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Purification and characterization of the D-mannose receptor from J774 mouse macrophage cells*,†

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ABSTRACT

Macrophages display on their cell surface a D-mannose-specific receptor which facilitates the scavenging of certain pathogens and deleterious macromolecules from the extracellular fluid as part of the host defense mechanism. The mouse D-mannose receptor was purified from J774 E macrophages and an antiserum was generated against the receptor protein. In mouse macrophages, the newly synthesized receptor has an M_r of 157 000 Da and rapidly matures to a protein with an M_r of 172 000 Da. Both forms of the receptor protein are tightly associated with cell membranes. The receptor is found in a number of mouse macrophage cell types but is not present in mouse fibroblasts. An assay was developed to characterize D-mannose receptor-ligand binding based on immunoprecipitation of the detergent-solubilized receptor protein. The dissociation constant, determined for receptor and the neoglycoprotein D-mannose-BSA, was 1.67nM. Receptor-ligand binding was calcium and pH dependent. Monosaccharides, such as D-mannose and L-fucose, partially inhibited receptor binding to the ligand D-mannose-BSA.

INTRODUCTION

Macrophages selectively express a cell surface receptor which binds microorganisms and glycoproteins with terminal D-mannosyl and L-fucosyl groups[1]. Following binding, the receptor promotes the internalization of ligands *via* both the endocytic and phagocytic pathways. In this respect, the D-mannose receptor may be important in the scavenger and immunoregulatory function of macrophages. A biologically important group of proteins which binds the D-mannose receptor includes lysosomal enzymes expressing D-mannosyl terminal-group oligosaccharides. Lysosomal enzymes are secreted during inflammatory responses and the retrieval of these enzymes by macrophages could serve to regulate tissue damage *in vivo*[2]. The lysosomal enzyme, β-D-glucuronidase, is bound by the receptor present on the plasma membrane of macrophages, delivered into endosomal vesicles, and subsequently transported to lysosomes[3,4]. The receptor also facilitates macrophage phagocytosis of pathogens such as yeast, leishmania, and certain bacteria[5,6]. The D-mannose receptor has been isolated

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from tissue homogenates, as well as from lung lavage fluid, which is rich in alveolar macrophages[7-9]. To elucidate the role of the receptor in endocytosis and phagocytosis, a macrophage cell line, J774 E, was selected for high levels of mannose-receptor expression[10]. The mouse mannose receptor was purified from J774 E cells, and receptor biosynthesis and ligand binding were characterized during the course of this study.

EXPERIMENTAL

Materials. — Mouse J774 E cell line was selected on the basis of its ability to internalize large quantities of fluoresceinated β-D-glucuronidase, a ligand for the mannose receptor[10]. These cells were cultured in minimum essential medium containing Earle's salts (αMEM), 10% fetal calf serum, 60μM thioguanine, penicillin, and streptomycin. For this study, the mouse macrophage cell line P388D1 was grown in RPMI 1640 medium, 10% fetal calf serum, mM sodium pyruvate, penicillin, and streptomycin. L929 mouse fibroblasts were grown in αMEM with 10% fetal calf serum, penicillin, and streptomycin.

Purification of D-*mannose receptor.* — The mouse mannose receptor was purified by use of D-mannose-Sepharose as previously described for rabbit alveolar macrophages[9]. D-Mannosyl groups were coupled to Sepharose with vinyl sulfone. Routinely, 10^{10} J774 E cells were grown to confluence (36 roller bottles, each 850 cm^2) and were used to isolate the receptor. Total cell membranes prepared from J774 E cells were solubilized and passed over a mannose-Sepharose column. The receptor was bound to the column in 10mM Tris, pH 7.4, 1.25M NaCl, 15mM $CaCl_2$, 0.1mM phenylmethylsulfonyl fluoride, pepstatin A (1 μg/mL), leupeptin (1 μg/mL), and 1% Triton-X 100, and was eluted with the same buffer containing 0.2M D-mannose. The preparation was dialyzed, passed a second time over mannose-Sepharose, and eluted in buffer containing 2mM EDTA. Following affinity chromatography on mannose-Sepharose, the mouse receptor was found to migrate as a protein of M_r equal to 172 000* in 8% sodium dodecyl sulfate (SDS)–poly(acrylamide) electrophoresis gels stained with silver[11,12]. In some instances, the receptor preparation contained a contaminating protein having a M_r equal to 45 000. This protein with an M_r of 45 000 was separated from the receptor by preparative SDS-poly(acrylamide) gel electrophoresis. A similar contaminant was observed in preparations of the receptor from rat liver[13].

Preparation of antiserum against D-*mannose receptor.* — The purified mouse receptor was used to immunize New Zealand white rabbits at 2 week intervals with 10 μg of protein used for each injection. The antiserum obtained was found to be specific for the mannose receptor by immunoprecipitation, Western blotting, and immunofluorescence. Purified human placental mannose receptor is detected by the antiserum raised against the mouse protein[8]. In addition, a rabbit antibody generated against the human placental mannose receptor cross-reacts with the mouse mannose receptor.

Biosynthesis studies. — For biosynthesis studies, the mouse receptor was isolated

* All M_r values are expressed in dalton units.

by immunoprecipitation from metabolically radiolabeled cells. Plated cells (2×10^6) were preincubated in methionine-free αMEM with dialyzed fetal calf serum and antibiotics for 30 min. Fresh methionine-free medium with 9 MBq Trans-^{35}S (ICN) was added to cells for 1 h. In pulse–chase experiments the radiolabel was removed from the medium after 1 h, and the cells were incubated for an additional 2 h in complete medium with a 10-fold excess of unlabeled methionine and cysteine. After biosynthetic radiolabeling, the cells were washed with phosphate-buffered saline solution (PBS) and frozen at −20°. To immunoprecipitate the receptor, the cells were scraped and lysed by Dounce homogenization in 5mM Tris, μM leupeptin, and μM pepstatin at pH 7.4. The cell lysate was centrifuged at 100 000*g* and 4° for 15 min to separate membranes and soluble fractions. Membranes were resuspended in PBS, pH 7.4, with 0.5% Triton X-100, μM pepstatin, and μM leupeptin. Triton X-100 was added to 0.5% final concentration to soluble fractions. Cell supernatants were pretreated with protein A-Sepharose for 30 min at 4°, followed by removal of the Sepharose by centrifugation. The supernatants were immunoprecipitated overnight at 4° with antibody to the mouse receptor or normal rabbit serum. Protein A-Sepharose was added to the supernatants and the precipitated immune complexes were isolated by centrifugation. The immunoprecipitates were washed with PBS and 0.1% Triton X-100, followed by distilled water. Immunoprecipitates were analyzed by electrophoresis in 8% SDS–poly(acrylamide) gels[11]. The electrophoresis gels were impregnated for autoradiography.

Receptor-binding activity. — Receptor-binding activity was characterized by use of ^{125}I-radiolabeled, D-mannose-derivatized bovine serum albumin (mannose-BSA) as a ligand. Bovine serum albumin (BSA, Fraction V; Sigma Chem. Co.) was conjugated *via* an iminomethoxyethyl linkage to D-mannose and subsequently iodinated with chloramine T[4,14]. The mannose receptor was isolated by immunoprecipitation from J774 E cells and used to measure binding activity. J774 E cells were lysed at 2.8×10^6 cells/mL in solubilization buffer (150mM NaCl, 10mM Tris, 1% Triton X-100, 1% bovine serum albumin, 10mM ethylene bis(oxyethylenenitrilo)tetraacetic acid (EGTA), 1 μg/mL of pepstatin, 1 μg/mL of leupeptin, pH 7.4) for 30 min at 4°. After centrifugation at 12 000*g* for 5 min, the cell lysate was incubated overnight at 4° with antibody directed against the human mannose receptor. The following day, IgGsorb (The Enzyme Center, Malden, MA) was added for 1 h to precipitate the receptor–antibody complex. The IgGsorb was washed two times with solubilization buffer and then two times with binding buffer (150mM NaCl, 10mM Tris, 1% Triton X-100, 1% bovine serum albumin, 20mM $CaCl_2$, 1 μg/mL of pepstatin, 1 μg/mL of leupeptin, pH 7.4). The receptor–antibody complex bound to IgGsorb was incubated in binding buffer for 1 h with D-[^{125}I]mannose-BSA (1×10^7 c.p.m./μg) at 37°. Nonspecific binding was measured in the presence of 2 mg/mL of yeast mannan. The IgGsorb was washed four times with binding buffer to remove unbound ligand. The IgGsorb pellets were counted to determine ligand binding. The number of cell surface mannose receptors and their ligand dissociation constants were also determined for J774 E cells by use of radiolabeled mannose-BSA[15]. Ligand dissociation constants were calculated by nonlinear regression analysis of the data fit to the first-order binding equation, $B = nF/(Kd + F)$, where B is the concentration of bound

ligand and F is the concentration of ligand free in the medium. Statistical analysis using the F ratio test indicated that the data were best fit by assuming only a single class of receptor-binding sites[16].

RESULTS

Identification of the D-*mannose receptor in mouse macrophages.* — J774 E macrophages expressed high levels of mannose-receptor activity with 9.8×10^4 active receptors being present on the cell surface at 4°. A yield of 60–70 μg of purified receptor protein was obtained from 10^{10} J774 E cells. The mannose receptor isolated from this mouse macrophage cell line had an M_r of 172 000 as determined by SDS–poly(acrylamide) gel electrophoresis (Fig. 1). Similar mannose-specific proteins with an M_r of 175 000 have been isolated from rabbit lung and human placenta.

The mannose receptor purified from J774 E cells was used to generate a specific antibody. The antibody was found to cross-react with the receptor protein from both mouse and human tissues. Studies were carried out using this antibody to characterize receptor biosynthesis and distribution in mouse cells. The mannose receptor was found to be associated only with membrane fractions of metabolically radiolabeled J774 cells (Fig. 2). Stringent washing of the membranes with buffers at pH 10.6 or pH 5 did not release the receptor, indicating that the receptor is tightly anchored to the membrane. As in human macrophages, the mouse receptor is translated as a precursor protein of lower molecular-weight. The receptor-precursor protein with an M_r equal to 157 000 is rapidly processed into the mature receptor with an M_r of 172 000. Studies with the

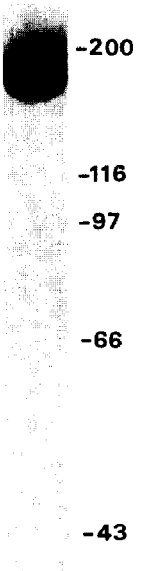

Fig. 1. Purified mouse mannose receptor analyzed by SDS-poly(acrylamide) gel electrophoresis. The mobility of molecular-weight-marker ($\times 10^3$) proteins is indicated.

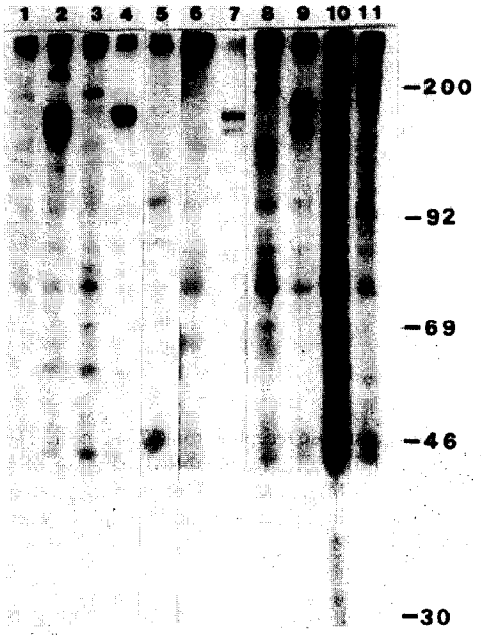

Fig. 2. Identification of the mannose receptor in mouse macrophages. Mouse cells were biosynthetically radiolabeled, and soluble and membrane fractions from these cells used for immunoprecipitation. J774 E cells were pulse radiolabeled for 1 h and harvested immediately (lanes 1, 2, and 5) or incubated in chase medium for 2 h (lanes 3 and 4). Peritoneal macrophages (lanes 6 and 7), P388D1 macrophages (lanes 8 and 9), and L929 fibroblasts (lanes 10 and 11) were biosynthetically radiolabeled for 1 h. Immunoprecipitates were analyzed by SDS–PAGE and autoradiography: Lane 1, membrane fraction J774 E (pulse), normal serum control; lane 2, membrane fraction J774 E (pulse), mannose receptor serum; lane 3, membrane fraction J774 E (chase), normal serum control; lane 4, membrane fraction J774 E (chase), mannose receptor serum; lane 5, soluble fraction J774 E (pulse), mannose receptor serum; lane 6, membrane fraction peritoneal macrophages, normal serum control; lane 7, membrane fraction peritoneal macrophages, mannose receptor serum; lane 8, membrane fraction P388D1, normal serum control; lane 9, membrane fraction P388D1, mannose receptor serum; lane 10, membrane fraction L929, normal serum control; and lane 11, membrane fraction, L929, mannose receptor serum. Protein-molecular-weight standards ($\times 10^3$) are indicated to the right.

human mannose receptor have shown that processing of both *N*-linked and *O*-linked oligosaccharides accounts for the shift in receptor size[17]. Following the radioiodination of whole cells, only the mature form of the mouse receptor was detected on the plasma membrane (data not shown). The macrophage cell line P388D1 contains low levels of mannose-receptor activity as measured with D-[^{125}I]mannose-BSA. The membrane-associated receptor protein is synthesized by P388D1 cells; however, the M_r of the mature receptor in this cell line is 174 000. The receptor-precursor protein appears similar in P388D1 and J774 cells, suggesting that posttranslational processing of the receptor differs in these cells. The receptor protein was not detected in mouse fibroblasts, nor did these cells express receptor activity. Mouse peritoneal and bone marrow-derived macrophages synthesize a mannose receptor that appears identical to the protein produced by J774 cells. The receptor was detected by Western blot analysis in whole tissue homogenates from mouse liver, spleen, and lung, but not brain.

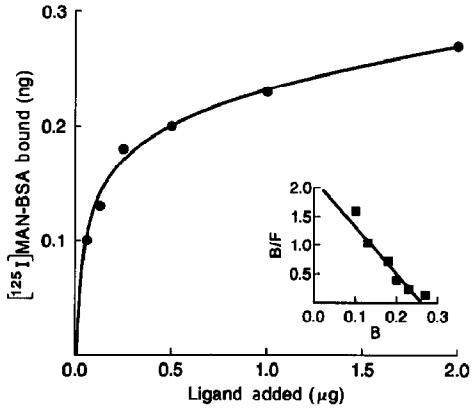

Fig. 3. Binding of mannose-BSA to detergent-solubilized mouse mannose receptor from J774 E cells. The mannose receptor was solubilized and isolated on antibody-coated IgGsorb. Radiolabeled mannose-BSA (1.2×10^7 c.p.m./μg) was added to the receptor–antibody complex for 1 h at 37°. Unbound ligand was removed by extensive washing. Nonspecific binding was determined in the presence of 2 mg/mL of yeast mannan. Scatchard analysis of the data is shown in the insert.

Characterization of receptor activity. — Studies of receptor–ligand interactions were hindered by the small amount of mouse receptor purified from J774 cells. A sensitive assay had been developed in this laboratory, in which binding of ligand to purified human mannose receptor was followed by immune precipitation of receptor–ligand complexes. This assay was modified in such a way that the mouse mannose receptor was first immunoprecipitated from solubilized J774 E cells. This semipurified receptor was then incubated with ligand and binding measured as shown in Fig. 3. Ligand binding was saturable when this assay was used and indicated a single class of receptor-binding sites. The receptor dissociation constant for mannose-BSA was 1.67nM, a value comparable to that observed with purified human placental receptor. During receptor–ligand binding studies using intact J774 E cells, a dissociation constant of 45nM was measured for mannose receptors present on the plasma membrane. These results indicate that receptor–antibody complexes retain full activity. Binding of the mouse receptor to mannose-BSA was significantly diminished in the presence of yeast

TABLE I

Inhibition of ligand binding to solubilized mouse mannose receptor[a]

Inhibitor	*Concentration*	*Inhibition (%)*
Yeast mannan	2 mg/mL	47
D-Mannose	100mM	19
L-Fucose	100mM	15
N-Acetyl-D-glucosamine	100mM	4
EDTA	10mM	100

[a] The mouse mannose receptor from J774 E cells was solubilized and binding assayed as described in the Experimental section. Inhibitors were added simultaneously with ligand to the receptor preparation.

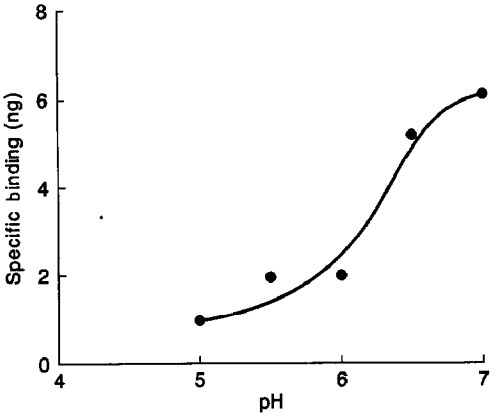

Fig. 4. pH profile of receptor-ligand binding. Mannose receptor solubilized from J774 E cells was incubated with 1 μg/mL of radiolabeled mannose-BSA (1.5×10^7 c.p.m./μg) in each of the following buffers: 30mM sodium acetate (pH 5 and 5.4), 30mM MES (pH 6 and 6.5), or 30mM HEPES (pH 7). Unbound ligand was separated from the receptor after 1 h. Nonspecific binding was determined in the presence of yeast mannan.

mannan and to a lesser extent by D-mannose and L-fucose (Table I). Both the human and rabbit mannose receptors require calcium for receptor activity[8,9]. Ligand binding to the mouse receptor was also calcium dependent and could be inhibited by the chelator, EDTA. Studies in whole cells using purified human and rabbit receptors have shown that receptor binding activity is pH dependent. Receptor-ligand binding for the mouse mannose receptor is also maximal at pH values >7 (Fig. 4).

DISCUSSION

A membrane-associated receptor with a high affinity for mannosylated proteins was isolated and characterized by use of a mouse macrophage cell line. The mouse mannose receptor was similar to mannose-specific receptors purified from rabbit, human, and rat tissues[7–13]. Specific antibodies generated against the purified receptor were used to study receptor–ligand binding and to characterize mannose-receptor biosynthesis.

In an *in vitro* assay, the detergent-solubilized mannose receptor was isolated by antibody precipitation and found to retain specificity and ligand binding. The assay for solubilized receptor described here is advantageous in that it does not require purified receptor, and a limited amount of cell tissue can be utilized. The antibody employed recognizes only the membrane-associated mannose receptor. Thus, this assay should be useful in studies of receptor specificity using homogenates of whole organs, such as liver, where a number of lectins with overlapping specificity have been observed. The dissociation constant for the detergent-solubilized mouse mannose receptor was found to be 1.67nM when the neoglycoprotein mannose-BSA was used. Binding experiments with intact mouse J774 E cells revealed a single class of receptor-binding sites with a dissociation constant of 45nM. In both assays, receptor-ligand binding was saturable

and inhibited by yeast mannan. Ligand binding to the solubilized receptor was partially inhibited by high concentrations of D-mannose and L-fucose. A similar sugar specificity for the receptor was observed in binding experiments using intact macrophages. Studies with neoglycoproteins and simple sugars suggested that mannose-receptor binding is influenced by the number of exposed D-mannose units present on a ligand[1,18]. This specificity may be important in the receptor's ability to mediate the phagocytosis of large, sugar-coated particles such as zymosan. The mouse receptor also required calcium for binding activity. The interaction of the mannose receptor and ligands was sensitive to acidic pH. Mannose receptor–ligand complexes internalize *via* the endocytic pahtway and dissociate with endosome acidification, and the released receptors are recycled to the cell surface[19]. The ligands released at low pH are subsequently transported from endosomes to lysosomes.

Mannose-receptor expression was observed in both primary macrophage cultures and established macrophage-like tumor cell lines. In biosynthetic studies, the receptor was found to be synthesized as a membrane-associated precursor protein with an M_r 157 000. The maturation of the receptor to a protein having M_r 172 000 occurs rapidly and only this form of the receptor is expressed on the plasma membrane. The shift in receptor size is most likely due to the addition of oligosaccharide chains as has been demonstrated for the human macrophage receptor[17]. The receptor protein was not detected in mouse fibroblasts by use of ligand binding, immunoprecipitation, or immunofluorescence techniques.

Mannose-receptor expression is highly regulated in macrophages. Activation of macrophages with lymphokines downregulates receptor activity, whereas anti-inflammatory glucocortocoids increase receptor activity in cells[1,2]. The expression of the 172 000-dalton receptor, as demonstrated here in both primary cultures of mouse macrophages and in mouse macrophage cell lines, will facilitate studies of receptor regulation. Macrophage-precursor cells can be isolated from mouse bone marrow and these cells differentiate in culture[20]. Mannose-receptor activity in these cells increases upon differentiation, and studies using antibodies to the receptor are in progress to define the mechanism of receptor regulation. In addition, clones of the J774 macrophage cell line have been isolated which express different levels of mannose-receptor activity[10]. In this study, the receptor was isolated from the J774 E cells which express high levels of functional mannose receptor. The mannose receptor in these cells internalizes ligands *via* both phagocytic and endocytic pathways. Antibodies specific for the mannose receptor will facilitate investigations of receptor synthesis and function in J774 cells with high and low levels of functional mannose receptors. Finally, the assay developed to measure solubilized-receptor activity will be useful in comparing mannose-receptor recognition of ligands which are internalized by endocytic *vs.* phagocytic routes.

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