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Purification of the human placental α_2-macroglobulin receptor

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The α_2-macroglobulin receptor was solubilized from human placental membranes, purified and characterized. Affinity cross-linking of labelled ligand to intact membranes showed a receptor size compatible with 400–500 kDa. The membranes were solubilized in 3-[(3-cholamidopropyl)dimethylammonio]propane sulfonate (CHAPS) and affinity chromatography was performed using Sepharose-immobilized α_2-macroglobulin-methylamine with elution in buffer containing 2 mM EDTA, pH 6.0. SDS-PAGE of the resulting receptor preparation showed a predominant approx. 440 kDa band (reducing conditions) and some minor accompanying proteins of 70–90 kDa and 40 kDa. The yield was 400–800 μg receptor preparation per placenta. The receptor preparation immobilized on nitrocellulose bound the α_2-macroglobulin-trypsin complex with a dissociation constant of about 400 pM. ^{125}I-iodinated receptor preparation bound almost quantitatively to Sepharose-immobilized α_2-macroglobulin-methylamine in the presence of CHAPS alone, and bound 70–80% in the presence of 0.2% SDS. The labelled proteins were separated in the presence of 0.2% SDS by gel filtration or SDS-PAGE (unboiled samples). The 440 kDa protein accounted for the major part of the binding, although some approx. 80 kDa proteins, perhaps proteolytic degradation products, also showed binding activity.

Macroglobulin receptor, α_2-; Placenta; Inhibitor-3, α_1-

1. INTRODUCTION

The α_2-macroglobulin (α_2M) receptor specifically binds complexes between α_2M and proteinases, whereas native (uncomplexed) α_2M does not. Treatment with methylamine also causes the conversion of α_2M to a receptor-active form. Previous studies have shown that α_2M receptors are abundant in rodent [1–3] and human [4] hepatocytes. In fact, receptor-mediated endocytosis in hepatocytes largely accounts for the rapid removal ($t_{1/2}$ about 2 min) of the α_2M-trypsin complex (α_2MT) in the rat [5]. Recently, the α_2M receptor was identified as a high molecular weight protein in solubilized rat liver membranes [6] and subsequently purified by affinity chromatography as an approx. 440 kDa single-chain protein [7].

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We have previously shown [8] that human placental membranes contain large quantities of α_2M receptor with affinities for the human α_2M-proteinase complex and the homologous pregnancy zone protein-proteinase complex similar to those described in rat and human hepatic membranes [6]. This communication describes the preparation of the placental α_2M receptor. We show that the human placental receptor has the same size as the rat hepatic receptor and that binding activity is preserved in the purified placental receptor protein.

2. MATERIALS AND METHODS

2.1. *Ligands*

Human α_2M was prepared from citrate plasma as described previously [9] and iodinated to a specific activity of about 0.5 mol ^{125}I (Amersham, UK) per mol α_2M using chloramine-T as the oxidizing agent [2]. Receptor-active α_2M was generated either by incubation for 5 min at 20°C with a 5 times molar excess trypsin followed by soybean trypsin inhibitor (yielding

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α_2MT [2]) or by incubation with 200 mM methylamine for 2 h at 20°C followed by dialysis. 0.9–1.0 mol of titrable SH groups were generated by both procedures [9]. Rat α_1-inhibitor-3 (α_1I_3), a 200 kDa homologue of the 180 kDa α_2M subunit which binds to the rat hepatic α_2M receptor [6,11], was prepared [11,12], iodinated and incubated with an equimolar concentration of chymotrypsin as described previously [6,7].

2.2. *Membranes*

Placental membranes were prepared essentially as described [8]. In brief, pieces (1–2 g) of villous tissue were washed in ice-cold phosphate-buffered saline and either frozen in liquid nitrogen or homogenized directly at 0°C in 120 ml of 250 mM sucrose, 10 mM Hepes, 5 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), pH 7.4 (45 s, 80 g wet tissue) using an ULTRA-TURRAX with a 525KG-256 head. Debris was removed by centrifugation first at 600 × *g* for 3 min, then at 3000 × *g* for 5 min. The supernatant was centrifuged at 48000 × *g* for 40 min and the membrane pellet rehomogenized in 140 mM NaCl, 0.6 mM $CaCl_2$, 10 mM Na-phosphate, 0.1 mM PMSF, pH 7.8. The membranes were again centrifuged at 48000 × *g* for 40 min and resuspended in the above buffer to a concentration of about 6 mg protein/ml. The yield from 80 g wet tissue was about 500 mg membrane protein (1.0–1.2 g per placenta). The membranes were either stored at −20°C or solubilized directly.

2.3. *Affinity chromatography*

8–10 mg α_2M-methylamine was coupled to about 1.2 g CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology, Sweden) according to the manufacturers' recommendations. The membranes (6 mg protein/ml) were solubilized by the addition of 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate dihydrate (CHAPS, Aldrich, FRG) to 1%. The suspension was stirred for 10 min at 0°C followed by centrifugation for 40 min at 48000 × *g* and incubation of the supernatant with the affinity gel for 18 h at 4°C. The gel was poured onto a column (bed volume about 4 ml) and washed extensively with 140 mM NaCl, 0.6 mM $CaCl_2$, 10 mM Na-phosphate, 1% CHAPS, pH 7.8. The receptor protein was eluted by reducing pH to 6.0 and adding EDTA to 2 mM. The eluted receptor preparation (4 μg protein) was iodinated by incubation for 3 min with 0.2 mCi $^{125}I^-$ and 16 μg/ml chloramine-T in buffer containing 0.1% CHAPS, pH 8.0 (final volume 55 μl), followed by filtration on Sephacryl S-300 to remove $^{125}I^-$ and minor degradation products. Incorporation of ^{125}I into protein was about 50%.

Rat liver membranes were prepared, solubilized and subjected to affinity chromatography as described previously [6,7].

2.4. *Assays for ligand binding*

Incubations (300 μg membrane protein/ml) were performed in 140 mM NaCl, 0.6 mM $CaCl_2$, 10 mM Na-phosphate, 1% bovine serum albumin, pH 7.8, for 18 h at 0°C. The membranes were pelleted by centrifugation in microfuge tubes [8]. Cross-linking of ^{125}I-α_1I_3-chymotrypsin was performed essentially as described for rat hepatic membranes using disuccinimidyl suberate (DSS), cf. legend to fig.1. Dot blot assay of the receptor protein eluted from the affinity column was performed as follows. Receptor solution (4 μl) was applied to nitrocellulose discs (Sartorius, FRG, diameter 5 mm) and dried at 4°C followed by incubation at 4°C for 1 h in the NaCl-$CaCl_2$-phosphate-CHAPS buffer with 1% bovine serum albumin followed by the addition of ^{125}I-α_2MT with or without unlabelled α_2MT and incubation for 18 h. The discs were then washed in 3 × 1.5 ml ice-cold buffer and counted. About 0.7% of the ^{125}I-α_2MT was associated with the filter when the procedure was performed with discs dotted with eluate not containing receptor protein and the same value was obtained when discs with receptor were incubated with a saturating concentration of unlabelled α_2MT, cf. fig.3.

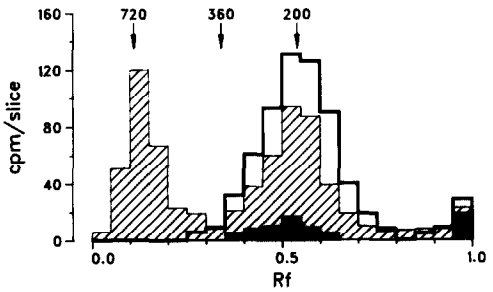

Fig.1. Cross-linking of ^{125}I-α_1I_3-chymotrypsin to placental membranes. Membranes (300 μg protein/ml) were incubated for 18 h at 4°C with about 20 pM ^{125}I-α_1I_3-chymotrypsin in the absence (open and hatched bars) or presence (filled bars) of 200 nM unlabelled α_2MT. DSS dissolved in DMSO was added to 0.2 mM and the incubation continued for 10 min (hatched and filled bars). The open bars (heavy line) show the result of the addition of DMSO only. The membranes were layered upon 60 mM Tris-HCl, 5% glycerol, pH 6.8, pelleted and dissolved in 200 ml of 5% SDS sample buffer. The samples were centrifuged at 100000 × *g* for 15 min followed by SDS-PAGE. The dried gel was sliced and pieces of about 4 mm were counted for radioactivity.

The ^{125}I-labelled receptor preparation was incubated with about 100 nM Sepharose-immobilized α_2M-methylamine. Sepharose-bound and unbound radioactivity was separated by centrifugation followed by four times wash in buffer at 0°C. The blank value was determined as the radioactivity associated with Sepharose-α_2M-methylamine in the presence of 2 mM EDTA (about 2%) and was subtracted from all measurements.

2.5. *Other methods*

Protein concentrations were determined according to Bradford [13] using bovine serum albumin as a standard.

SDS-PAGE with 4–8% or 8–16% polyacrylamide gradients was performed according to Laemmli [14] using 80 mm long, 0.4 mm thick slab gels. Molecular size markers were for non-reduced samples: cross-linked α_2M [6], 720 kDa; α_2M, 360 kDa; α_1I_3, 200 kDa; and for reduced samples: laminin, heavy chain 400 kDa; light chains 220 and 210 kDa [7,15]; and low molecular weight markers from Pharmacia, Sweden. Autoradiography of the ^{125}I-labelled receptor preparation was carried out on dried gels using preflashed Hyperfilm MP (Amersham, UK) with exposure between enhancing screens at −80°C.

3. RESULTS

We first tested that rat α_1I_3-chymotrypsin was bound to the α_2M receptors in human placental membranes as previously shown for rat and human hepatic membranes [6]. Table 1 shows that 20 pM ^{125}I-labelled α_1I_3-chymotrypsin was bound nearly as well as labelled human α_2M-trypsin and that binding of both labelled ligands was almost entirely inhibited by a saturating concentration of α_2M-trypsin. The addition of 2 mM EDTA abolished the binding within 10 min. Thus, α_1I_3-chymotrypsin binds to the placental α_2M receptors with an affinity of the same magnitude as those of human α_2M or pregnancy zone protein complexes [8].

Fig.1 shows the electrophoretic migration of ^{125}I-labelled α_1I_3 cross-linked to placental membranes followed by solubilization. The binding to the membranes in the absence of cross-linker is shown by the open bars (heavy line). The radioactive material migrates corresponding to a protein of about 200 kDa since α_1I_3-chymotrypsin dissociates from the receptor in SDS. An additional peak not distinguishable from a protein of about 700 kDa appears when the labelled ligand is cross-linked to the membranes (hatched bars). The ligand therefore binds to a large molecule compatible with a 400–500 kDa receptor protein. The filled bars show that the large MW species is absent when the membranes are incubated with ^{125}I-α_1I_3-chymotrypsin plus a saturating concentration of unlabelled α_2MT and cross-linked.

The placental membranes were solubilized in 1% CHAPS and applied to affinity chromatography

Table 1

Binding of α_1I_3-chymotrypsin to human placental membranes

	Tracer alone	Plus 100 nM α_2MT	Plus 2 mM EDTA
^{125}I-α_1I_3-chy	11.2 ± 0.9	0.6 ± 0.1	0.9 ± 0.2
^{125}I-α_2MT	16.1 ± 1.3	0.4 ± 0.1	0.7 ± 0.1

The incubations (300 μg membrane protein/ml) were carried out for 18 h with 20 pM ^{125}I-labelled α_1I_3-chymotrypsin or α_2MT. Unlabelled α_2MT, when present, was added together with the tracer. EDTA was added to 2 mM after 18 h followed by further incubation for 10 min. The numbers represent membrane-bound tracer as a % of the total tracer added, mean ± 1 SD, $n = 4$

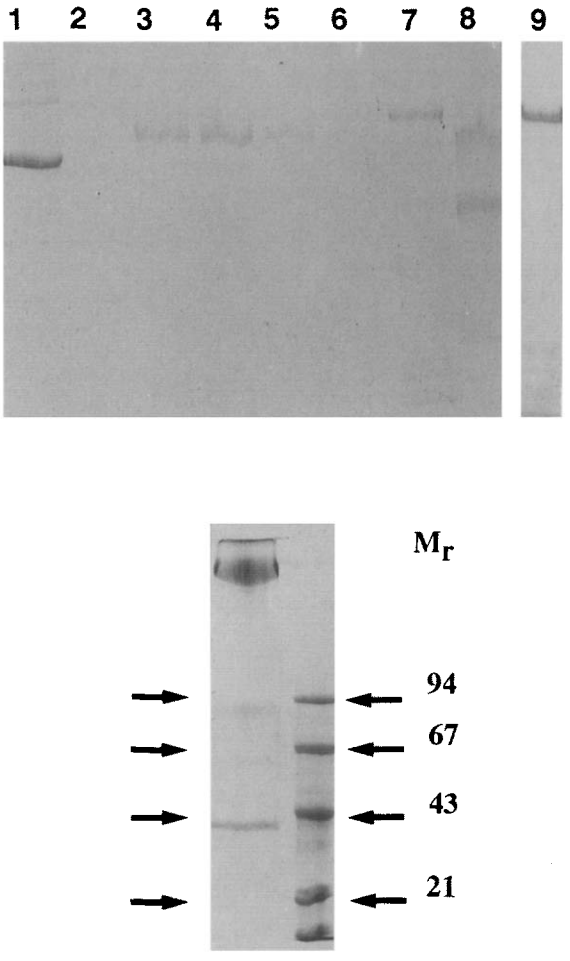

Fig.2. SDS-PAGE of affinity-purified placental α_2M receptor preparation. (Upper panel) 4–8% polyacrylamide. Lanes 1–6, non-reduced samples; lanes 7–9, reduced samples. Lanes: 1, α_2M half molecule (360 kDa); 2, eluate before the pH shift; 3–6, samples (40 μl) of 1.0 ml fractions eluted consecutively; 7, reduced eluted protein; 8, reduced laminin (heavy chain 400 kDa, light chains, 220 and 210 kDa); 9, reduced rat hepatic receptor. (Lower panel) 8–16% polyacrylamide. (Left) About 20 μg non-reduced placental α_2M receptor preparation; (right) molecular size markers. Stain, Coomassie brilliant blue.

using Sepharose-immobilized α_2M-methylamine. Fig.2, upper panel, shows the results using SDS-PAGE. Lanes 1–6 represent non-reduced samples. Lane 1 shows human α_2M used as a molecular size marker. Lane 2 illustrates the absence of protein in the column eluate before the shift to elution buffer with 2 mM EDTA, pH 6.0. The eluted receptor preparation is seen in lanes 3–6 with the pH shift

corresponding to lane 3. The non-reduced large receptor protein shows a slightly slower migration than the 360 kDa α_2M half molecule as previously shown for the rat hepatic α_2M receptor [7]. Dot blot assay (not demonstrated) showed that α_2MT-binding activity coeluted with the large protein as previously shown for the rat hepatic receptor preparation [7]. The yield was 150–300 μg large protein per 500 mg solubilized placental membrane protein.

Lane 7 shows that reduction of the α_2M receptor preparation caused a decreased mobility of the large receptor protein with an estimated size of about 440 kDa when using reduced laminin as a standard (lane 8). This apparent size is not distinguishable from that previously determined [7] for the reduced rat hepatic receptor displayed in lane 9.

Fig.2, lower panel, shows a 8–16% polyacrylamide gel, overloaded with regard to the 440 kDa protein to show the presence of some minor coeluting proteins. A distinct band is seen at about 40 kDa and a smear at 70–90 kDa.

Fig.3 shows that the receptor preparation eluted from the affinity column and immobilized on nitrocellulose bound ^{125}I-labelled α_2MT. The binding was abolished by 500 nM unlabelled α_2MT and the apparent K_d was estimated as about 400 pM (inset). Thus, the α_2MT-binding affinity of the human placental receptor preparation is similar to that of the rat hepatic receptor [7].

The following experiments were performed to ascertain that the 440 kDa protein was the binding species. Preliminary experiments (not demonstrated) showed that the receptor preparation chromatographed as a single peak in buffer with 1% CHAPS on Sephacryl S-300. On the other hand, separation of the minor proteins from the 440 kDa species was achieved in the presence of 2% SDS, but binding activity was abolished. We therefore labelled the receptor preparation in the presence of 1% CHAPS. The labelled preparation was almost quantitatively bound to Sepharose-immobilized α_2M-methylamine and increasing concentrations of SDS caused a progressive decrease of the binding (not shown). Binding of the labelled receptor preparation was about 80% when SDS was added to 0.2%.

Fig.4, upper panel, shows that a partial separation was achieved when the labelled receptor

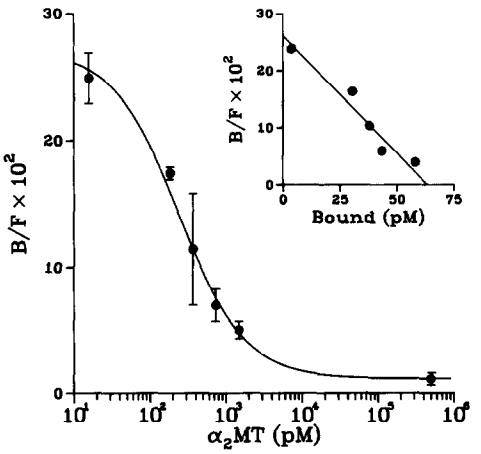

Fig.3. α_2MT binding to nitrocellulose-immobilized α_2M receptor preparation. The nitrocellulose discs with about 70 ng α_2M receptor preparation were incubated for 18 h at 4°C with 15 pM ^{125}I-labelled α_2MT and total α_2MT concentrations as indicated. The inset shows a Scatchard plot of the same data after subtraction of the B/F value at 500 nM α_2MT. The apparent K_d was calculated as 413 pM.

preparation was chromatographed on Sephacryl S-300 in the presence of 0.2% SDS. The peak fraction (A) showed 72% binding to Sepharose-methylamine, whereas only 4–5% of the shoulder fraction (B) was bound. Fig.4, lower panel, shows autoradiograms of gels performed with samples (not boiled) of fractions A and B with 0.2% SDS. Lanes 1 and 3 show that 85–90% of the radioactivity in fraction A was in the 440 kDa band and 10–15% in an approx. 80 kDa band corresponding to the 70–90 kDa smear shown in fig.2B. This shows that the 440 kDa protein accounts for the major part of the binding activity of fraction A. Lanes 2 and 4 show that fraction B had about 10% of the radioactivity in the approx. 80 kDa band and about 90% in the 40 kDa band.

We attempted to obtain more direct data by extracting the labelled proteins into buffer containing 1% CHAPS from the wet gel slices run in parallel to the gels used for autoradiography. The 440 kDa protein bound 15.2 ± 0.5% (n = 3). Thus, the SDS-PAGE and extraction procedure had reduced the ability of the molecules to bind to α_2M-methylamine. The approx. 80 kDa proteins also bound, although to a varying degree (6–16% in three experiments), whereas the 40 kDa protein did not bind.

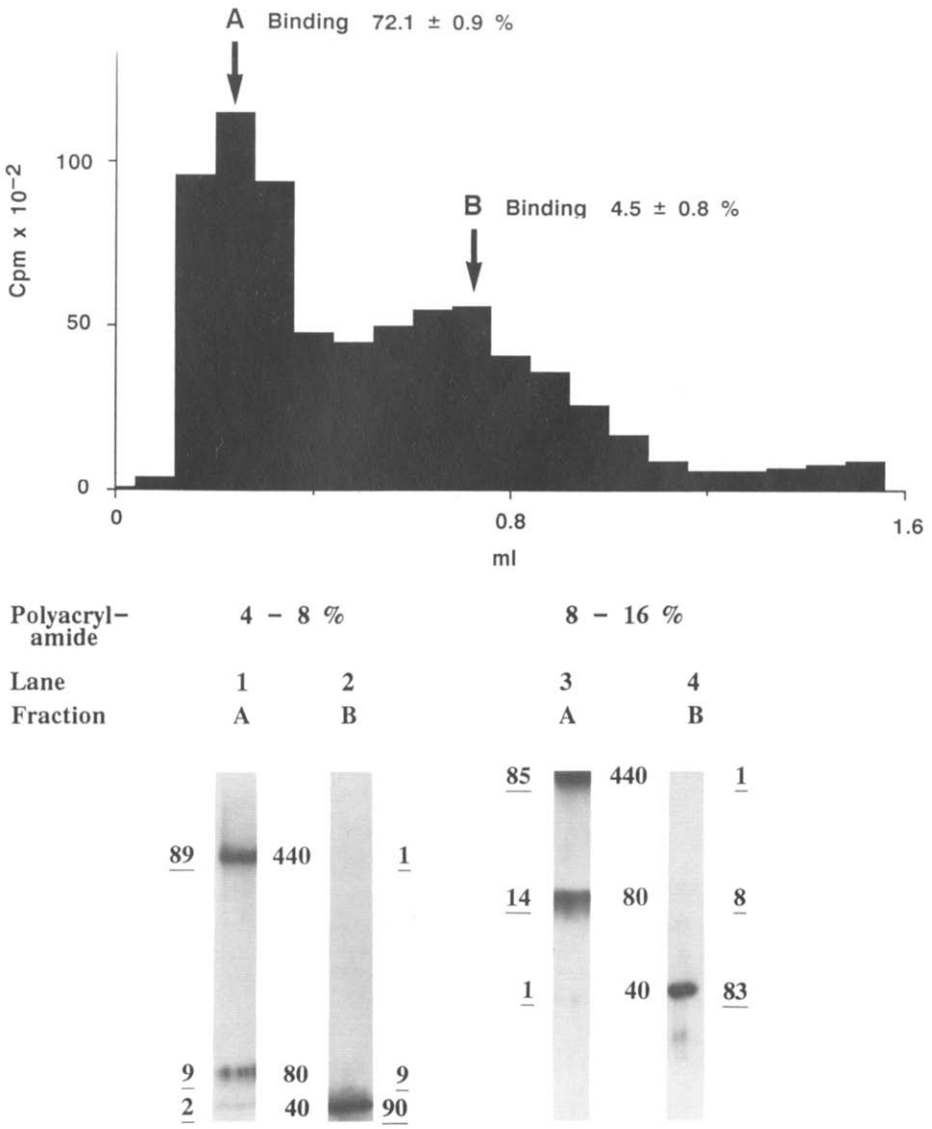

Fig.4. Separation of the ^{125}I-labelled α_2M receptor preparation in the presence of 0.2% SDS. (Upper panel) Sephacryl S-300 chromatography in 140 mM NaCl, 0.6 mM $CaCl_2$, 10 mM Na-phosphate, 1% CHAPS, 0.2% SDS, pH 8.0. The numbers show the % radioactivity of fractions A or B bound to 100 nM Sepharose-immobilized α_2M-methylamine (n = 3). (Lower panel) Autoradiograms (PAGE: 0.2% SDS, unboiled, non-reduced samples) of fractions A and B. The numbers between lanes indicate molecular sizes (kDa) of the radioactive bands. The underlined numbers show the radioactivity in each of the bands, expressed as % of total radioactivity in the lane. The numbers were determined by counting slices of the dried gel.

4. DISCUSSION

The conclusion that the 440 kDa protein is an α_2M receptor is based on the following findings. (i) Affinity cross-linking of the α_1I_3-chymotrypsin complex to intact membranes is compatible with a 400–500 kDa receptor. (ii) The 440 kDa species is by far the dominating protein of the affinity-purified receptor preparation with preserved binding affinity. (iii) Binding of radiolabelled receptor preparation to Sepharose-immobilized α_2M-methylamine is largely accounted for by the

440 kDa protein. These results are similar to those previously described for the rat hepatic α_2M receptor [7].

The origin of the co-purifying minor proteins is uncertain. The simplest explanation is perhaps that they are proteolytic products which bind non-covalently to the 440 kDa receptor in the absence of SDS. If so, the 40 kDa fragment does not contain the ligand-binding domain, whereas one or more of the 70–90 kDa fragments do. However, it cannot be excluded that the 70–90 kDa proteins represent low molecular weight receptors. If so, they appear quantitatively unimportant as judged both from the cross-linking experiments to placental membranes (fig.2) and their low content in the affinity-purified receptor preparation (fig.3). The approx. 440 kDa protein is therefore the major and perhaps the only placental α_2M receptor.

In summary, the human placental α_2M receptor has been purified as an approx. 440 kDa single-chain protein with, at least partially, preserved ligand-binding activity. The affinity-purified receptor preparation contains traces of minor, accompanying proteins, perhaps proteolytic degradation products, and at least one of the 70–90 kDa proteins can bind to the ligand.

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