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Identification of the macrophage mannose receptor as a 175-kDa membrane protein

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ABSTRACT **Mannose-lactoperoxidase, a neoglycoprotein prepared by reaction of lactoperoxidase with cyanomethyl 1-thiomannoside, bound to alveolar macrophages at 4°C (K_d = 5.8×10^{-8} M) and was rapidly internalized at 37°C (K_{uptake} = 2×10^{-8} M). Mannose-lactoperoxidase binding and uptake were blocked by yeast mannan, and mannose-lactoperoxidase inhibited uptake of ^{125}I-labeled mannose-BSA (bovine serum albumin). Radioiodination of cells with surface-bound mannose-lactoperoxidase was carried out in the presence of glucose and glucose oxidase. A major polypeptide (175 kDa) was radioiodinated by this procedure. Iodination of the 175-kDa polypeptide appeared to be receptor-mediated, since it was blocked by the presence of yeast mannan. Specific iodination was absent from receptor-negative cells. To demonstrate that the 175-kDa species is a ligand-binding protein, cells were iodinated by the standard lactoperoxidase method. Washed cells were then allowed to bind mannose-BSA. Receptor–ligand complexes, prepared by detergent extraction, were passed over anti-BSA IgG affinity columns. Mannose, but not mannose 6-phosphate or galactose, eluted a radioactive protein from the column that migrated with an apparent molecular mass of 175 kDa on NaDodSO$_4$/PAGE. Detergent extracts of crude membranes prepared from macrophage-enriched whole rabbit lung were adsorbed to mannose-Sepharose; the fraction obtained by elution with mannose contained two protein components of 175 and 55 kDa. Subsequent chromatography on *N*-acetylglucosamine-agarose yielded a single protein of 175 kDa. The 175-kDa polypeptide was shown to bind ^{125}I-labeled mannose-BSA in a precipitation assay. This binding could be blocked with mannan or mannose-BSA. The results indicate that the cell-surface mannose receptor is a 175-kDa protein.**

Many ligands are transported into cells by a process referred to as receptor-mediated endocytosis (1). Mammalian lung macrophages express a receptor that binds and internalizes mannosylated glycoproteins, including a variety of lysosomal enzymes (2, 3). The receptor is macrophage-specific and recognizes oligosaccharide chains terminating in mannose, fucose, or *N*-acetylglucosamine (4). Lysosomal enzymes internalized by the macrophage via the mannose receptor enter an endosomal compartment where ATP-dependent receptor–ligand dissociation and segregation occur (5). The enzymes are subsequently delivered to secondary lysosomes (6, 7), while the receptor returns to the cell surface ready to initiate another round of internalization (8). Although the ligand-binding specificity and the kinetics of ligand uptake via the mannose receptor have been studied extensively in intact cells (2–8), the receptor protein has not yet been identified.

In this study we have synthesized mannosylated lactoperoxidase as a ligand for the mannose receptor. The modified enzyme binds the mannose receptor with high specificity and affinity and is taken up into the macrophage by the mannose-receptor pathway. Vectorial labeling using receptor-bound enzyme, ^{125}I, and glucose oxidase results in radioiodination of a single polypeptide. This peptide can be identified as a component of macrophage plasma membrane by use of antiligand-affinity chromatography. Using crude membranes from whole rabbit lung, we isolated a 175-kDa protein by affinity chromatography. This protein retained the ability to bind radiolabeled ligands. This evidence suggests that the 175-kDa mannose-binding protein is the macrophage mannose receptor.

MATERIALS AND METHODS

Lactoperoxidase from bovine milk, glucose oxidase type V, Sepharose 4B, 3,3′-diaminobenzidine, *N*-acetylglucosamine-agarose, and goat anti-bovine albumin were purchased from Sigma. The IgG fraction of rabbit anti-bovine albumin was purchased from Cappel Laboratories (Malvern, PA). Rabbit alveolar macrophages (9) and rat bone marrow macrophages (10) were prepared as described. Mannose-Sepharose was prepared as described (11). Protein was measured by the method of Bradford (12).

Ligand Binding and Uptake Studies. Mannosylated bovine serum albumin (Man-BSA) and lactoperoxidase (Man-peroxidase) were prepared by the method of Lee *et al.* (13). The extent of mannosylation was determined using the phenol/sulfuric acid assay (14). β-Glucuronidase was isolated from rat preputial glands by the method of Keller and Touster (15). Man-BSA, Man-peroxidase, and β-glucuronidase were radioiodinated by use of chloramine-T as described (3). Cell binding and uptake studies were performed using rabbit alveolar macrophages in Hanks' balanced salts solution supplemented with 1% BSA (Hanks'/BSA). The precipitation assay of Wileman *et al.* (5) was used to demonstrate specific binding of Man-BSA to the purified receptor preparation. Nonspecific binding and uptake were determined in the presence of yeast mannan (1 mg/ml) or Man-BSA.

Cell Surface Iodination. Rabbit alveolar macrophages (10^7 per ml in Hanks'/BSA) were incubated with Man-peroxidase (5 μg/ml) at 4°C for 90 min. The cells were washed twice with ice-cold Hanks'/BSA and suspended (2×10^7 per ml) in 10 mM Tris Cl, pH 7.4/150 mM NaCl at 4°C. Glucose (2 mg/ml), glucose oxidase (3 units/ml), and Na^{125}I (1 mCi/ml; 1 Ci = 37 GBq) were then added and radioiodination was allowed to proceed for 60 min at 4°C. The cells were washed extensively in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salts solution containing 1 mM EDTA at pH 6.0 to dissociate receptor-bound Man-peroxidase. The cells were then disrupted by nitrogen cavitation [300 psi (2067 kPa), 15 min at 4°C], and a particulate membrane fraction was prepared. These membranes were either fractionated on Percoll gradients as described previously (5) or dissolved in sample preparation buffer containing 2-mercaptoethanol and processed for NaDodSO$_4$/PAGE (16).

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Abbreviations: BSA, bovine serum albumin; Man-BSA, mannose-BSA; Man-peroxidase, mannose-lactoperoxidase.

Antiligand-Affinity Chromatography. An IgG fraction was prepared by ammonium sulfate precipitation of rabbit antiserum raised against BSA. Sepharose 4B was activated using the cyanotransfer method of Kohn and Wilchek (17). Packed activated beads (2 ml) were incubated at pH 9.0 in 0.1 M bicarbonate buffer (5 ml) containing 60 mg of rabbit IgG. After overnight incubation and extensive washing with 200 mM Tris Cl buffer (pH 8.0), 6 mg of IgG remained bound per milliliter of Sepharose beads. Each milliliter of Sepharose beads bound 250 μg of Man-BSA that could be eluted by glycine acetate buffer (pH 3.0). Nonspecific radioiodination of rabbit alveolar macrophage plasma membrane proteins was performed using the method of Mellman *et al.* (18).

Isolation of Mannose-Binding Proteins from Rabbit Lung. Macrophage infiltration into rabbit lungs was induced by prior intravenous injection of an emulsion containing equal parts 0.9% NaCl and Freund's complete adjuvant (0.25 ml/kg of body weight). Lungs were removed intact and used immediately. All procedures were carried out at 4°C unless otherwise specified. In a typical binding-protein preparation, six rabbit lungs (200 g) were shredded by passage through a Cuisinart food processor. The tissue was then suspended in 1.2 liters of dissociation buffer (10 mM Tris Cl, pH 6.0/1.25 M NaCl/15 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride) and homogenized twice (45 sec each) at low speed in a Waring blender. An additional 1.2 liters of dissociation buffer was added and the homogenate was stirred for 30 min. Membranes were pelleted by centrifugation (10,000 rpm, 15 min, 4°C, Beckman JA-14 rotor) and washed once by resuspension in 1.3 liters of dissociation buffer. The pellet was then resuspended in loading buffer (10 mM Tris Cl, pH 7.4/1.25 M NaCl/15 mM $CaCl_2$/0.1 mM phenylmethylsulfonyl fluoride) containing 200 mM mannose and pelleted as above. Residual mannose was removed from the membranes by sedimentation from 1.2 liters of loading buffer. Washed membranes were solubilized by an overnight incubation in 1 liter of loading buffer containing 1% (vol/vol) Triton X-100. The Triton-resistant residue was removed by centrifugation (40,000 rpm, 15 min, Beckman Ti-50.2 rotor), and the supernatant was added to 50 ml of packed mannose-Sepharose beads. After overnight incubation at 4°C, the beads were washed by sedimentation with 10 volumes of loading buffer containing 1% (vol/vol) Triton X-100 and then were loaded into a column. Mannose-binding proteins were eluted with 150 ml of 200 mM mannose in loading buffer containing 1% Triton X-100. After extensive dialysis against 10 mM Tris Cl, pH 7.4/0.5 mM NaCl, the eluate was incubated overnight with 5 ml of *N*-acetylglucosamine-agarose. The resin was then poured into a column and washed with 40 ml of loading buffer containing 1% Triton X-100. Bound material was eluted with 15 ml of dissociation buffer containing 1% Triton X-100. Samples were precipitated with 15% trichloroacetic acid. The precipitate was dissolved in a minimal amount of methanol to give a total volume of ≈10 ml. The samples were prepared for electrophoresis by a modification of the method of Wessel and Flügge (19). Aliquots (0.5 ml) were extracted with 0.1 ml of chloroform plus 0.3 ml of water. The protein precipitated at the phase interface. The upper (organic) phase was removed, 0.3 ml of methanol was added, and the protein was precipitated by centrifugation (2 min, Beckman Microfuge B). The pellets were washed twice with 0.3 ml of methanol to remove residual trichloroacetic acid. The resultant pellets were dissolved in 50 μl of electrophoresis buffer before $NaDodSO_4$/PAGE according to Laemmli (16).

RESULTS

Binding and Uptake of Man-Peroxidase. The neoglycoprotein Man-peroxidase was prepared by reaction of the enzyme with cyanomethyl 1-thio-α-D-mannopyranoside (11). Lactoperoxidase bearing 30 mannose residues per molecule retained ≈75% of its original enzyme activity. Rabbit alveolar macrophages were incubated with various concentrations of ^{125}I-labeled Man-peroxidase at 4°C (Fig. 1*A*). Man-peroxidase binding to macrophages was both concentration-dependent and saturable. Scatchard analysis of the binding curve indicated the presence of approximately 100,000 high-affinity (K_d 5.8 × 10^{-8} M) surface binding sites per cell. Uptake of Man-peroxidase at 37°C was concentration-dependent (Fig. 1*B*) and saturable. The concentration of ligand required to produce half-maximal uptake (K_{uptake}) was estimated graphically as 2 × 10^{-8} M. Incubation of cells with Man-peroxidase

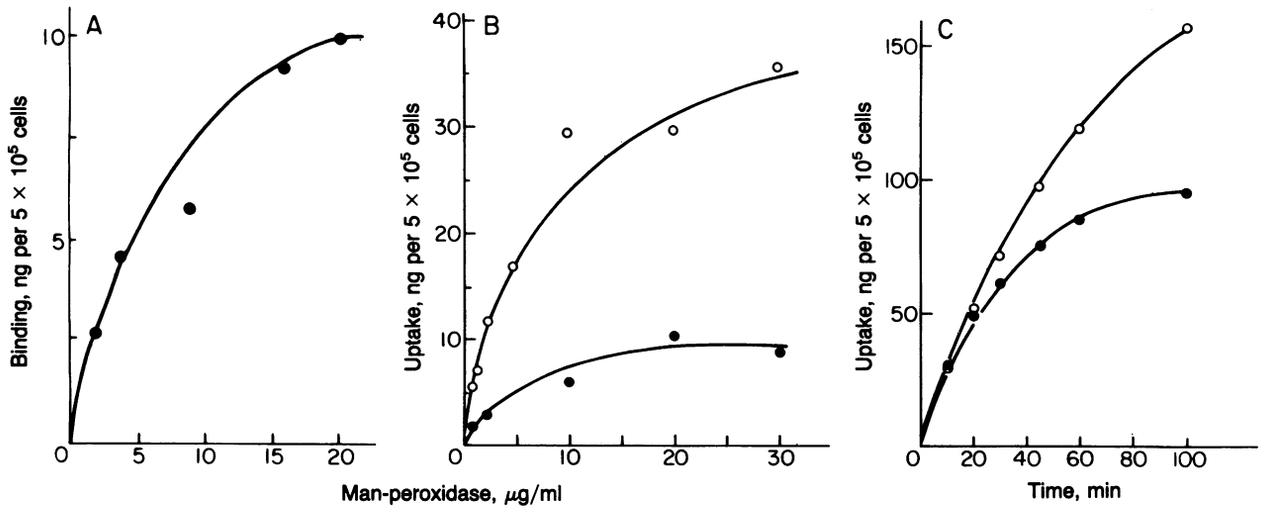

FIG. 1. Binding and uptake of Man-peroxidase by rabbit alveolar macrophages. Cells (5 × 10^6 per ml) were incubated at 4°C (binding) or 37°C (uptake) in Hanks' balanced salts solution containing 1% BSA. Cells were separated from the medium by sedimentation through oil (8). Nonspecific binding and uptake were determined in the presence of excess yeast mannan (1 mg/ml); both were <20% of total. (*A*) Concentration-dependence of cell-surface binding was determined by incubating cells with ^{125}I-labeled Man-peroxidase for 90 min at 4°C. (*B*) Concentration-dependence of Man-peroxidase uptake; cells were incubated at 37°C for 15 min with ^{125}I-labeled Man-peroxidase (2.5 × 10^3 cpm/ng) (○) or with Man-peroxidase that had been oxidized with sodium periodate before iodination (●). (*C*) Time course of Man-peroxidase uptake; cells were incubated with ^{125}I-labeled Man-peroxidase (20 μg/ml, 2.5 × 10^3 cpm/ng) at 37°C. ○, Cell-associated radioactivity. ●, Specific uptake adjusted for degradation.

in the presence of excess yeast mannan substantially reduced binding (75%) and ligand uptake (80%). Moreover, periodate oxidation of carbohydrate residues of Man-peroxidase markedly reduced the endocytosis of the ligand. Uptake of Man-peroxidase was linear for at least 60 min at 37°C (Fig. 1*C*) and trichloroacetic acid-soluble radioactivity could be detected in cell supernatants after ≈20 min of incubation, suggesting transport of Man-peroxidase to secondary lysosomes. Unlabeled Man-peroxidase inhibited the uptake of ^{125}I-labeled Man-BSA by rat bone marrow macrophages; half-maximal inhibition of Man-BSA uptake was observed at 2.6×10^{-8} M Man-peroxidase (data not shown).

Identification of the Mannose Receptor. Man-peroxidase was bound to macrophage surface receptors at 4°C. The cells were then washed extensively and radioiodinated by addition of ^{125}I, glucose, and glucose oxidase. Iodination was quenched by addition of KI (40 mM) followed by extensive washing in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salts solution (pH 6.0) containing EDTA. EDTA was included to dissociate Man-peroxidase from the receptor. A postnuclear membrane fraction was prepared from the cells, and a sample of membrane vesicles was analyzed by Percoll gradient centrifugation. The data (not shown) indicated that all the incorporated radiolabel migrated with fractions rich in the plasma-membrane marker enzyme alkaline phosphodiesterase (EC 3.1.4.1). No radioactivity could be detected in fractions containing secondary lysosomes or in endosomes labeled in a parallel gradient using a brief pulse of ^{125}I-labeled β-glucuronidase (5). The remaining membranes were extracted using 1% Triton X-100, and trichloroacetic acid-precipitated material from the extract was analyzed by NaDodSO$_4$/PAGE. Fig. 2 (lane A) shows that the bulk of the ^{125}I was incorporated into a polypeptide of ≈175 kDa. When surface iodination was repeated in the presence of excess yeast mannan (lane B) or EDTA (data not shown), incorporation of ^{125}I into this band was substantially reduced. When the same protocol was applied to the macrophage cell line J774, which does not express a mannose receptor, the 175-kDa band was not observed (lane C).

Isolation of the 175-kDa Polypeptide from the Macrophage Plasma Membrane. Rabbit alveolar macrophages were radioiodinated at 4°C, using unmodified lactoperoxidase in the presence of EDTA to prevent preferential iodination of the mannose receptor. The cells were washed as described above, suspended in Hanks' balanced salts solution containing 1% BSA, and then allowed to bind Man-BSA at 4°C for 1 hr. After removal of unbound ligand, the cells were solubilized and passed over a column of Sepharose-conjugated anti-BSA (Fig. 3). The radioactivity remaining on the column was resistant to removal by galactose or by mannose 6-phosphate but was readily eluted by mannose or EDTA. When analyzed by NaDodSO$_4$/PAGE, the radiolabeled material that passed through the column was found in many polypeptides. The radioactivity eluted by mannose or EDTA migrated as a single band at 175 kDa.

Isolation of Active Mannose-Binding Protein from Rabbit Lung. Rabbit lung, taken from animals that had been injected intravenously 3 weeks earlier with complete Freund's adjuvant, was chosen as a potentially rich source of mannose receptor. Since mannose-binding proteins, present in serum (20), liver (11, 21–23), and the macrophage plasma membrane (8) bind ligands in a Ca^{2+}- and pH-dependent manner, membranes from whole rabbit lung were washed, prior to affinity chromatography, in a dissociation buffer (pH 6.0) containing EDTA and then in a mannose-containing buffer to elute any endogenous ligands. Membrane proteins solubilized in 1% Triton X-100 were subsequently passed over mannose-Sepharose, and mannose-binding proteins were eluted with 200 mM mannose. The eluate from mannose-Sepharose contained two major and several minor proteins. These were visualized as bands of 175 and 55 kDa when analyzed by NaDodSO$_4$/PAGE under reducing conditions

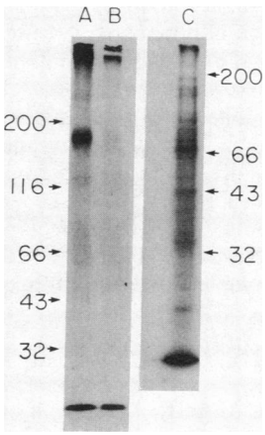

FIG. 2. Radioiodination of macrophages by receptor-bound Man-peroxidase. Rabbit alveolar macrophages (10^7 per ml) were allowed to bind Man-peroxidase (20 μg/ml) at 4°C for 90 min. The cells were washed and suspended (2×10^7 per ml) in 10 mM Tris Cl, pH 7.4/150 mM NaCl at 4°C. Glucose (2 mg/ml), glucose oxidase (3 units/ml), and Na^{125}I (1 mCi/ml) were then added and radioiodination was allowed to proceed for 60 min at 4°C. The cells were washed extensively in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salts solution containing 1 mM EDTA to remove Man-peroxidase from the receptor. The cells were then solubilized in electrophoresis sample buffer containing 2-mercaptoethanol and were analyzed by NaDodSO$_4$/PAGE followed by autoradiography. Lane A: macrophages iodinated as described above; 5% gel. Lane B: macrophages iodinated in the presence of yeast mannan (1 mg/ml); 5% gel. Lane C: radioiodination of J774 macrophages; 5–10% gradient gel. Markers were myosin heavy chain (200 kDa), β-galactosidase (116 kDa), BSA (66 kDa), ovalbumin (43 kDa), and carbonic anhydrase (32 kDa).

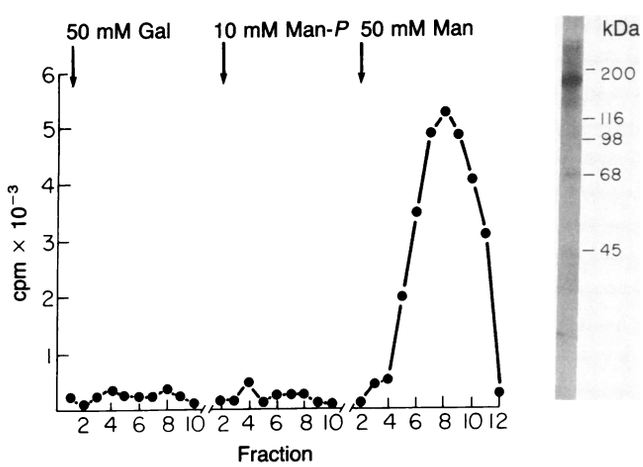

FIG. 3. Elution of mannose receptor from anti-BSA-Sepharose. Rabbit alveolar macrophages (2×10^7 per ml) were surface-labeled at 4°C, using unmodified lactoperoxidase (30 μg/ml). The cells were washed and incubated with Man-BSA (20 μg/ml) at 4°C for 90 min and then were washed again and solubilized in 0.5% Triton X-100/25 mM Tris Cl, pH 7.4/150 mM NaCl/15 mM $CaCl_2$/0.1 mM phenylmethylsulfonyl fluoride. After 30 min at 4°C, the cell debris was removed by centrifugation (100,000 × *g*, 15 min), and the supernatant was passed over anti-BSA-Sepharose. After extensive washing with loading buffer, the column was washed sequentially with 50 mM galactose, 10 mM mannose 6-phosphate, and 50 mM mannose. Mannose removed all the remaining radioactivity from the column; these radioactive fractions were pooled and dialyzed extensively against distilled water, and proteins were precipitated with trichloroacetic acid prior to analysis by NaDodSO$_4$/5% PAGE (autoradiograph at right).

(Fig. 4). The presence of phenylmethylsulfonyl fluoride was required throughout the procedure to prevent generation of multiple degradation bands in the purified product. The macrophage mannose receptor binds *N*-acetylglucosamine, and this property was exploited to purify the protein further. Chromatography of mannose-eluted material on *N*-acetylglucosamine-agarose allowed separation of the two proteins, since the 175-kDa protein was retained by the resin (Fig. 5). This purified material was subjected to a precipitation assay designed to detect mannose-binding activity. The protein was able to bind ^{125}I-labeled Man-BSA (Table 1); binding was specific, since it was substantially reduced by excess unlabeled ligand (Table 1) or yeast mannan (data not shown).

DISCUSSION

Mannosylated lactoperoxidase was synthesized as a ligand for the macrophage mannose endocytosis receptor with the intention of using the enzyme to specifically radioiodinate the receptor. Preliminary studies showed that Man-peroxidase bound with high affinity and specificity to the macrophage mannose receptor. Binding and uptake were both concentration-dependent and saturable and could be inhibited by yeast mannan. Man-peroxidase also inhibited the uptake of Man-BSA, a neoglycoprotein with a high affinity for the mannose receptor (4). Endocytosis of Man-peroxidase was linear for nearly 2 hr, suggesting that its endocytosis requires the reuse of cell surface receptors, indicative of its uptake via receptor-mediated endocytosis. Within 20–30 min, trichloroacetic acid-soluble digestion products were released into the cell medium, indicating that the ligand had been delivered to secondary lysosomes.

Vectorial radioiodination of the macrophage plasma membrane using Man-peroxidase bound to the mannose receptor resulted in preferential iodination of a 175-kDa polypeptide. Percoll gradient centrifugation showed that this polypeptide was a plasma membrane component. This polypeptide is unlikely to be autoradioiodinated Man-peroxidase, since the molecular mass of Man-peroxidase is ≈80 kDa. Moreover, receptor bound Man-peroxidase was washed from the surface of the cells using a dissociating medium (EDTA, pH 6.0) before analysis by electrophoresis. These results may, however, reflect radioiodination of a plasma membrane component rich in tyrosine residues. Indirect evidence to the contrary is provided by the following observations. First, incorporation of ^{125}I into the 175-kDa polypeptide was substantially reduced in the presence of yeast mannan or EDTA. Second, the 175-kDa polypeptide was absent following vectorial labeling of a cell line that does not express a mannose receptor. Unfortunately these observations do not completely rule out the possibility of an artifactual result, since in the absence of a mannose receptor or under dissociating conditions the enzyme cannot bind to the macrophage.

Direct evidence that the 175-kDa polypeptide is the mannose receptor comes from two experiments. First, of the many polypeptides present in the macrophage plasma membrane that can be radioiodinated nonspecifically by use of unmodified lactoperoxidase, only the 175-kDa protein subsequently binds Man-BSA. This protein can be captured as a receptor–ligand complex within Triton extracts of macro-

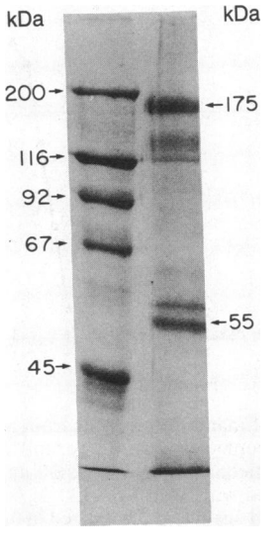

FIG. 4. Polyacrylamide gel electrophoresis of lung membrane proteins eluted from mannose-Sepharose. Membranes prepared from rabbit lungs were extracted with Triton X-100. Solubilized proteins were incubated overnight with mannose-Sepharose. After extensive washing, the resin was loaded into a column and mannose-binding proteins were eluted with 200 mM mannose. The figure shows the reduced proteins resolved in a 7.5% acrylamide gel run in the presence of $NaDodSO_4$ (right lane), along with marker proteins (left lane) as indicated for Fig. 2. Proteins were visualized with Coomassie blue R-250.

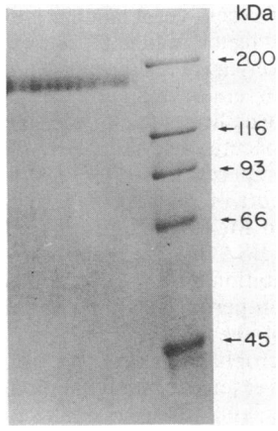

FIG. 5. Polyacrylamide gel electrophoresis of lung membrane protein eluted from *N*-acetylglucosamine-agarose. Protein eluted from mannose-Sepharose (Fig. 4) was adsorbed to *N*-acetylglucosamine-agarose. The protein eluted with EDTA elution buffer was resolved by $NaDodSO_4$/7.5% PAGE under reducing conditions. Marker proteins (right lane) were as indicated for Fig. 2. Proteins were visualized with Coomassie blue.

Table 1. Binding activity of purified lung mannose receptor

Receptor	Unlabeled Man-BSA	^{125}I-labeled Man-BSA bound, cpm	Specific binding,* cpm
–	–	70,903	
–	+	35,612	35,000
+	–	177,602	
+	+	21,000	156,000

Fractions eluted from the GlcNAc-agarose column were assayed for protein (12) and binding activity (5). The binding assay was carried out in a total volume of 300 μl at room temperature for 60 min. The reaction mixture contained 1.5 μg of ^{125}I-labeled Man-BSA (1.3×10^6 cpm/μg) plus or minus receptor protein (8 μg). To estimate nonspecific binding, excess unlabeled Man-BSA (76 μg) was added. To precipitate receptor–ligand complexes, gamma globulin (1 mg) and saturated ammonium sulfate were added in a total volume of 370 μl. After 10 min on ice, the samples were filtered on GF/C filters and washed before measurement of radioactivity with a gamma counter.
*Estimated by subtracting ligand binding in the presence of excess Man-BSA from binding in its absence.

phages by using an antibody to BSA. The polypeptide is eluted from the ligand by EDTA or mannose but not by galactose or by mannose 6-phosphate. Second, the macrophage mannose receptor has relatively broad binding specificity and will also bind glycoproteins that terminate in *N*-acetylglucosamine (2). This property was shared by a 175-kDa protein isolated from detergent extracts of rabbit lung. The protein was eluted from mannose-Sepharose by mannose and could be adsorbed selectively from the dialyzed eluate by an *N*-acetylglucosamine affinity resin. A 55-kDa contaminant eluted from mannose-Sepharose did not adsorb to *N*-acetylglucosamine-agarose. Since the extract derived from whole lung, neither the nature nor the origin of this 55-kDa polypeptide is understood. When the protein that did adsorb to *N*-acetylglucosamine-agarose was reduced and analyzed by $NaDodSO_4$/PAGE, it appeared as a single band at 175 kDa. This ostensibly pure protein of 175 kDa was able to bind ^{125}I-labeled Man-BSA specifically (Table 1). Although further structural studies are required, these observations strongly suggest that the 175-kDa protein is the macrophage mannose endocytosis receptor.

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