Isolation and characterization of fibronectin–α_1-microglobulin complex in rat plasma

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Molecules containing the 28 kDa immunoregulatory protein α_1-microglobulin (α_1-m), also known as protein HC, were isolated from rat plasma or serum by immunoaffinity chromatography. Three molecular species were distinguished on the basis of non-denaturing PAGE. Two of these have been described previously: uncomplexed α_1-m, and the complex of α_1-m with α_1-inhibitor-3. The third species was analysed by denaturing PAGE, immunoblotting, proteinase digestion and N-terminal-sequence analyses, and shown to consist of a complex between α_1-m and fibronectin. This complex, with a mass of about 560 kDa, was resistant to dissociation in the presence of denaturants, but not in the presence of reducing agents in combination with denaturants, and we conclude that the two components are linked by disulphide bonds. About 60% of the total detectable plasma α_1-m exists as high-molecular-mass complexes distributed approximately evenly between fibronectin and α_1-inhibitor-3. Immunochemical analyses were used to determine the proportion of the total plasma pools of fibronectin and α_1-inhibitor-3 that circulate in complex with α_1-m. About 3–7% of the total plasma fibronectin from three different rat strains contained α_1-m, whereas 0.3–0.8% of the total plasma α_1-inhibitor-3 contained α_1-m. Complexes were found at similar levels in plasma and serum, indicating that coagulation is not responsible for complex formation. Moreover, immunochemical analyses of human plasma revealed small amounts of α_1-m in complex with fibronectin and α_2-macroglobulin (an α_1-inhibitor-3 homologue). The existence of a complex between α_1-m and fibronectin in rats and humans suggests a mechanism for the incorporation of the immunoregulatory molecule α_1-m into the extracellular matrix.

INTRODUCTION

α_1-Microglobulin (α_1-m), a 26–28 kDa plasma protein first isolated from human urine (Ekström et al., 1975; Ekström and Berggård, 1977), is involved in the immune response of the organism (for a review, see Åkerström and Lögdberg, 1990). It has regulatory, mainly suppressive, effects on lymphocytes and granulocytes (Lögdberg and Åkerström, 1981; Mendez et al., 1986), and receptors for α_1-m have been identified on white blood cells (Fernandez-Luna et al., 1988; Babiker-Mohamed et al., 1990). It is synthesized in rat hepatocytes, and secretion from liver cells is stimulated by cytokines activated during the immune response and inflammation (Pierzchalski et al., 1992). The gene for α_1-m also encodes bikunin, a Kunitz-type protease inhibitor (Diarra-Mehrpour et al., 1990; Vetr and Gebhard, 1990). A precursor protein, α_1-m–bikunin, is expressed by the gene and cleaved in the trans-Golgi network, and free α_1-m is released from the liver cells (Kaumeyer et al., 1986; Bratt et al., 1993). In the blood, however, only about 50% of α_1-m is found in the free form, and the remaining portion is found as high-molecular-mass complexed forms (Ekström et al., 1975; Tejler and Grubb, 1976).

It is not yet known why α_1-m and bikunin are encoded by the same mRNA. No functional connection between the two proteins has so far been reported, and no circulating α_1-m–bikunin form has been found. Instead, α_1-m complexes formed with human albumin (Tejler and Grubb, 1976), human IgA (Grubb et al., 1986) and the rat α-macroglobulin α_1-inhibitor-3 (α_1-I_3) (Falkenberg et al., 1990) have been demonstrated. Bikunin, on the other hand, is found as a constituent of the plasma proteins inter-α-inhibitor (Salier, 1990), pre-α-inhibitor (Enghild et al., 1989) and inter-α-like inhibitor (Enghild et al., 1993).

In the pursuit of a molecular species containing both α_1-m and bikunin, we studied the high-molecular-mass forms of rat plasma α_1-m. We found no evidence for the presence of this species, but α_1-m was instead found to be linked by disulphide bonds to fibronectin, a cell-adhesive extracellular-matrix glycoprotein which occurs in a soluble form in plasma (for a review, see Yamada, 1991). The complex was isolated and found to be circulating in normal rat plasma at similar levels to the α_1-m–α_1-I_3 complex.

EXPERIMENTAL

Materials

Frozen rat plasma was obtained from Pel-Freeze Biologicals (U.S.A.). Fresh rat plasma (using EDTA) or serum was drawn from Sprague–Dawley, Wistar–Furth or Copenhagen rats (B&K Universal AB, Sweden) which had been anaesthesized with ether. In some experiments blood was drawn directly into a solution of *N*-ethylmaleimide to a final concentration of 10 mM. Human plasma was obtained from a healthy blood donor. Monoclonal mouse anti-α_1-m, BN11.2, binds to human and rabbit α_1-m, BN11.3, BN11.7 and BN11.10 to rat and human α_1-m, and BN11.6 to rat and rabbit α_1-m (Babiker-Mohamed et al., 1991). Rat serum 28 kDa α_1-m (Falkenberg et al., 1990) and human urinary α_1-m (Ekström and Berggård, 1977) were isolated as described. α_1-I_3 was purified from rat plasma as reported earlier (Enghild et al., 1989). Human fibronectin was purchased from Sigma Chemical Co. and also generously provided by Dr. Bo

Abbreviations used: α_1-m, α_1-microglobulin; α_1-I_3, α_1-inhibitor-3; PVDF, poly(vinylidene difluoride); V8 proteinase, *Staphylococcus aureus* V8 proteinase.

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Cederholm (Department of Nephrology, University of Lund, Sweden). Rat fibronectin was bought from Sigma and *Staphylococcus aureus* V8 proteinase (V8 proteinase) from Boehringer–Mannheim. Rabbit antisera against rat α_1-I_3, and against human and rat α_1-m, and goat antisera against human α_1-m and rabbit IgG, were prepared and characterized in this laboratory, and purified by ion-exchange chromatography (Nilson et al., 1986). Rabbit anti-(rat fibronectin) antibody was from Calbiochem–Behring and chicken anti-(human fibronectin) and rabbit anti-(chicken IgG) antibodies were from Immunsystem AB (Sweden). Rabbit anti-(human fibronectin) and rabbit anti-(mouse IgG) were obtained from Dakopatts, Denmark.

Chromatography methods

Immunosorbent chromatography was performed using monoclonal anti-α_1-m antibodies BN11.3 and BN11.10 immobilized to Affi-gel Hz (40 mg/2 ml of gel), according to the manufacturer's instructions (Bio-Rad Laboratories, Richmond, CA, U.S.A.). Plasma samples (5 ml) from Sprague–Dawley rats were centrifuged for 10 min at 10000 ***g***. After adding EDTA to a final concentration of 10 mM, the plasma was filtered through a 0.22 μm pore diam. membrane, diluted with 5 ml of 20 mM Tris/HCl, pH 8.0, containing 0.15 M NaCl, 0.02 % NaN_3 and 10 mM EDTA (Tris/NaCl/NaN_3/EDTA), and finally applied to the affinity column which had been equilibrated in the Tris/NaCl/NaN_3/EDTA buffer. After application of the sample and washing with Tris/NaCl/NaN_3/EDTA buffer, the column was eluted with 4 M $MgCl_2$ and the eluate immediately dialysed against 20 mM Tris/HCl, pH 8.0, containing 0.15 M NaCl and 0.02 % NaN_3 (Tris/NaCl/NaN_3). The eluates from a total of 15 ml of rat plasma samples were pooled and concentrated by ultrafiltration and then applied to a column (100 cm × 1.4 cm) packed with Sephacryl S-300 (Pharmacia-LKB Biotechnology AB, Sweden). The column was equilibrated in, and eluted with, Tris/NaCl/NaN_3 buffer, and had been calibrated with Blue Dextran (Pharmacia-LKB Biotechnology AB), human IgG (Sigma), ovalbumin (Sigma) and dinitrophenylalanine (Sigma).

Comparative studies on plasma and serum from different rat strains were performed as described above with a few changes. Samples (2 ml) of plasma or serum were applied to the Affi-gel-coupled monoclonal mouse anti-α_1-m. The column was eluted with 0.1 M glycine/HCl, pH 2.5, and the acidic fractions were immediately neutralized by the addition of 1 M Tris/HCl, pH 8.0. When serum was applied, EDTA was excluded from the sample and dilution buffers. α_1-m-containing fractions were pooled and concentrated.

The following purification protocol was carried out before amino acid sequence analysis. Rat plasma (180 ml, Pel-Freeze Biologicals) was centrifuged for 30 min at 10000 ***g***, and adjusted to 20 mM sodium phosphate, pH 7.0, 10 mM EDTA and 0.5 M NaCl in a total volume of 540 ml. The plasma was then filtered through a 0.22 μm pore diam. membrane, and Nonidet P-40 (NP-40) was added to 1 % before application to the column (2 ml) packed with Sepharose-coupled monoclonal anti-α_1-m antibodies BN11.3 and BN11.6. The column was washed with the dilution buffer, and then eluted with 4 M $MgCl_2$. The eluted sample was dialysed against 50 mM Tris/HCl/50 mM NaCl, pH 7.4. Following concentration the sample was further purified by anion-exchange chromatography employing a Pharmacia f.p.l.c. system and a 1 ml Mono Q column. The elution from this column was done with a linear gradient from 0 to 1.0 M NaCl in 50 mM Tris/HCl, pH 7.4.

Human plasma (1 ml) was subjected to affinity chromatography on a BN11.2 anti-(α_1-m) Affi-gel Hz column (80 mg/2 ml). The column was eluted with 0.1 M glycine/HCl, pH 2.5, acidic fractions were immediately neutralized, and α_1-m-containing fractions in the eluate pooled and concentrated by ultrafiltration as described for rat plasma.

PAGE analysis

PAGE in the presence of SDS, with or without 2-mercaptoethanol in the sample buffer, was performed according to the method of Laemmli (1970). For molecular-mass standards we used high-molecular-mass Rainbow markers from Amersham International (Amersham, Bucks., U.K.). The procedure for performing non-denaturing pore-limit PAGE in 4–20 % gradient gels was modified from the description of Manwell (1977). The gels were run for 17 h at 135 V with continuous circulation of the 89 mM Tris/boric acid, pH 8.34, 2 mM EDTA (Tris/EDTA/boric acid buffer) between the upper and lower buffer reservoirs. Pharmacia standards, thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa) and albumin (67 kDa), were used as markers for molecular masses. Some samples were first separated by the non-denaturing pore-limit PAGE system and then as a second step applied to SDS/PAGE, according to Enghild et al. (1989). After completed pore-limit PAGE, the gels were stained for 5 min, rinsed in distilled water, and protein bands cut out. SDS-containing sample buffer (60 μl), with or without 2-mercaptoethanol, and 1 μl of 1 M NaOH were added to the gel slices, which then were incubated for 30 min at 37 °C. The gel slices were then transferred to the sample application wells of an SDS/PAGE gel and electrophoresis was performed as usual.

Western-blot analysis

Proteins separated by SDS/PAGE or pore-limit PAGE were transferred to poly(vinylidene difluoride) (PVDF) membranes (Immobilon, Millipore, U.S.A.) as described (Madsudaira, 1987). The membranes were incubated with different rabbit or mouse antibodies (10 mg/l), and probed with ^{125}I-labelled goat anti-(rabbit IgG) or rabbit anti-(mouse IgG) (approx. 10 kBq/ml). For a detailed description of the procedure, see Falkenberg et al. (1992). After washing and drying, the membranes were autoradiographed or developed using a Fujix BAS 2000 Bio-imaging analyser (Fuji Films Co., Japan).

Radiolabelling of proteins

Proteins were labelled with ^{125}I (Bio-Nuclear AB, Sweden) by the chloramine-T method (Greenwood et al., 1963) to a specific radioactivity of between 0.3 and 1 MBq/μg of protein.

Determination of specific protein concentrations

Specific rat or human α_1-m concentrations were determined by r.i.a. as described (Åkerström, 1985). Specific concentrations of rat fibronectin or α_1-I_3 were measured by solid-phase r.i.a. Microtitre plate wells (Falcon 3912, Becton Dickinson and Co., U.S.A.) were coated by incubation overnight at room temperature with 50 μl of rabbit anti-(rat fibronectin) serum (diluted 20000-fold in PBS: 8 mM sodium phosphate, 1.5 mM potassium phosphate, pH 7.4, 0.12 M NaCl, 2.7 mM KCl) or rabbit anti-(rat α_1-I_3) IgG (3 mg/l). The wells were washed three times with 0.15 M NaCl/0.05 % Tween-20, and incubated overnight at room temperature with 50 μl of a mixture of radioiodinated rat fibronectin or α_1-I_3 (20 or 40 kBq/ml in PBS/0.05 % Tween-20) and standard or unknown amounts of unlabelled rat fibronectin or α_1-I_3. This mixture had been preincubated overnight at room

temperature in a total volume of 200 μl, using a fibronectin or α_1-I_3 concentration range of 4–2000 μg/l. After a final wash, each well was counted separately for radioactivity.

The relative contents of fibronectin or α_1-I_3 in affinity- or gel-chromatography fractions were also followed by solid-phase r.i.a. Briefly, the microtitre plate wells were coated with proteins by incubating overnight with 50 μl of the diluted chromatographic fractions, washed and incubated overnight with 50 μl of antibody (diluted to 50 mg/l), washed again and incubated for 2 h with ^{125}I-labelled goat anti-(rabbit IgG) (20 kBq/ml). The elution volumes of the α_1-m–fibronectin and α_1-m–α_1-I_3 complexes, estimated from the relative contents of fibronectin and α_1-I_3 respectively, were identical using different dilutions (10-, 50- or 200-fold) of the chromatographic fractions for coating of the microtitre plate wells.

Binding between α_1-m and fibronectin

The binding between α_1-m and fibronectin was studied by solid-phase r.i.a. The wells were coated with different concentrations of rat or human fibronectin, washed and incubated with ^{125}I-labelled rat or human low-molecular-mass α_1-m (0.1 MBq/ml) in PBS and 0.05 % Tween-20, with or without the additions of 6 M guanidine hydrochloride, 2 M, 4 M, or 6 M urea and/or 1 mM 2-mercaptoethanol. Alternatively, the wells were coated with rat low-molecular-mass α_1-m, washed and then incubated with ^{125}I-labelled rat fibronectin.

Preparing samples for amino-acid-sequence analysis

Rat serum samples were purified by anti-α_1-m affinity chromatography and anion-exchange chromatography as described above. Individual fractions from the latter chromatography were separated by SDS/PAGE using the buffer system described by Bury (1981), and bands of interest were electro-eluted from the gels according to Hunkapiller et al. (1983). The electro-eluted proteins were then digested in NH_4HCO_3 with V8 proteinase for 4 h at 37 °C employing an enzyme:substrate ratio of 1:50 (w/w). The digest was made 1 M in guanidine hydrochloride and the peptides were separated in an Applied Biosystems Model 130A h.p.l.c. system using an Ultrasphere C8 column (2.1 cm × 25 cm, Beckman, U.S.A), eluting with 0.1 % trifluoroacetic acid and a linear gradient of 0–90 % CH_3CN. The absorbance was monitored at 220 nm and the peptides were collected manually. The peptides were analysed by Edman degradation and the resulting sequences compared with the Protein Identification Resource Database (National Biomedical Research Foundation) (PIR, NRBF).

Amino-acid-sequence analysis

Automated Edman degradation was carried out in an Applied Biosystems Model 477A sequencer with on-line phenylthiohydantoin analysis using an Applied Biosystems Model 120 h.p.l.c. system. The samples were applied to peptide-sample support discs and sequenced employing the modified cycles PI-BGN and PI-1 recommended by Porton Instruments.

RESULTS

Isolation of an α_1-m–fibronectin complex

The initial aim of this work was to analyse high-molecular-mass complexes of α_1-m in rat plasma, including the previously discovered α_1-m–α_1-I_3 complex (Falkenberg et al., 1990). All α_1-m-containing molecules were isolated from rat plasma by affinity chromatography on immobilized monoclonal anti-α_1-m antibodies. Bound proteins were eluted with 4 M $MgCl_2$, separated by Mono Q ion-exchange chromatography and SDS/PAGE (results not shown). Protein bands with a molecular mass of around 220 kDa were eluted from the polyacrylamide gels, digested by V8 proteinase and the resulting peptides separated by reversed-phase h.p.l.c. Isolated peptides were then submitted to N-terminal amino-acid-sequence analysis. The resulting sequences from three peptides are shown in Figure 1. All three

1 ANPDTGVLTV (residues 1151–1160)

2 GLNQPTDDSCFDPY (residues 2255–2268)

3 LTNLLVRYSPVKNEE (residues 1353–1367)

Figure 1 Amino acid sequences of peptides derived from a rat plasma α_1-m complex

Rat plasma was subjected to anti-α_1-m affinity chromatography, Mono Q-Sepharose anion-exchange chromatography, and SDS/PAGE in the presence of mercaptoethanol. A band around 220 kDa was electro-eluted (Hunkapiller et al., 1983), digested with V8 proteinase and separated by h.p.l.c. on a reversed-phase C column. Three peaks were subjected to N-terminal amino-acid-sequence analysis. After homology searches, all three sequences were found to be identical with rat fibronectin internal amino acid sequences at the positions shown in the Figure.

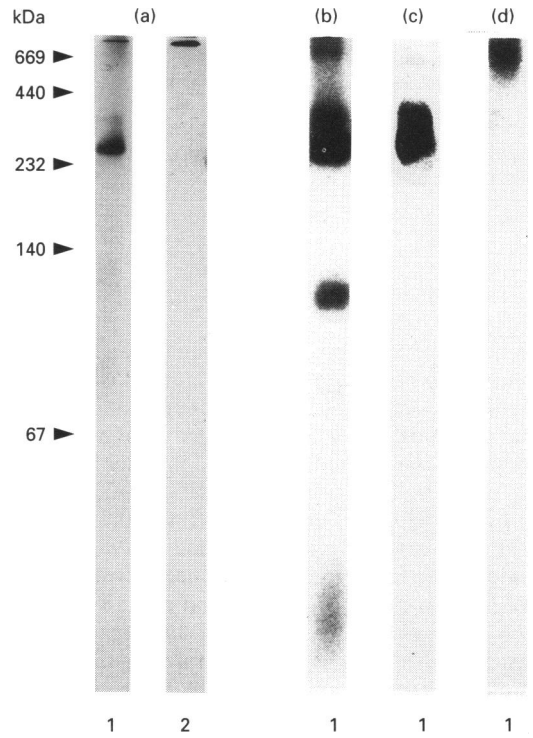

Figure 2 Pore-limit gel electrophoresis of α_1-m-containing proteins in rat plasma

Rat plasma was subjected to affinity chromatography on an anti-α_1-m Affi-gel column. The proteins eluted with 4 M $MgCl_2$ were separated under non-denaturing conditions by pore-limit PAGE (4–20% polyacrylamide), and stained for protein (**a**). In lane 1, approx. 5 μg of proteins of the anti-α_1-m eluate were applied, and in lane 2, 10 μg of rat fibronectin. Lane 1 was also transferred to PVDF membranes (**b–d**). The membranes were incubated with monoclonal anti-α_1-m antibodies (**b**), rabbit anti-(rat α_1-I_3) antibodies (**c**) or rabbit anti-(rat fibronectin) antibodies (**d**), and then probed with ^{125}I-labelled rabbit anti-(mouse IgG) (**b**) or ^{125}I-labelled goat anti-(rabbit IgG) (**c** and **d**). Autoradiography was performed at −30 °C. Molecular-mass markers are shown in kDa.

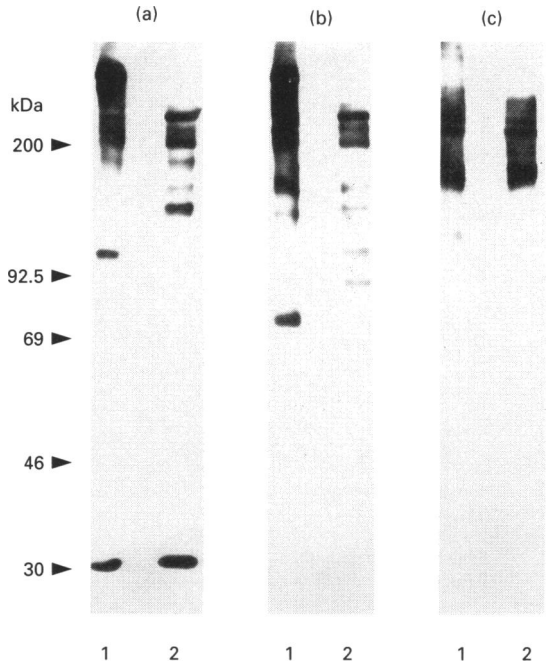

Figure 3 SDS/PAGE of α_1-m-containing proteins in rat plasma

Rat plasma was subjected to affinity chromatography on an anti-α_1-m Affi-gel column. The proteins eluted with 4 M $MgCl_2$ were separated by SDS/PAGE (8.5% polyacrylamide, 2.5% bis-acrylamide) in the absence (lane 1) or presence (lane 2) of mercaptoethanol, and were transferred to PVDF membranes (**a–c**). The membranes were incubated with monoclonal anti-α_1-m antibodies (**a**), rabbit anti-(rat α_1-I_3) antibodies (**b**) or rabbit anti-(rat fibronectin) antibodies (**c**), and then probed with ^{125}I-labelled rabbit anti-(mouse IgG) (**a**) or ^{125}I-labelled goat anti-(rabbit IgG) (**b** and **c**). The radioactivity on the membranes was analysed by digital imaging in a Fujix Bio-imaging analyser.

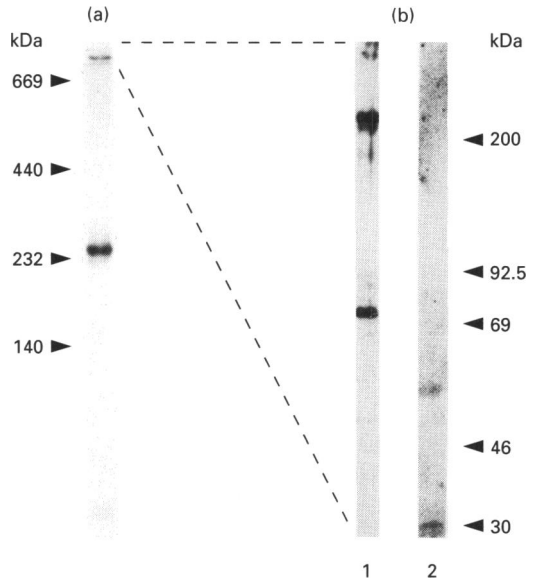

Figure 4 Separation of α_1-m-containing proteins in rat plasma by non-denaturing pore-limit PAGE, followed by SDS/PAGE

Rat plasma was subjected to affinity chromatography on an anti-α_1-m Affi-gel column. The proteins eluted with 4 M $MgCl_2$ were separated under non-denaturing, non-reducing conditions by pore-limit PAGE (see legend to Figure 2), and stained (**a**). A high-molecular-mass anti-fibronectin-binding band, identified in a separate experiment by immunoblotting as described in the legend to Figure 2, was cut out from two equivalent lanes. After equilibration with buffer containing SDS (lane 1) or buffer containing mercaptoethanol and SDS (lane 2), the gel slices were applied to SDS/PAGE (8.5% polyacrylamide, 2.5% bis-acrylamide). After electrophoresis, the gel was blotted on to PVDF membranes (**b**), which were incubated with a mixture of three monoclonal anti-α_1-m antibodies (30 mg/l), and probed with ^{125}I-labelled rabbit anti-(mouse IgG).

sequences were identical with sequences in rat fibronectin [see for instance Hynes (1990)].

The proteins which were eluted from the anti-α_1-m column were subjected to pore-limit PAGE (Figure 2), which separates native molecules according to size. Protein staining of the anti-α_1-m eluate (Figure 2a, lane 1) revealed two strongly stained bands migrating as 795 and 250 kDa. Immunoblotting demonstrated α_1-m in both of these bands (Figure 2b), α_1-I_3 in the 250 kDa band (Figure 2c) and fibronectin in the 795 kDa band (Figure 2d). The fibronectin/α_1-m-containing 795 kDa band was slightly larger than the purified rat fibronectin which was applied in Figure 2(a) (lane 2). A 115 kDa band and an heterogeneous 20–30 kDa band, not visible by protein staining, were seen after blotting with anti-α_1-m. The latter migrated as free, monomeric α_1-m (results not shown), and most likely represents free plasma α_1-m. These results thus suggest that an α_1-m–fibronectin complex, with a molecular mass slightly larger than free fibronectin, is present in rat plasma, as well as free α_1-m and the previously described α_1-m–α_1-I_3 complex (Falkenberg et al., 1990).

The mass of the α_1-m–fibronectin complex was also estimated by gel chromatography. The material which was eluted from the anti-α_1-m affinity column was applied to a Sephacryl S-300 column (not shown). The fractions were analysed for total protein by u.v.–light absorbance and SDS/PAGE, and for reactivity with anti-fibronectin and anti-α_1-I_3 by solid-phase r.i.a. The fibronectin and α_1-I_3 complexes eluted as two incompletely separated peaks, with the top fractions appearing around 560 and 300 kDa respectively.

Disulphide bond formation between α_1-m and fibronectin

The binding between α_1-m and its complexing partners was studied by SDS/PAGE under non-reducing and reducing conditions and immunoblotting of the eluate from the affinity chromatography column (Figure 3). In the absence of reducing agents, most of the α_1-m was found in high-molecular-mass bands above 200 kDa (Figure 3a, lane 1), suggesting that the binding between α_1-m and fibronectin or α_1-I_3 is mostly covalent. The presence of small amounts of free α_1-m, however, indicate that non-covalent forces may be involved to some extent, though base-catalysed hydrolysis of disulphide bonds cannot be ruled out completely. More of the free 28 kDa α_1-m was liberated in the presence of reducing agents (Figure 3a, lane 2), indicating that disulphide bonds are partly responsible for the covalent binding between α_1-m and its complex partners. However, blotting with anti-α_1-I_3 yielded a similar pattern to blotting with anti-fibronectin, both under non-reducing and reducing conditions (Figures 3b and 3c respectively), making it impossible to distinguish between the α_1-I_3 and fibronectin complexes. It was thus difficult to determine whether the 28 kDa α_1-m was liberated from the α_1-I_3 or fibronectin complex. To overcome this problem, the α_1-m–fibronectin complex was isolated by pore-limit PAGE (Figure 4a), and then analysed by SDS/PAGE under non-reducing or reducing conditions, followed by immunoblotting with anti-α_1-m (Figure 4b). All α_1-m was associated with high-molecular-mass bands around 200 kDa when the separation was done without reducing agents (Figure 4b, lane 1), but dissociated completely in the presence of reducing agents (Figure 4b, lane 2).

Table 1 Amounts of fibronectin and α_1-I_3 in the eluates from anti-α_1-m affinity chromatography of plasma and serum from different rat strains

α_1-m-containing proteins were purified from rat plasma or serum by anti-α_1-m affinity chromatography. The concentrations of fibronectin and α_1-I_3 in the original plasma and serum samples (total) and in the eluates (α_1-m-bound) were determined by solid-phase r.i.a. Abbreviations: SpD, Sprague–Dawley; WF, Wistar–Furth; Cop, Copenhagen.

	Fibronectin			α_1-I_3		
		α_1-m-bound			α_1-m-bound	
Source	Total (g/l)	(mg/l)*	(%)	Total (g/l)	(mg/l)*	(%)
SpD plasma	0.46	23	5.0	6.7	42	0.63
SpD serum	0.36	25	7.0	8.0	59	0.74
WF plasma	0.36	23	6.4	7.9	27	0.33
WF serum	0.32	12	3.8	7.2	56	0.77
Cop plasma	0.45	22	4.8	7.8	44	0.57
Cop serum	0.34	12	3.6	7.8	49	0.62

*The values for α_1-m-bound fibronectin and α_1-I_3 were adjusted to compensate for an α_1-m recovery of less than 100% in the affinity chromatography step.

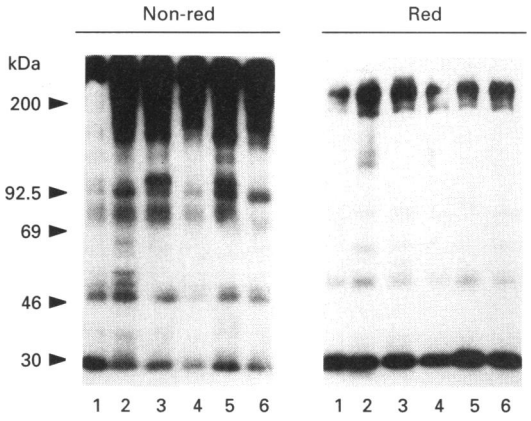

Figure 5 Comparison of α_1-m-containing proteins in plasma and serum from different rat strains

Plasma or serum from three different rat strains were subjected to affinity chromatography on an anti-α_1-m Affi-gel column. The proteins eluted with 0.1 M glycine/HCl, pH 2.5, were separated by SDS/PAGE (8.5% polyacrylamide, 2.5% bis-acrylamide) under non-reducing or reducing conditions. The proteins were then transferred to PVDF membranes, which were incubated with polyclonal rabbit anti-α_1-m antibodies and ^{125}I-labelled goat anti-(rabbit IgG). Lane 1, Sprague–Dawley plasma was applied; lane 2, Sprague–Dawley serum; lane 3, Wistar–Furth plasma; lane 4, Wistar–Furth serum; lane 5, Copenhagen plasma; lane 6, Copenhagen serum.

This suggests that α_1-m and fibronectin are linked together by disulphide bonds. The dissociated α_1-m migrated upon SDS/PAGE as two bands, 28 kDa and 58 kDa.

α_1-m–fibronectin complex in plasma or serum from different rat strains and human plasma

The two α_1-m complexes were quantified in plasma and serum from three different rat strains. Small samples of plasma or serum were applied to anti-α_1-m affinity chromatography columns. No α_1-m was found in the effluents from the column, as

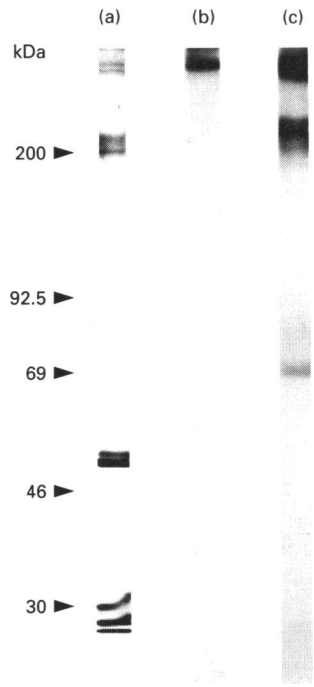

Figure 6 Western blotting of α_1-m-containing proteins in human plasma

Human plasma was subjected to affinity chromatography on an anti-α_1-m Affi-gel column. The proteins eluted with 0.1 M glycine/HCl, pH 2.5, were separated by SDS/PAGE (8.5% polyacrylamide, 2.5% bis-acrylamide) under non-reducing conditions. The gel was protein stained (**a**) or transferred to PVDF membranes, which were incubated with rabbit anti-(human α_2-macroglobulin) (**b**) or anti-(human fibronectin) (**c**) and ^{125}I-labelled goat anti-(rabbit IgG).

determined by r.i.a. The bound α_1-m was eluted with 0.1 M glycine buffer, pH 2.5, and the contents of fibronectin and α_1-I_3 in the eluate and the original plasma or serum samples were estimated by solid-phase r.i.a. (Table 1). Thus approx. 0.3–0.8% of the plasma or serum α_1-I_3 and 3–7% of the plasma or serum fibronectin was bound to the anti-α_1-m column, presumably because of complex formation to α_1-m. No consistent differences were seen between the plasma and serum samples, or between different strains. In one set of experiments, the proteins eluted from the anti-α_1-m affinity column were separated into high-molecular-mass and low-molecular-mass fractions by gel chromatograpy on Sephadex G-200. Approx. 60% of the total α_1-m found by r.i.a. was in the high-molecular-mass fraction, and 40% in the low-molecular-mass fraction.

Some differences were observed when the plasma and serum samples were compared by SDS/PAGE (Figure 5). A band with an apparent mass of 115 kDa was present in plasma samples but not in serum, and another band with an apparent mass of 165 kDa was more pronounced in plasma than in serum. Furthermore, free monomeric α_1-m dissociated from the high-molecular-mass bands when SDS/PAGE was performed in the presence of a reducing agent, supporting the finding that disulphide bonds are involved in the formation of the α_1-m–fibronectin complex.

In an attempt to generalize the findings, human plasma was applied to an anti-α_1-m affinity chromatography column. After washing, bound proteins were eluted with glycine buffer, pH 2.5, analysed by SDS/PAGE under non-reducing conditions and blotted with anti-(human α_2-macroglobulin) or anti-(human fibronectin) (Figure 6). Bands with apparent molecular masses

> 400 kDa were stained by anti-α_2-macroglobulin and bands with apparent molecular masses of > 300 kDa and 210 kDa by anti-fibronectin, indicating the presence of α_1-m–α_2-macroglobulin and α_1-m–fibronectin complexes respectively in human plasma.

DISCUSSION

This work describes the identification, isolation and partial characterization of a complex formed between the two plasma proteins α_1-m and fibronectin. The α_1-m–fibronectin complex was isolated from rat plasma and characterized as a molecule with a mass of 500–600 kDa, which was stabilized by one or more disulphide bonds between the two proteins, and present in the circulation at approx. 20 mg/l.

Another α_1-m complex in rat plasma, formed with the protease inhibitor α_1-I_3, was described in a previous report (Falkenberg et al., 1990). This complex appeared upon SDS/PAGE as several bands with molecular masses around 200–220 kDa which react with antibodies against α_1-m and α_1-I_3. The lack of detection of the α_1-m–fibronectin complex during the purification of the α_1-m–α_1-I_3 complex is partly explained by the almost identical behaviour of the two complexes on SDS/PAGE under reducing conditions (Figure 3). However, anti-α_1-m affinity chromatography of commercial rat plasma, which contained mostly degraded α_1-I_3 (not shown), revealed bands upon SDS/PAGE with molecular masses around 220 kDa that did not react with α_1-I_3, indicating a novel, non-α_1-I_3–α_1-m complex. Elution of the 220 kDa bands from the polyacrylamide gels, followed by protease digestion and purification of the resulting peptides by h.p.l.c., yielded sequences which were identical with those reported for rat fibronectin (see Hynes, 1990), suggesting that the novel complex was α_1-m–fibronectin. No sequences were achieved when analysing the bands before proteolytic cleavage, in accordance with reports from other investigators that the N-termini of human fibronectin are blocked (Garcia-Pardo et al., 1983). The presence of another α_1-m complex besides the α_1-I_3 complex was also suggested by analysis of the anti-α_1-m eluate by non-denaturing pore-limit PAGE. The identity of the complex as α_1-m–fibronectin was then confirmed with immunochemical methods.

It was estimated that 3–7 % of total plasma or serum fibronectin was linked to α_1-m. An immunochemical competitive assay was used, and it was assumed that the α_1-m–fibronectin complex and free fibronectin interacted with anti-fibronectin antibodies on equal terms. The plasma and serum concentrations of fibronectin, 0.3–0.5 g/l, agreed well with reports from other investigators (Sochorova et al., 1983). In parallel, it was estimated that 0.3–0.8 % of total plasma or serum α_1-I_3 was linked to α_1-m. The previously reported value of 1–3 % (Falkenberg et al., 1990) was probably in error because it was based upon a mixture of the α_1-I_3 and fibronectin complexes. Our specific reason for examining differences between the plasma and serum distribution of α_1-m relates to the possible ways in which the protein could incorporate into fibronectin and α_1-I_3. Blood coagulation converts plasma into serum by a proteolytic cascade system during which some fibronectin becomes covalently cross-linked to fibrin by the action of Factor XIII (Mosher, 1975). The proteinases active in this cascade are potentially able to activate the thiol ester of α_1-I_3 and cause it to covalently incorporate proteins via their primary amino groups. If these events were responsible for incorporation of α_1-m, we should observe increased binding of α_1-m to the two proteins by covalent links that do not involve disulphides. Although small structural differences were seen in SDS/PAGE, no major quantitative differences were found in the distribution of α_1-m between its binding proteins in plasma or serum. This rules out blood coagulation as a stimulation or mechanism of α_1-m incorporation into fibronectin and α_1-I_3.

Native fibronectin has been described as a heterodimer composed of two similar disulphide-linked polypeptides, each around 250 kDa (for a review, see Mosher, 1992). The results in the present work are consistent with a model of the α_1-m–fibronectin complex in which the two fibronectin polypeptides and one or more of the 28 kDa α_1-m polypeptides are interconnected by disulphide bonds. The calculated molecular mass of this model, 500–600 kDa depending on the number of α_1-m molecules, is consistent with the value for the isolated α_1-m–fibronectin complex, 560 kDa, achieved by gel chromatography. Larger values were obtained by pore-limit PAGE, 795 kDa for α_1-m–fibronectin and slightly less for uncomplexed fibronectin, most likely due to an elongated shape and low net charge of the molecules.

Fibronectin is described as a very fibrous molecule, with many structurally and functionally independent domains arranged in a linear fashion (for a review, see Hynes, 1990). To a large extent, the domains consist of homologous internal repeats of three types, I, II and III. Several protocols describe the isolation of such domains after proteolytic cleavage of the fibronectin molecule. Thus it should be possible to locate the α_1-m molecule along the fibronectin polypeptide following proteolytic cleavage and isolation of α_1-m-containing fragments. Assuming that the disulphide bond arrangement of rat α_1-m is homologous to its human counterpart, rat α_1-m has an unpaired cysteine residue at position 33 (Lindqvist et al., 1992). Obviously, this residue is a candidate for the disulphide interaction with fibronectin as it is not involved in an intra-chain disulphide loop. Each polypeptide of the fibronectin dimer has two free sulphydryl groups (Hynes, 1990), both of which are located in type III repeats, one near the middle and another near the C-terminal end of the polypeptide chain. Naturally, all four free sulphydryl groups of the fibronectin dimer are candidates for the binding of α_1-m. As mentioned in the introduction to this article, other circulating α_1-m complexes have been described. Thus α_1-m is found complexed to human albumin (Tejler and Grubb, 1976), human IgA (Grubb et al., 1986) and rat α_1-I_3 (Falkenberg et al., 1990). Interestingly, all four complex partners have free sulphydryl groups. So far only α_1-m–fibronectin has been shown to be connected by disulphide bonds, and from a structural point of view it would be interesting to know if the other complexes are also joined via disulphide bonds, and if the complex partners of α_1-m share a similar structure, primary or three-dimensional, around the bonds.

The site of formation of the α_1-m–fibronectin complex is as yet unknown. To exclude the possibility that it was formed during the sample preparation or the purification procedure by an *in vitro* disulphide exchange between free sulphydryl groups on α_1-m and fibronectin respectively, the rat blood was drawn in the presence of *N*-ethylmaleimide, which alkylates reactive sulphydryl groups. However, similar amounts of the α_1-m–fibronectin complex could be purified under these conditions (not shown), indicating that the complex is formed *in vivo*. α_1-m, α_1-I_3 and fibronectin are synthesized by hepatocytes (Tamkun and Hynes, 1983; Pierzchalski et al., 1992), and it could be suspected that the α_1-m–α_1-I_3 and α_1-m–fibronectin complexes are formed in these cells. However, no high-molecular-mass α_1-m was detected in rat hepatocyte culture medium (Pierzchalski et al., 1992). To test whether the complexes are formed after the entry of α_1-m into the vascular compartment, radiolabelled 28 kDa α_1-m was incubated with rat plasma or serum, or was injected into the bloodstream of whole rats (not shown). No

radiolabelled high-molecular-mass α_1-m could be detected after anti-α_1-m affinity chromatography of the plasma or serum, contra-indicating vascular complex formation. However, an alternative explanation is that the free sulphydryl group of the 28 kDa α_1-m, which was purified from rat plasma, is more or less irreversibly blocked by unknown structures, as suggested by Escribano et al. (1991). Thus it can be speculated that *in vivo*, the α_1-m complexes are formed after secretion of free α_1-m into the bloodstream, but before blocking of the sulphydryl group by other structures.

The results in this study have interesting biological implications. α_1-m regulates immune and inflammatory responses, and suppresses antigen-induced lymphocyte proliferation, granulocyte migration and chemotaxis (for a review, see Åkerström, 1992). Fibronectin binds to the extracellular matrix molecules collagen and heparin/heparan and to cell-surface integrin receptors, and acts as a ligand between cells and extracellular matrix during development and organization of tissues. It has binding sites for fibrin and is important for haemostasis and wound healing (Yamada, 1991). Hence, the discovery that 3–7 % of the fibronectin molecules carry α_1-m suggests a mechanism for local immunoregulation during episodes such as wound healing and tissue morphogenesis.

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