The Human α_2-Macroglobulin Receptor: Identification of A 420-kD Cell Surface Glycoprotein Specific for The Activated Conformation of α_2-Macroglobulin

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Abstract. Ligand affinity chromatography was used to purify a cell surface α_2-macroglobulin (α_2M) receptor. Detergent extracts of human placenta were applied to an affinity matrix consisting of α_2M, previously reacted with methylamine, coupled to Sepharose. Elution with EDTA specifically released polypeptides with apparent molecular masses of 420 and 39 kD. In some preparations, small amounts of a 90-kD polypeptide were observed. The 420- and 39-kD polypeptides appear specific for the forms of α_2M activated by reaction with proteinases or methylamine and do not bind to an affinity matrix consisting of native α_2M coupled to Sepharose. Separation of these two polypeptides was accomplished by anion exchange chromatography, and binding activity was exclusively associated with the 420-kD polypeptide. The purified 420-kD protein binds to the conformationally altered forms of α_2M that are known to specifically interact with α_2M receptors and does not bind to native α_2M. Binding of the 420-kD polypeptide to immobilized wheat germ agglutinin indicates that this polypeptide is a glycoprotein. The cell surface localization of the 420-kD glycoprotein was confirmed by affinity chromatography of extracts from surface radioiodinated fibroblasts. These properties suggest that the 420-kD polypeptide is a cell surface receptor for the activated forms of α_2M.

α_2-MACROGLOBULIN (α_2M)[1] is a 718,000–mol wt glycoprotein that is capable of inhibiting enzymes from all four classes of proteinases (2) in a reaction that is essentially irreversible. The reaction of proteinases or methylamine with α_2M "activates" the molecule by inducing conformational changes in α_2M (3, 4, 16, 49) that, in the case of the proteinase reaction, reduce the activity of the bound enzyme toward large molecular weight substrates. These conformational changes also generate sites on the inhibitor that result in specific recognition by cell surface receptors (31, 52, 54). It has been possible to prepare monoclonal antibodies that selectively recognize the activated forms of α_2M (34, 50) by binding to these newly generated regions (25).

In addition to regulating proteinase activity, studies have suggested that α_2M binds to TGFβ (41), PDGF (22), and bFGF (7). The association of these molecules with α_2M results in a reduction of their activity, suggesting that α_2M may serve not only to modulate proteinase activity but also the activity of these molecules. Interestingly, it appears that several tumor-derived or virus-transformed cell lines are devoid of specific cell surface receptors for α_2M–proteinase complexes (52).

The process of receptor-mediated endocytosis has been the subject of extensive study, and the mechanisms involved in endocytosis have been investigated using a variety of labeled ligands (6), including transferrin (20), low density lipoprotein (1), EGF (20), and α_2M (8, 37, 54, 55). Early studies reported the specific binding of α_2M to fibroblasts (9, 52), and it was recognized that the binding of α_2M to cells was specific for the forms of α_2M activated either by proteolysis or by reaction with methylamine (23, 32, 52). Despite the widespread use of α_2M in studying the process of endocytosis, little is known about the polypeptide composition and structure of the α_2M receptor. A more comprehensive understanding of the function of the α_2M receptor requires a knowledge of the structure of this molecule. The present investigation was initiated to characterize the α_2M receptor.

Materials and Methods

Proteins

α_2M was prepared according to the method of Imber and Pizzo (23) followed by immunoaffinity chromatography (50) to remove trace amounts of the cleaved form when necessary. α_2M-trypsin complexes were prepared by reacting α_2M with a 1.8-fold molar excess (active site concentration) of trypsin. Trypsin activity was then inhibited with 20 mM diisopropyl fluorophosphate. α_2M was iodinated with Enzymobeads (Bio-Rad Laboratories, Richmond, CA). The specific radioactivity of the labeled protein was 1 μCi/μg protein. Upon activation with trypsin or with methylamine, SDS-PAGE followed by autoradiography of this material showed a distribution of products that was essentially identical to that obtained for unlabeled acti-

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1. *Abbreviations used in this paper:* α_2M, α_2-macroglobulin; α_2M:Me, methylamine-treated α_2-macroglobulin; NRK, normal rat kidney.

vated α_2M. Monoclonal antibody 7H11D6 was prepared as described (25, 50). Mouse laminin was provided by Dr. Albert Chung (Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA).

Preparation of Methylamine-treated α_2M (α_2M:Me)-Sepharose

Native α_2M (8.6 mg/ml) was incubated with 200 mM methylamine in 50 mM sodium phosphate, 150 mM NaCl, pH 7.4, for 1 h at room temperature. The α_2M:Me was then dialyzed extensively against 0.1 M NaHCO$_3$, 0.5 M NaCl, pH 8.3. After dialysis, the protein was coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) as recommended by the manufacturer using 10 mg α_2M:Me/ml resin. The coupling was allowed to proceed for 2 h at room temperature using end-over-end mixing. After coupling, the solution was removed and replaced with 0.1 M Tris, pH 8.0, for an additional 2 h at room temperature. The resin was then washed extensively with alternating buffers of 0.1 M sodium acetate, 0.5 M NaCl, pH 5.0, and 0.1 M sodium bicarbonate, pH 8.3, 0.5 M NaCl.

Purification of the α_2M Receptor

All procedures, unless indicated, were carried out at 4°C. Fresh placenta, obtained from Montgomery General Hospital (Olney, MD) was washed with cold TBS (50 mM Tris, 150 mM NaCl, pH 7.4) and 200 mM sucrose. The fetal membranes and umbilical cord were removed, and the placenta was ground and either used immediately or stored at −20°C until needed. The tissue was suspended in an equal volume of TBS containing 0.005% digitonin, 1 mM each of MgCl$_2$ and CaCl$_2$, and the following proteinase inhibitors: 1 mM PMSF, 0.02 mg/ml leupeptin, and 0.02 mg/ml D-Phe-Pro-Arg-Ch$_2$Cl. After stirring for 15 min on ice, the mixture was homogenized in a blender (three times for 30 s each) and then centrifuged at 5,000 g for 20 min. The supernatant was discarded, and the pelleted tissue was suspended in an equal volume of extraction buffer (50 mM octyl-β-D-glucopyranoside in TBS containing 1 mM each of MgCl$_2$ and CaCl$_2$, 1 mM PMSF, 0.02 mg/ml leupeptin, 0.02 mg/ml D-Phe-Pro-Arg-Ch$_2$Cl for 1 h at 4°C or on ice. The suspension was subjected to centrifugation at 5,000 g for 20 min, and the supernatant from this step was subjected to additional centrifugation at 11,000 g for 20 min. The resultant supernatant was then applied to a 120-ml Sepharose CL-4B column. The unabsorbed material from this column was mixed overnight at 4°C with 40–60 ml of the α_2M:Me-Sepharose column. The resin was then washed with eight column volumes of 25 mM octyl-β-D-glucopyranoside in TBS containing 1 mM CaCl$_2$, 1 mM MgCl$_2$, 1 mM PMSF. Elution was achieved using TBS containing 20 mM EDTA, 25 mM octyl-β-D-glucopyranoside. Further purification was achieved by applying the eluate from this column to a Mono Q anion exchange column (Pharmacia Fine Chemicals) at room temperature previously equilibrated with 20 mM octyl-β-D-glucopyranoside in 50 mM Tris, pH 8.2, at a flow rate of 0.5 ml/min. After washing the column, a linear gradient from 0 to 1 M NaCl was applied over 40 min using a Waters Instruments, Inc. (Rochester, MN) 650E advanced protein purification system. Protein was monitored at 280 nm, and 1-ml fractions were collected. Fractions containing the receptor were stored at 4°C and used for further studies. The concentration of the receptor was estimated by absorbance measurements at 280 nm assuming an $E_{1\%}^{280 \text{ nm}}$ of 10.0.

SDS-PAGE

A discontinuous pH gel system (Laemmli) was used with 4% polyacrylamide stacking gel and a 5–15% separating gel (1.5 mm × 16 cm × 13.5 cm). Proteins were stained with Coomassie blue R-250. Before SDS-PAGE, samples were either reduced in SDS sample buffer with 50 mM DTT at 50°C for 10 min or in SDS sample buffer with 1% β-mercaptoethanol at 100°C for 5 min.

Surface Labeling of Fibroblasts

Normal rat kidney (NRK) fibroblasts (CRL 1570), obtained from the American Type Culture Collection (Rockville, MD) were grown in a 75-ml flask in DME containing 5% heat-inactivated FCS with 1% penicillin/streptomycin and 1% L-glutamine. Cells (2.5 × 10^7) were removed from the flask with 2 mM EDTA, pH 7.4, counted, washed twice with serum-free DME, and then resuspended in 3–4 ml of 20 mM glucose in Dulbecco's PBS. The cells were concentrated by centrifugation and resuspended in 1 ml of 20 mM glucose/Dulbecco's bufer. Glucose oxidase (0.4 U), lactoperoxidase (200 μg), and Na^{125}I (1 mCi) were then added and incubated with the cells for

20 min on ice with frequent mixing. After this period, the cells were washed three times (3 ml each wash) with cold PBS, and resuspended in 1 ml of extraction buffer. After stirring for 1 h at 4°C, the supernatant was mixed with 1 ml of α_2M:Me-Sepharose overnight. After washing, the column was eluted with a buffer containing 20 mM EDTA.

Binding of α_2M to the Purified 420-kD Protein

Two assays were used to measure the binding activity of various fractions. The first assay measured the binding of affinity-purified (50) ^{125}I-labeled α_2M:Me (or α_2M) to microtiter wells coated with either the purified receptor or with various column fractions. Coating was carried out in 50 mM Tris, 150 mM NaCl, 5 mM CaCl$_2$, pH 7.4, and was allowed to proceed overnight at 4°C. After washing, the wells were blocked with 3% BSA and incubated with ^{125}I-labeled α_2M:Me (5–7 nM) in the presence of increasing concentrations of unlabeled α_2M or α_2M:Me. The buffer used was 50 mM Tris, 150 mM NaCl, 5 mM CaCl$_2$, 3% BSA, pH 7.4. After a 2-h incubation at 4°C, the wells were washed and counted. Binding data were analyzed as described by Munson and Rodbard (39) using the computer program LIGAND. In some experiments, ^{125}I-labeled α_2M-trypsin was used in place of α_2M:Me. The binding assay was shown to depend upon the concentration of added ligand, upon the mass of protein coated to the microtiter wells, and, further, on the form of α_2M used. An ELISA (10) was used to measure the specificity of various α_2M derivatives for the purified 420-kD polypeptide. This assay was performed by coating wells of microtiter plates (Immulon II; Dynatech Laboratories, Inc., Alexandria, VA) with 100 μl purified 420-kD polypeptide (5 μg/ml) in coating buffer (Dulbecco's PBS plus 1 mM CaCl$_2$) for 6 h at room temperature. After washing with PBS, the plate was blocked with 10 mg/ml BSA in coating buffer for 1 h at room temperature. The plates were then washed with PBS containing 0.05% Tween 20, and various amounts of α_2M or α_2M-trypsin complexes diluted in PBS containing 0.05% Tween 20, 10 mg/ml BSA, and 1 mM CaCl$_2$ were added. Incubation was carried out overnight at 4°C. After washing, rabbit anti-human α_2M (Boehringer Mannheim Biochemicals, Indianapolis, IN) diluted 1:10,000 was added, and detection was accomplished using an anti-rabbit IgG-alkaline phosphatase conjugate. All experiments included control wells that contained no receptor coating. The data were analyzed according to the following relationship (24):

$$A = A_{max} \left(\frac{[L]}{K_d + [L]} \right),$$

where A is the absorbance at 410 nm, A_{max} is the maximum absorbance at saturation and is treated as a variable parameter, $[L]$ is the molar concentration of free α_2M or α_2M-trypsin, and K_d is the dissociation constant. Application of this analysis to the present data assumes that the amount of added ligand is greater than the amount of receptor bound to the microtiter wells. This is especially true at higher ligand concentrations. Under these conditions, the concentration of free α_2M or α_2M-trypsin is approximately equal to the total concentration of α_2M or α_2M-trypsin.

Results

Affinity Chromatography of Octyl-β-D-glucopyranoside Extracts from Placenta

Binding studies have suggested that relatively large quantities of α_2M receptor are present in placenta (28). This tissue is an abundant source of receptors for fibronectin (43), vitronectin (43), and transferrin (51). Consequently, studies were initiated to isolate the α_2M receptor from octyl-β-D-glucopyranoside extracts of human placenta. All procedures were carried out in the presence of proteinase inhibitors, and solubilization was performed in a buffer containing 50 mM octyl-β-D-glucopyranoside, 1 mM Ca^{2+}, and 1 mM Mg^{2+}. The extract was first applied to a Sepharose CL-4B column to remove any protein with an affinity for this matrix and then applied to an affinity column prepared by coupling α_2M previously reacted with methylamine (α_2M:Me) to Sepharose CL-4B. After extensive washing, the column was eluted with metal-free buffer containing 20 mM EDTA. The elution
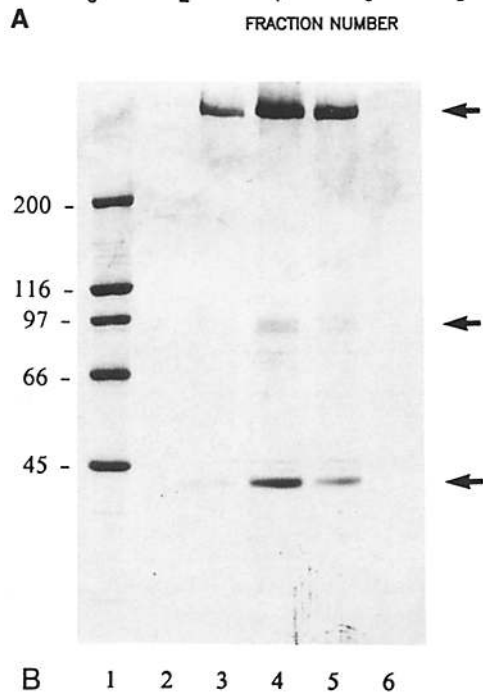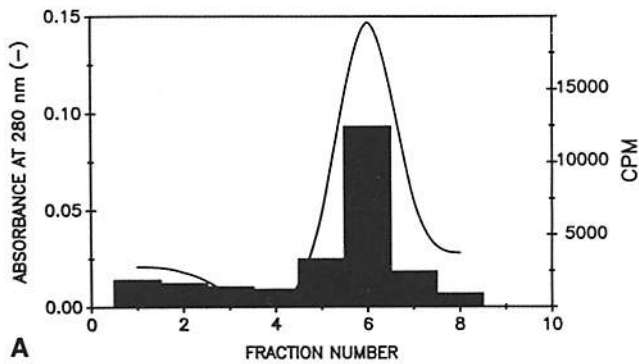
Figure 1. Affinity chromatography of placental extract over Sepha-rose–α_2M:Me. (*A*) Elution profile after addition of buffer containing 20 mM EDTA (*solid line*). Individual fractions were diluted 20-fold and coated to microtiter wells, and 5.0 nM ^{125}I–α_2M–trypsin was added. After incubation and washing, the wells were counted (*bars*). Nonspecific binding was determined by adding a 200-fold molar excess of unlabeled α_2M–trypsin. (*B*) Polypeptide composition of fractions eluted from the α_2M:Me–Sepharose affinity column assessed by SDS-PAGE. Aliquots (20 μl) from each fraction were mixed with SDS sample buffer containing 50 mM DTT. After incubation, the samples were subjected to electrophoresis on a 5–15% gradient gel with a 4% stacking gel using the Laemmli buffer system. (Lane *1*) Standards; (lanes *2–6*) fractions 4–8 from the affinity column.

column was assessed by SDS-PAGE. Representative data, shown in Fig. 1 *B*, reveals that three polypeptides (Fig. 1 *B*, *arrows*) are eluted from the affinity column. Polypeptides with apparent molecular mass of 420 and 39 kD are the major proteins eluted from the affinity column. A third polypeptide, with an apparent molecular mass of 90 kD is present in variable amounts in different preparations. In some preparations, this polypeptide can be readily detected by SDS-PAGE (e.g., Fig. 1 *B*), while in other preparations (e.g., Fig. 2, lane *1*) only trace levels are present. The variable amounts of the 90-kD polypeptide in different preparations support the contention that this protein is not exclusively responsible to the binding activity. The data shown in Fig. 1 *B* represent SDS-PAGE under reducing conditions. Analysis of the samples under nonreducing conditions revealed that none of the polypeptides are covalently linked by disulfide bonds.

Binding studies have documented that cells bind specifically to the activated forms of α_2M (32, 52). These forms occur after reaction of α_2M with either methylamine or proteinases. To examine the specificity of the 420- and 39-kD polypeptides for various α_2M conformers, a portion of a placental extract was applied to an affinity column consisting of native α_2M coupled to Sepharose. In a control experiment, an equal portion was applied to an α_2M:Me affinity column. Fig. 2 shows an analysis of the eluted proteins by SDS-PAGE and demonstrates that the 420- and 39-kD polypeptides (Fig. 2, *arrows*) are specific for the activated forms of α_2M (Fig. 2, lane *1*) and do not bind to native α_2M (Fig. 2, lane *2*). Preincubation of the α_2M:Me affinity column with stoichiometric amounts of monoclonal antibody 7H11D6 reduces the amount of both the 420- and 39-kD polypeptides recovered from the column (data not shown). This monoclonal antibody binds to the region of α_2M that is involved in cell recognition (25, 50).

The requirement of metal ions for the binding of this protein to immobilized α_2M:Me is in agreement with the known requirement of metal ions for the binding of α_2M:Me and α_2M–proteinase complexes to cells (11, 13, 18, 32, 40, 42, 52). Many ligands, such as α_2M–proteinase complexes, that enter the cell by receptor-mediated endocytosis are delivered to lysosomes, while their receptors are recycled back to the cell surface (5). This mechanism requires that the receptor-ligand complex dissociate, which for a number of receptor systems is presumably the result of lowering of the pH. The interaction of activated α_2M with cells (36) and with solubilized membranes (15) is pH dependent, with maximal binding occurring between pH 7 and 8 and a reduction in affinity
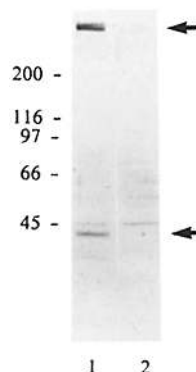
profile from the affinity column is depicted in Fig. 1 *A*. Dilute aliquots of each fraction were adsorbed onto microtiter wells and tested for their ability to bind ^{125}I-labeled α_2M–trypsin complexes. Nonspecific binding, determined by including a 200-fold molar excess of unlabeled α_2M–trypsin with the labeled ligand, was ∼9% of total binding. The binding activity measured by this assay correlated with the total protein eluted from the column, and the presence of specific binding in these fractions indicates that it is possible to reconstitute binding activity.

The polypeptide content of each fraction from the affinity

Figure 2. Analysis of proteins derived from affinity chromatography of placental extracts on immobilized α_2M:Me (lane *1*) or immobilized native α_2M (lane *2*) by SDS-PAGE. 15-ml extracts were applied to 1-ml columns, incubated overnight at 4°C, and washed. After washing, the columns were eluted in a buffer containing 20 mM EDTA. Equal volumes (65 μl) of the eluted fractions were analyzed by SDS-PAGE under reducing conditions on 5–15% gradient gels.
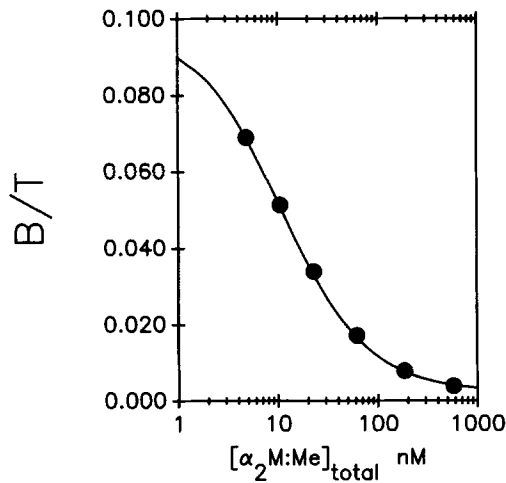
Figure 3. Binding of ^{125}I-labeled α_2M:Me to the immobilized receptor preparation obtained from the α_2M:Me affinity column. Microtiter wells, coated with receptor, were incubated with 4.9 nM ^{125}I-labeled α_2M:Me in the presence of increasing concentrations of unlabeled α_2M:Me. The curve displays the best fit of the data to a single class of sites with a K_d of 10 nM. Each point represents the average of duplicates.

as the pH decreases below 6.0. Consistent with this is the observation that the 420- and 39-kD polypeptides could be eluted from the affinity resin with a pH 5.0 sodium acetate buffer.

Since it is possible to reconstitute binding activity after elution from the immobilized α_2M:Me column, experiments were conducted to measure the affinity of this material for α_2M:Me. Fig. 3 demonstrates the results of an experiment in which a constant amount of ^{125}I-labeled α_2M:Me, with increasing amounts of unlabeled α_2M:Me, is added to microtiter wells coated with the material eluted from the affinity column. The curve in Fig. 3 represents the best fit of the data to a single class of sites using the procedure described by Munson and Rodbard (39), and a K_d of 10 nM was derived from the fit. In several different α_2M receptor preparations, values of the K_d ranged from 2 to 14 nM. These values are relatively close to those values of 0.2–10 nM reported for the binding of α_2M:Me to various cells (11, 13, 15, 17, 48).

### Demonstration That the Binding Activity Is Associated with the 420-kD Polypeptide

The following experiments were conducted to determine which of the polypeptides eluted from the α_2M:Me affinity column is responsible for the binding activity. No binding of ^{125}I-α_2M:Me to any polypeptide was detected after SDS-PAGE and transfer to immobilon. An additional chromatography step was used to separate the components eluted from the affinity column. This was accomplished by anion exchange chromatography on a Mono-Q column (Fig. 4 A). The α_2M-trypsin binding activity was associated with those fractions that were eluted with 0.5–0.6 M NaCl. Examination of individual fractions by SDS-PAGE (Fig. 4 B) revealed that these fractions contain the 420-kD polypeptide (Fig. 4 B, lanes 4–7). The 39-kD fragment does not bind to the column (Fig. 4, lane 2). Additional experiments revealed that the

majority of the 90-kD polypeptide, when present, does not bind to the Mono Q resin, and is present with the 39-kD polypeptide.

A comparison of the binding of ^{125}I-α_2M-trypsin to the various fractions derived from the anion exchange column is shown in Fig. 5. In these experiments, the purified 420-kD polypeptide and a pool of the unabsorbed fractions from the Mono Q column containing the 39-kD polypeptide were coated to microtiter wells. As a control, material recovered from the α_2M:Me affinity column was also included. ^{125}I-α_2M-trypsin (5 nM) was then added in the absence (Fig. 5, solid bars) or presence (Fig. 5, open bars) of a 200-fold molar excess of unlabeled α_2M-trypsin. After incubation and washing, the amount of labeled α_2M-trypsin bound to each well was measured. The results confirm that the 420-kD polypeptide specifically binds α_2M-trypsin complexes. Very
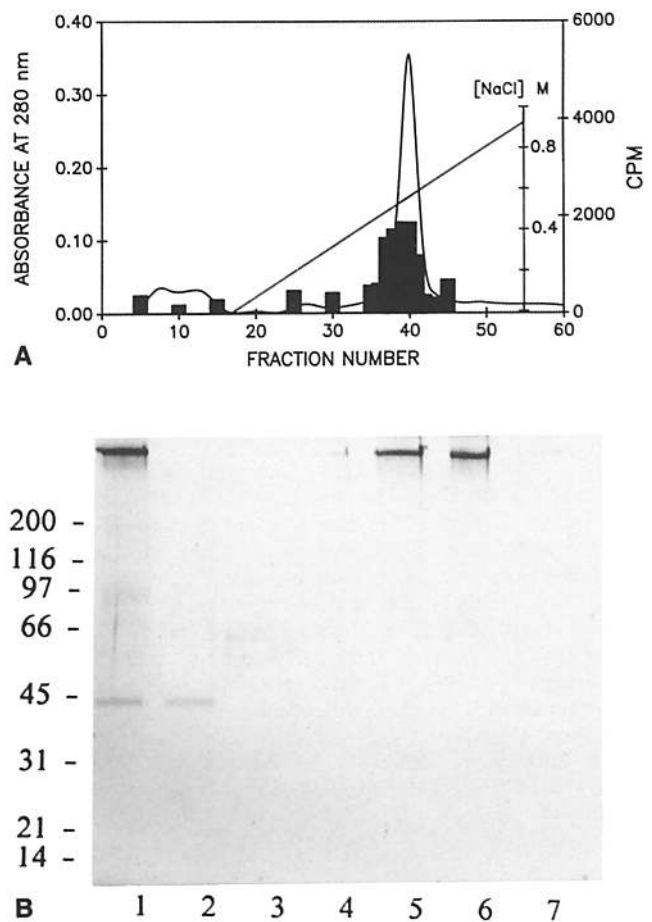

Figure 4. (A) Separation of the 420- and 39-kD polypeptides by anion exchange chromatography on a Mono Q column. The flow rate was 0.5 ml/min, and 1-ml fractions were collected. For assay, fractions were diluted 40-fold, coated to microtiter wells, and incubated with 5 nM ^{125}I-α_2M-trypsin. After incubation and washing, the wells were counted (bars). Nonspecific binding was determined by adding a 200-fold molar excess of unlabeled α_2M-trypsin. (B) Analysis of selected fractions by SDS-PAGE on 5–15% gradient gels. (Lanes 1–7, respectively) Material loaded on the column, pool of fractions 7–14, fraction 35, fraction 37, fraction 39, fraction 41, and fraction 43. In lanes 1 and 2, 40-μl aliquots were applied to the gels, while, in lanes 3–7, 10-μl aliquots were applied.
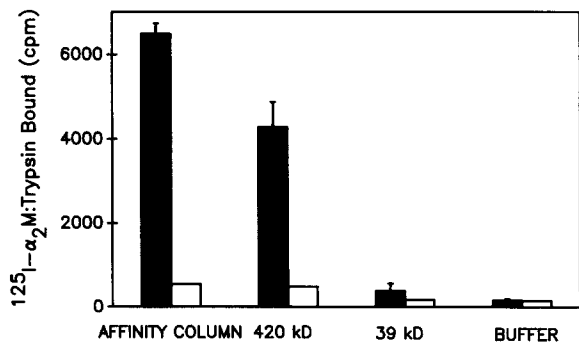
Figure 5. Comparison of the binding of ^{125}I-labeled α_2M–trypsin to the purified 420-kD polypeptide and the 39-kD polypeptide. Microtiter wells were coated with 100 μl of 420-kD polypeptide (21 nM) or 100 μl of 39-kD polypeptide (34 nM). After washing, the wells were incubated with 5 nM ^{125}I-α_2M–trypsin in the absence (solid bars) or presence of a 1 μM unlabeled α_2M–trypsin (open bars). After incubation overnight at 4°C, the wells were washed and counted. As a control, binding to the material derived from the α_2M:Me affinity column is also shown.

little binding of these complexes to microtiter wells coated with the 39-kD polypeptide occurs.

The experiments presented in Fig. 5 indicate that the purified 420-kD polypeptide specifically binds ^{125}I-α_2M–trypsin complexes whereas the 39-kD polypeptide does not bind these complexes. A comparison of the binding activity of the purified 420-kD receptor with the material eluted from the affinity resin is best accomplished by a comparison of the K_d values for these two samples. Fig. 6 demonstrates the results of a binding experiment using the purified 420-kD polypeptide. A K_d of 57 nM was determined from a best fit of the data to a single class of sites. This value is slightly higher than the K_d measured for the material recovered from the affinity column and most likely reflects the fact that optimal
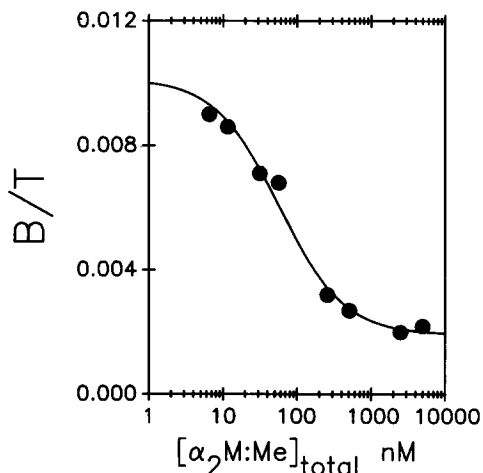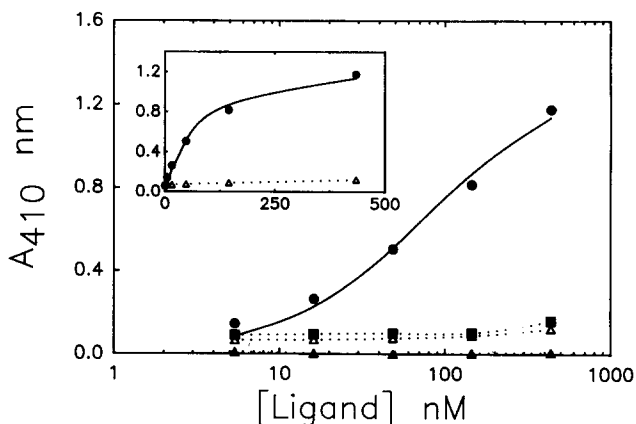
reconstitution conditions have not been achieved. Binding experiments, in which the purified 39- and 420-kD polypeptides were mixed before assay, suggest that the 39-kD polypeptide has little influence on the affinity of the 420-kD polypeptide for ^{125}I-labeled α_2M–trypsin.

Specificity of the Purified Placental Protein for α_2M-Proteinase Complexes

An ELISA (10) was used to measure the specificity of the purified 420-kD polypeptide. For these experiments, affinity-purified native α_2M (50) was used to ensure no contamination of this form with the proteolyzed form. The results of these experiments (Fig. 7) demonstrate the specific binding of activated α_2M to microtiter wells coated with the purified 420-kD polypeptide. Very little binding of native α_2M to the 420-kD protein was detected. Furthermore, no binding of any form of α_2M to wells coated with BSA or with the purified human fibronectin receptor was observed. Thus, the purified placental protein demonstrates a similar specificity to that documented in studies examining the binding of derivatives of α_2M to cells. The data reveal that the solid phase binding approaches saturation (Fig. 7, inset) and when fit to a single class of binding sites (Fig. 7, solid line) yielded an estimate for the K_d of 70 nM. This value is in good agreement with the K_d determined from the displacement experiments described earlier.

Estimate of the Size of the Purified α_2M Receptor and Demonstration That This Polypeptide Is a Glycoprotein

Fig. 8 A shows an analysis of the purified polypeptide by SDS-PAGE. Comparison of its relative mobility with that of the reduced laminin A chain ($\sim$400 kD) confirm that this protein is unusually large. Extrapolation of the molecular

Figure 6. Binding of ^{125}I-labeled α_2M:Me to the purified receptor preparation obtained after anion exchange chromatography. Microtiter wells, coated with receptor, were incubated with 7 nM ^{125}I-labeled α_2M:Me in the presence of increasing concentrations of unlabeled α_2M:Me. The curve displays the best fit of the data to a single class of sites with a K_d of 57 nM. Each point represents the average of duplicates.

Figure 7. Specificity of the purified α_2M receptor for activated α_2M. The binding of native α_2M ($\triangle$) or α_2M–trypsin complexes ($\bullet$) to mirotiter wells coated with purified 420-kD polypeptide were measured. Control experiments show the binding of α_2M–trypsin complexes to microtiter wells coated with BSA ($\blacktriangle$) or with the fibronectin receptor ($\blacksquare$). The binding of native α_2M to BSA and the fibronectin receptor was identical to that shown for α_2M–trypsin complexes. (Inset) Replot of the data in the form used for the fitting process. Solid line in both graphs represents the best fit of the data to a single class of sites, giving a K_d of 70 nM and A_{max} of 1.33.

mass standard calibration curve suggests that this protein has an apparent molecular mass of 420 kD under reducing conditions. Under nonreducing conditions, the mobility of the α_2M receptor increases slightly, suggesting that the polypeptide has several internal disulfide bonds. In one experiment (not shown), the 420-kD polypeptide was transferred to immobilon and stained with anti-human α_2M or anti-human laminin antibodies. These studies revealed that the placental protein is not related to any form of α_2M or laminin. The presence of carbohydrate on the 420-kD polypeptide was determined by chromatography over immobilized wheat germ agglutinin. The 420-kD polypeptide was bound to this resin and could be eluted with 0.5 M N-acetyl-D-glucosamine. All of the α_2M-proteinase binding ability was eluted with the 420-kD polypeptide.

Identification of a 420-kD Polypeptide from Affinity Chromatography of Surface-labeled Proteins from NRK Fibroblasts

Surface labeling experiments were performed to determine if the 420-kD protein is located on the cell surface. NRK fibroblasts were chosen for these experiments since the binding of α_2M-proteinase complexes to this cell line is well characterized. Cell surface proteins were iodinated using lactoperoxidase. After washing, the cells were solubilized and applied to the α_2M:Me–Sepharose affinity column. Fig. 8 B demonstrates an analysis of the material eluted from the affinity resin after SDS-PAGE and autoradiography. These results indicate that NRK fibroblasts contain a surface protein with a similar electrophoretic mobility to the human placental protein.

Figure 8. (*A*) Estimate of the size of the purified receptor by SDS-PAGE under reducing conditions on a 5% gel. (Lane *1*) Standards, which include myosin, β-galactosidase, phosphorylase b, BSA, and ovalbumin; (lane 2) purified α_2M receptor. The migration position of the α_2M subunit and the A and B chains of laminin are also shown. (*B*) SDS-PAGE analysis of the material eluted from α_2M:Me affinity chromatography after surface labeling of proteins from NRK fibroblasts. The migration position of standard proteins are shown.

Discussion

The general interest in the mechanism of receptor-mediated endocytosis has prompted a number of studies describing the uptake, internalization, and degradation of various derivatives of α_2M (8, 14, 27, 29, 30, 37, 54, 55). These investigations have yielded considerable insight into mechanisms involved in endocytosis. Despite some insight into the role of the α_2M receptor in this process, information on its structure is extremely limited. The present studies were initiated with the goal of deriving information on this receptor system. Binding studies have reported the existence of a large number of α_2M receptors in placental syncytiotrophoblasts (28). This observation, combined with the fact that the placenta has large quantities of receptors for a variety of proteins (43, 46, 51), suggested that this tissue might be an excellent source for the isolation of relatively large quantities of this receptor. Affinity chromatography was used to investigate the α_2M receptor from this tissue.

This approach identified two polypeptides, with apparent molecular masses of 420 and 39 kD, that are specifically bound to an affinity column prepared by reacting native α_2M with methylamine. The specificity of these two components for the conformationally altered form of α_2M was documented by demonstrating that these polypeptides did not bind to a column prepared by coupling native α_2M to Sepharose. Additional chromatography steps were used to separate these two components, and the results of these experiments indicated that the binding activity was associated
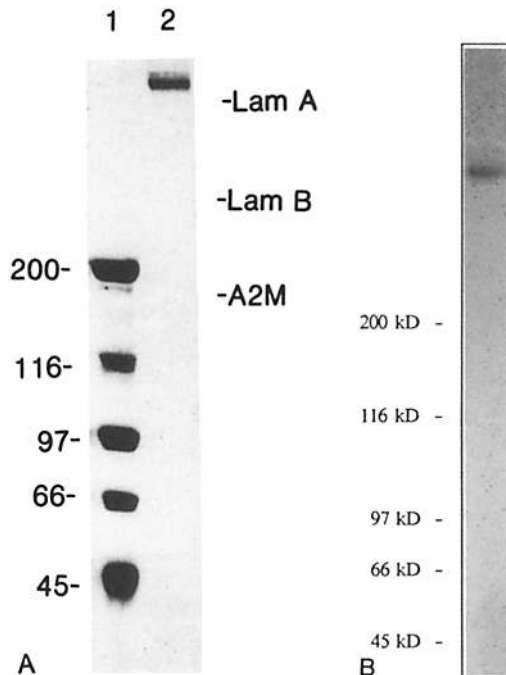
with the 420-kD polypeptide. Considerable evidence has been obtained that indicates that this polypeptide has properties of the α_2M receptor. Most important, the purified glycoprotein exhibits an identical specificity and a similar high affinity for the activated form of α_2M that is displayed by the binding of α_2M derivatives to cells. Very little, if any, binding of the purified protein to the native molecule was observed in the present study. Second, the purified protein demonstrates a similar requirement for metal ions, and a similar pH dependence for binding, that is found for the binding of proteolyzed α_2M to cells. Third, surface labeling experiments indicate that the 420-kD polypeptide is located on the surface of fibroblasts. All of these data provide compelling evidence that the 420-kD glycoprotein is an α_2M receptor.

At this time, the relationship between the 420- and 39-kD polypeptides is not known. The 39-kD polypeptide, like the 420-kD polypeptide, does not bind to native α_2M immobilized on Sepharose and thus is specific for the affinity column containing activated α_2M. Interestingly, this polypeptide does not appear to bind directly to ^{125}I-labeled α_2M-trypsin or α_2M:Me. These results suggest that the 39-kD polypeptide is associated with the 420-kD polypeptide and could represent either a subunit of the α_2M receptor or, possibly, a cytosolic protein that binds to the α_2M receptor. Further experiments are required to determine the potential interaction between these two components and the significance of this interaction.

Most studies examining the binding of α_2M–proteinase complexes (or the methylamine-reacted form) to cells have resulted in nonlinear Scatchard plots. The data have been interpreted to represent two classes of binding sites. At this time it is not clear if these two binding sites represent distinct molecules or, as suggested (18), whether they represent ligand binding to aggregated receptor molecules with high affinity and to single receptor molecules with a lower affinity. The binding affinity for the purified receptor recovered from the affinity column is similar to that measured for the binding of ^{125}I-labeled α_2M–trypsin complexes to solubilized membranes (15, 18). Further purification of the α_2M receptor by anion exchange chromatography results in a molecule with a reduced binding affinity. It is quite likely that the methods used in the present study, in which microtiter wells are coated with purified receptor, are not optimal for receptor–ligand binding. This is particularly true if aggregation of the receptor is required for high affinity binding. Further, reconstitution of the receptor into appropriate phospholipid vesicles would likely result in an increased affinity of the 420-kD polypeptide for ligand. Studies are currently in progress to examine these and other possibilities. The fact that α_2M contains four identical subunits and the observation that α_2M appears to be a multivalent ligand for the receptor (53) further complicates interpretation of the binding interaction.

The large size of the α_2M receptor in the present study differs from some previous reports. Three reports in the literature have described the partial purification of the α_2M receptor from membranes of spontaneously transformed fibroblasts (NIH-3T3 cells) (17) and from human diploid fibroblasts (12, 35). There does not appear to be any general consensus as to the identity of this molecule from these reports. Hanover et al. (17, 19) identified two polypeptides (90 and 180 kD) as potential candidates for the α_2M receptor. A second study (12) identified a single 125-kD component that appeared to bind to ^{125}I-α_2M–trypsin complex after transfer of the polypeptide to nitrocellulose. Using affinity chromatography over immobilized α_2M:Me, Marynen et al. (34) identified three components from ^{35}S-labeled normal human fibroblasts with molecular masses of 360, 130, and 83 kD as the putative α_2M receptor. The largest of these polypeptides has a molecular mass close to that obtained in the present study. The relationship between these proteins and the large single chain molecule identified in the present study remains to be determined. It is likely that some or all of these polypeptides may represent degradation products of the larger polypeptide, and it does not appear likely that fibroblasts contain a different and much smaller molecular mass receptor than that isolated in the present study since studies with surface-labeled fibroblasts identified a large molecular mass component as the major protein eluted from an α_2M:Me affinity column. An 85-kD polypeptide was noted in some of the receptor preparations in the present studies. The presence of only trace amounts of this polypeptide in most preparations suggest that it is not responsible for the binding activity. It is possible that this polypeptide arises as a proteolytic product of the larger 420-kD polypeptide. This proposal is supported by Jensen and Pizzo (26) who noted an accumulation of an 80–90-kD polypeptide upon storage of their receptor preparation for several days. Further, incubation of the purified 420-kD polypeptide with elastase

(Cravens, J. L., and D. K. Strickland, unpublished observation) in the present study yielded several polypeptides with apparent molecular masses in the 66–90-kD range in addition to other distinct polypeptides. These results indicate that it is possible to generate fragments with similar molecular masses by proteolysis of the purified 420-kD polypeptide.

In excellent agreement with the present work is a report (15) examining the functional molecular mass of the α_2M receptor by radiation inactivation. This approach, in conjunction with cross-linking studies, concluded that the α_2M receptor is a large polypeptide with a molecular mass ranging from 420 to 500 kD. During the preparation of the present manuscript, two studies appeared in the literature describing large molecular mass polypeptides as components of the α_2M receptor. The size of these polypeptides, isolated from human, baboon, rat, and mouse liver (26) and from rat liver membranes (38), agree with the size of the α_2M receptor in the present investigation.

Few receptors have subunits as large as the α_2M receptor identified in the present study. It is considerably larger than other well characterized receptors involved in receptor-mediated endocytosis. For example, the transferrin receptor is a dimer of 90 kD (44), while the low density lipoprotein receptor appears to be a single chain of 164 kD (45). The CR1 (C3b/C4b) receptor, a molecule that binds to proteins closely related to α_2M (47), is a polymorphic polypeptide (33) with a relatively large molecular mass (ranging from 160 to 250 kD). This receptor was found to contain 30 domains containing the short consensus repeat units also found in C4b/C3b binding protein (33). Herz et al. (21) have identified a large 500-kD liver membrane protein, closely related to low density lipoprotein receptor, whose function remains undefined. It is quite possible that a family of large molecular mass receptors exists on a variety of cells. The availability of large amounts of homogenous α_2M receptor will facilitate structural determination of this molecule which will be of great value in establishing the role and function of this receptor.

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