributed in the mast cells of lung, skin and peritoneal fluid [29,34] and it is localized in secretory granules of mast cells and released from activated mast cells. So far proteoglycan [38] and trypstatin [29] are proposed to be regulators of tryptase. Our results suggested that α_1 -macroglobulin plays roles both as an inhibitor and scavenger of tryptase released from activated mast cells.

It has been thought until now, that α_2 -macroglobulin does not participate in the regulation of mast-cell tryptase activity. This assumption was based mainly on the studies by Smith et al. [34], who examined the effects of human α_2 -macroglobulin on human lung tryptase activity using azocasein as the substrate, and reported that tryptase was not inhibited by α_2 -macroglobulin. However, we have shown previously that the α_2 -macroglobulin-thrombin complex has activity towards [14 C]methylated casein [12,13]. Therefore it is difficult to conclude that α_2 -macroglobulin does not bind to tryptase from an inhibition study only. Differences of proteinase-binding specificity between human α_2 - and rat α_1 -macroglobulin may exist.

The rat has two related macroglobulins, α_1 - and α_2 -macroglobulin. In human plasma, however, α_1 -macroglobulin is absent [5]. Human α_2 -macroglobulin is not classified as an acute-phase reactant as is α_1 -macroglobulin in the rat, although human α_2 -macroglobulin is immunologically [39] and structurally [40] more closely related to rat α_2 -macroglobulin than to rat α_1 -macroglobulin. In normal rat plasma, α_2 -macroglobulin is present at very low concentrations compared with α_1 -macroglobulin. Rat α_1 - and human α_2 -macroglobulins are present at similar concentrations in plasma (2–4 mg/ml). Therefore it might be thought that rat α_1 - and human α_2 -macroglobulin have similar functions *in vivo*.

In this paper, we proposed the physiological function of rat α_1 -macroglobulin as a tryptase inhibitor. Further detailed analysis is necessary to clarify the physiological function of α -macroglobulins.

We wish to thank Dr. Hiroshi Kido and Dr. Takae Towatari (Division of Enzyme Chemistry, The Institute for Enzyme Research, University of Tokushima, Tokushima, Japan) for supplying antibodies to rat mast-cell tryptase, and rat liver cathepsins B and L. We thank Yuka Nishine, Tomomi Katsuta and Tokue Kuroda for excellent secretarial help. This work was supported by a grant from the Ministry of Education, Science and Culture of Japan.

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