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Rat plasma α_1-inhibitor$_3$ binds to receptors for α_2-macroglobulin

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The cellular binding and uptake was studied for α_1-inhibitor$_3$, a monomeric 200 kDa proteinase inhibitor present in rat plasma. After intravenous injection in the rat the inhibitor disappeared from the circulation with a half-time of 2.5 min when complexed with chymotrypsin, whereas the half-time for uncomplexed inhibitor was more than 60 min. 6 min after the injection of labelled complex, 83% was in the liver and 2.5% in the spleen. In vitro experiments at 4°C with isolated hepatocytes and peritoneal macrophages showed binding to the previously described receptors which bind and internalize the tetrameric rat and human α_2-macroglobulin-proteinase complexes. The binding affinities were similar for the two types of complexes and binding was followed by uptake and degradation of the labelled complex when the cells were warmed to 37°C. The binding of uncomplexed α_1-inhibitor$_3$ was low and did not increase following treatment with methylamine in spite of cleavage of the internal thiol ester. α_1-Inhibitor$_3$-methylamine was changed to the receptor binding form when treated with chymotrypsin which caused the cleavage of at least one peptide bond in the bait region.

α_1-Inhibitor$_3$; α_2-Macroglobulin; Cellular receptor; Thiol ester

1. INTRODUCTION

The proteinase inhibitor designated α_1-inhibitor$_3$ (α_1I_3) was first isolated from rat plasma by Gauthier and Ohlsson [1]. The molecular mass is about 200 kDa [2] and the concentration in normal rat plasma is about 30 μM. It is capable of interacting with elastase, chymotrypsin and trypsin [3-5]. It was recently reported that 1 mol of SH groups is generated when 1 mol of α_1I_3 interacts with 1 mol of chymotrypsin or trypsin, that at least one peptide bond near the middle of the polypeptide chain is cleaved and that the formation of a stable complex is associated with a conformational change of the α_1I_3 molecule. Moreover, treatment with methylamine also generated SH groups [6-9].

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The results are analogous to those obtained when human α_2-macroglobulin interacts with a proteinase (review [10]). These features strongly suggest that α_1I_3 is homologous to α_2-macroglobulin. In addition, recent partial sequence data from the N-terminus [10] and the C-terminus of α_1I_3 [11] clearly demonstrate that α_1I_3 is a member of the macroglobulin family of proteinase binding proteins. The present paper shows that α_1I_3 is also a homologue of α_2-macroglobulin in the sense that when complexed with chymotrypsin it binds to the same receptors in the two major target cells, hepatocytes and macrophages.

2. MATERIALS AND METHODS

Rat α_1I_3 was prepared from pooled EDTA plasma obtained from 200 g male Wistar rats essentially according to [8] employing polyethylene glycol precipitation, DEAE-Sephacel chromatography,

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Zn-chelate chromatography and Sephacryl S-300 gel chromatography. Approximately 170 mg α_1I_3 was obtained from 76 ml plasma. The preparation contained 0.9 mol active β-cysteinyl-γ-glutamyl thiol ester as revealed by titration with 2,2'-dithiobis-5-nitrobenzoic acid of samples that had been incubated with an equimolar amount of trypsin, chymotrypsin, elastase or *S. aureus* protease. Non-denaturing PAGE and HPLC gel chromatography using columns of Superose 12 or TSK G-3000 SW demonstrated that freshly prepared α_1I_3 migrated as a protein of approx. 200 kDa, consistent with earlier results [7-9]. After proteinase complex formation, minor amounts (approx. 5-10%) of dimers and tetramers were observed.

Human [12] and rat [13] α_2-macroglobulin were prepared as described previously. The ligands were iodinated using 0.5 mol ^{125}I per mol 200 kDa α_1I_3 monomer or 180 kDa α_2-macroglobulin subunit as described in [14]. Labelled or unlabelled α_1I_3 was complexed with a 1.5-times molar excess of bovine chymotrypsin (Worthington, three times crystallized) for 3 min at 20°C followed by the addition of PMSF (phenylmethanesulfonyl fluoride) to a final concentration of 1 mM. α_2-Macroglobulin was complexed with trypsin as described [12]. The tracers were passed over a 0.5 × 3 cm Sephacryl S 200 column before each experiment to remove low molecular mass decay products [14].

The disappearance of intravenously injected labelled α_1I_3 from the blood and the uptake in various organs of the rat were measured as described in [15]. Rat hepatocytes were prepared by perfusion of livers from 200–300 g ad lib fed male Wistar rats with collagenase followed by centrifugation in a preformed linear gradient of Percoll to remove Kupffer cells and damaged hepatocytes [16]. Peritoneal macrophages were elicited by intraperitoneal injection of 3 ml thioglycolate (260 mg/ml). The rat was killed after 4 days by cervical dislocation and the peritoneum was flushed with 4°C incubation buffer containing 5 U/ml heparin. 3–5 × 10^7 cells were obtained per rat and at least 87% were esterase positive [17], i.e. macrophages. Separate experiments showed that binding was trivial or absent in cells not adhering to plastic at 37°C, i.e. non-macrophages.

Cells (1.6 × 10^6/ml) were incubated at 4°C with shaking in buffer containing 25 mM Hepes, 1% (w/v) bovine serum albumin and Krebs' salts, pH 7.6 [14]. The incubations were terminated by the addition of 200 μl cell suspension to microfuge tubes containing 100 μl dibutylphthalate/dinonylphthalate (3 : 1) followed by centrifugation for 40 s in a Beckman microfuge and the cell pellets were recovered by cutting through the oil layer. About 0.35% of the incubation buffer was trapped in the cell pellet when using this procedure [14]. The same percent of radioactivity was present in the cell pellet when a very large concentration of unlabelled α_1I_3-chymotrypsin was present together with the labelled ligand (cf. fig.2). Thus, all binding was displaceable or 'specific'.

3. RESULTS

While it has been indicated earlier that complexes between α_1I_3 and proteinases are eliminated from the circulation in rats with a half-time of about 15 min [4], no attempt was made to identify the cells responsible for this. The recent realization that α_1I_3 is structurally related to the α-macroglobulins suggested that α_1I_3-proteinase complexes would bind to rat hepatocytes and macrophages. These cells are known from previous studies to contain receptors which recognize and internalize not only rat α_2-macroglobulin and α_1-macroglobulin but also human α_2-macroglobulin and pregnancy zone protein [13–16,18,19]. α_1I_3-chymotrypsin was used as a standard preparation for the following studies in the rat.

In vivo experiments showed that intravenously injected ^{125}I-labelled α_1I_3-chymotrypsin disappeared from the blood with a half-time of 2.5 ± 0.6 min (n = 6) whereas the half-time of uncomplexed α_1I_3 was more than 60 min. By 6 min, 10–15% of the labelled α_1I_3-chymotrypsin remained in the blood and 83 ± 3% (n = 3) of the radioactivity taken up by the tissue was in the liver, whereas 2.5 ± 0.3% was in the spleen. These numbers are indistinguishable from those obtained when ^{125}I-labelled human α_2-macroglobulin-trypsin [15] or the homologous pregnancy zone protein-chymotrypsin [18] are injected intravenously into rats. This suggested that the cell types responsible for removal of these complexes from the circulation might also remove α_1I_3-chymotrypsin.

Table 1 shows the binding of α_1I_3-proteinase

complexes to hepatocytes and macrophages as compared with the binding of α_2-macroglobulin-trypsin. α_1I_3 complexed with chymotrypsin or trypsin showed the same specific binding to hepatocytes, and it was similar to that of human α_2-macroglobulin-trypsin. Uncomplexed α_1I_3 showed much lower binding. Surprisingly, treatment with methylamine did not increase this binding in spite of the complete cleavage of the thiol esters. However, incubation of methylamine-treated α_1I_3 for 5 min with a 1.5-times molecular excess of chymotrypsin caused an increase in binding to the same level as that of α_1I_3-chymotrypsin. SDS-PAGE in the presence of mercaptoethanol demonstrated that this chymotrypsin treatment caused a fragmentation of α_1I_3-methylamine similar to that obtained after treatment of native α_1I_3 [6], i.e. into a 96 kDa fragment and several fragments of low molecular mass (not shown). This indicates that α_1I_3-methylamine, like native α_1I_3, is cleaved in the bait region and subsequently at several other sites [6].

Binding of α_1I_3-chymotrypsin to hepatocytes was analyzed further at 4°C. Fig.1 shows a slow time course of binding at this temperature as previously observed for α_2-macroglobulin and pregnancy zone protein complexes [14,18]. The binding was almost identical for α_1I_3-chymotrypsin and human α_2-macroglobulin-trypsin at tracer concentrations. Specific binding of labelled α_1I_3-chymotrypsin was completely abolished by

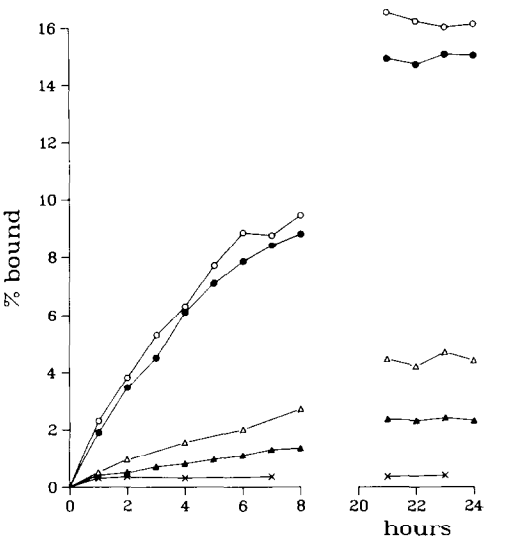

Fig.1. Time course of α_1I_3-chymotrypsin binding to rat hepatocytes. The cells were incubated at 4°C for the times indicated. 40 pM ^{125}I-labelled α_1I_3-chymotrypsin alone (●—●), labelled α_1I_3-chymotrypsin plus 200 nM unlabelled α_1I_3-chymotrypsin (×—×), labelled α_1I_3 plus 100 nM α_2-macroglobulin-trypsin (▲—▲), 40 pM ^{125}I-labelled uncomplexed α_1I_3 (△—△), 10 pM ^{125}I-labelled human α_2-macroglobulin-trypsin (○—○).

200 nM unlabelled homologous ligand and markedly inhibited by 100 nM α_2-macroglobulin-trypsin. Similar data were obtained with macrophages (not shown). This suggested that α_1I_3 com-

Table 1

Binding of α_1I_3 complexes to rat hepatocytes and peritoneal macrophages

Labelled complex	Percent specifically bound	
	Hepatocytes	Macrophages
α_1I_3-chymotrypsin[a]	17.0 ± 1.6	7.1 ± 0.7
α_1I_3-trypsin[b]	15.7 ± 1.1	6.5 ± 0.4
α_1I_3 (uncomplexed)	2.1 ± 0.6	0.5 ± 0.1
α_1I_3-methylamine	2.8 ± 0.3	1.4 ± 0.3
α_1I_3-methylamine-chymotrypsin[c]	20.7 ± 1.9	8.0 ± 0.6
α_2-Macroglobulin-trypsin	18.2 ± 2.1	11.3 ± 0.9

[a] 1.5-times molecular excess of chymotrypsin for 3 min

[b] 50-times molecular excess of trypsin for 5 min; 3-times excess for 5 min gave only partial effect

[c] 1.5-times molecular excess for 5 min

All complexes were prepared at 20°C as explained in the footnotes. Cells were incubated for 20 h at 4°C with approx. 20 pM of the indicated labelled ligands. The results are the mean values of 4 experiments ± 1 SD

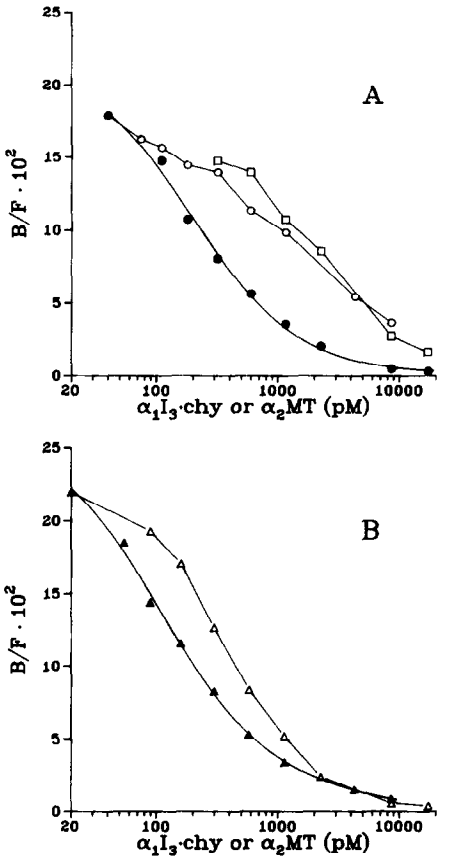

Fig.2. Concentration dependence of α_1I_3-chymotrypsin binding. Hepatocytes were incubated for 22 h at 4°C. Panel A shows the binding of 80 pM ^{125}I-labelled α_1I_3-chymotrypsin plus unlabelled α_1I_3-chymotrypsin to give total concentrations as indicated on the abscissa (●—●). This curve represents the best least-square fit using a one-receptor model for reversible binding. The apparent K_d was 480 pM and R_o was 96 pM. Competition with human α_2-macroglobulin-trypsin (○—○) and with uncomplexed α_1I_3 (□—□) are also shown. Panel B shows the binding of 20 pM ^{125}I-labelled α_2-macroglobulin-trypsin plus unlabelled homologous ligand (▲—▲). This curve is computed using the best least-square fit for a two-receptor model. The inhibition by unlabelled α_1I_3-chymotrypsin is also shown (△—△).

plexes, at least in part, were bound to the receptors for α_2-macroglobulin-proteinase complexes.

Fig. 2 shows the concentration dependence of α_1I_3-chymotrypsin binding, and the apparent dissociation constant was about 500 pM. Similar results were obtained when α_1I_3 was complexd with trypsin, elastase or *S. aureus* protease (not shown). The inhibition constant of uncomplexed α_1I_3 was approximately 4 nM. The mean apparent K_d value for α_1I_3-chymotrypsin binding in this and 3 similar experiments was 492 ± 36 pM. Human α_2-macroglobulin-trypsin was less efficient and the inhibition curve was not parallel with that obtained when using unlabelled α_1I_3-chymotrypsin, a phenomenon which was reproduced in several experiments. On the other hand, the 18 kDa receptor binding fragment obtained by digestion of α_2-macroglobulin-methylamine with papain [20] inhibited ^{125}I-labelled α_1I_3 or α_2-macroblobulin equally well (not shown).

Fig. 2B shows, in agreement with previous results [14], that binding of human α_2-macroglobulin-trypsin kinetically behaves according to a two-receptor model. This binding was completely abolished with high concentrations of of α_1I_3-chymotrypsin confirming that this ligand is bound to the entire population of α_2-macroglobulin complex receptors. This result was also obtained in several experiments with macrophages (not shown). Other results may be summarized as follows (not shown). The dissociation of labelled α_1I_3-chymotrypsin was very slow and similar to that obtained with human pregnancy zone protein complex [18]. α_1I_3-chymotrypsin was taken up and degraded by hepatocytes at 37°C in a way similar to that observed for human [14] and rat [13] α_2-macroglobulin-trypsin. This process was poorly competed for by human α_2-macroglobulin-proteinase complex and complete inhibition was not achieved even with 1 μM complex. On the other hand, α_1I_3-chymotrypsin readily inhibited uptake of labelled α_2-macroglobulin complex and half-maximal inhibition was obtained with about 10 nM ligand, a value similar to that obtained with unlabelled α_2-macroglobulin-trypsin [15].

4. DISCUSSION

Previous results have shown that α_1I_3 is a member of the macroglobulin family in the sense that proteinase, e.g. chymotrypsin, splits a peptide bond in the bait region, that an SH group is generated and that a stable α_1I_3-proteinase complex is formed [6–9]. The present results demonstrate that the monomeric [8,9] α_1I_3-chymotrypsin binds to and is internalized via those recep-

tors in rat hepatocytes and macrophages which recognize both the tetrameric (e.g. human and rat α_2-macroglobulin) and dimeric (pregnancy zone protein) α-macroglobulins.

The receptor binding domain of human α_2-macroglobulin is located in the C-terminal residues 1314–1451 [20,21]. The recently determined partial sequence of the C-terminal 225 residues of α_1I_3 revealed several stretches with sequences nearly identical to the equivalent stretches in α_2-macroglobulin [11]. It is therefore highly probable that α_1I_3 is a close homologue of α_2-macroglobulin with respect to sequence and location of the receptor binding determinants.

The native α-macroglobulins all show very low receptor binding affinities in contrast to the high affinities exhibited by the α-macroglobulin-proteinase complexes. It is well established that cleavage of the four thiol esters in the tetrameric human α_2-macroglobulin, for instance by incubation with methylamine, is necessary and sufficient for obtaining optimal binding affinity [22,23]. It has therefore been proposed as a general mechanism for α-macroglobulins that thiol ester cleavage leads to the conformational change necessary for high receptor binding affinity [21–23]. However, the cleavage of at least one peptide bond was necessary to bring about the high affinity conformation in α_1I_3, in which the thiol esters were cleaved due to incubation with methylamine. This extends the previous observation that tetrameric rat α_2-macroglobulin was not converted to the high affinity form by incubation with methylamine [24]. In our hands, results were similar to the pregnancy zone protein: treatment with methylamine to cleave the two thiol esters failed to increase its affinity (Moestrup et al., unpublished).

It has been proposed that the disulfide-bridged dimers might constitute the receptor binding unit of tetrameric or dimeric α-macroglobulins [20,21]. The present finding that the monomeric α_1I_3 binds with nearly the same affinity as the tetrameric human α_2-macroglobulin and dimeric pregnancy zone protein makes such a hypothesis unlikely. The following model may be proposed based on the present and previous data. The C-terminal approx. 140 residue domain of an α-macroglobulin monomer contains the necessary information for receptor binding. However, the affinity of the entire monomer is, when complexed with a proteinase, about 100-times higher, probably because other parts of the monomer are necessary for maintaining the right conformation of the binding domain. Cleavage of the thiol esters is a necessary condition for obtaining the high affinity conformation. In some cases (e.g. human α_2-macroglobulin) it is also a sufficient condition whereas in other cases (e.g. rat α_1I_3 and rat α_2-macroglobulin) cleavage of at least one bait region peptide bond is required. The presence of two or four domains in dimers and tetramers will cause quite subtle changes in the binding kinetics [18], as will the extent of bait region cleavage in tetrameric human α_2-macroglobulin [25], but high affinity binding is not critically dependent on the quaternary structure.

In conclusion, the binding of the monomeric α_1I_3 to the α_2-macroglobulin receptors indicates that dimeric or tetrameric structure is not necessary for high affinity receptor binding. This binding only occurs when at least one peptide bond in α_1I_3 is cleaved in the bait region and cleavage of the thiol ester is not sufficient to elicit the high affinity conformation of the binding domain.

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