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Characterization and isolation of an intracellular D-mannose-specific receptor from human promyelocytic HL60 cells*

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ABSTRACT

Most mammalian macrophages express D-mannose-specific receptor (membrane lectin, M_r 175 000) allowing endocytosis of their ligands, but cells of the monocytic lineage (HL60, U937, monocyte) lack this receptor. However, after permeabilization, promyelocytic, promonocytic cells and monocytes bound fluoresceinylated D-mannose-terminated neoglycoproteins as evidenced by flow cytometry. Under these conditions, confocal analysis confirmed the intracellular membrane localization of the labeling and the absence of nuclear binding. An intracellular D-mannose-specific receptor was isolated from the human promyelocytic cell line HL60, by affinity chromatography on 4-isothiocyanatophenyl α-D-mannopyranoside-substituted Affi-gel as a 60 000-M_r membrane protein requiring divalent cations for the ligand binding. Under the same conditions, mouse macrophages were shown to express a 175 000-M_r D-mannose-specific receptor but not the 60 000-M_r receptor.

INTRODUCTION

Mammalian macrophages express cell surface receptors that specifically bind and internalize glycoproteins bearing terminal D-mannosyl, L-fucosyl, or 2-acetamido-2-deoxy-D-glucosyl groups[1,2]. This receptor was identified and isolated, as a 175 000-M_r** membrane glycoprotein, from different species, such as rabbit[3,4], human[5,6], and rat[7]. More recently, Lennartz *et al.*[8] showed that the receptor is synthetized as a 154 000-M_r precursor protein which matures in 90 min to give a 162 000-M_r protein. It is known that the D-mannose receptor mediates endocytosis of its ligands[1,9] into acidic compartments (endosomes) where dissociation occurs[10]; then, the receptor is rapidly recycled to the cell surface[9]. Expression of the receptor is closely regulated; monocytes or bone marrow precursor cells do not express D-mannose receptors[11], but their expression occurs after three days in culture[11,12]. Likewise, the receptor expression changes according to the macrophage type[13] and downregulation occurs upon macrophage activation[14,15]. Moreover, it was shown that most macrophage cell lines, such as J774, P388D_1, or HL60, are devoid of cell surface D-mannose receptors[11,13,16]. Recently, Diment *et al.*[17] selected a

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** Molecular mass values (M_r) are expressed in dalton units.

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J774 clone which expresses D-mannose receptors at the cell surface upon 5-azacytidine exposure.

Permeabilization studies indicated that, in differentiated macrophages, 80% of the D-mannose receptors are localized intracellularly[18]. The intracellular pool may include newly synthesized receptors migrating to the endosome compartments and receptors recycled from the intracellular pool to the cell surface, or may represent other receptors with unknown functions[8]. We were interested to know whether macrophage precursors that lack cell surface D-mannose receptors (such as HL60 promyelocytic cells, U937 promonocytic cells, or monocytes) express an intracellular receptor. Paietta *et al.*[19] demonstrated the presence of a 17 000-M_r intracellular membrane lectin specific for both *N*-acetylneuraminic acid- and D-galactose-terminated, biantennary oligosaccharides in HL60. The D-mannose 6-phosphate receptor, which binds lysosomal enzymes and allows their trafficking between cellular compartments[20], has also been evidenced inside several cell types, such as U937 and monocytes[21].

We describe herein, by use of flow cytometry and confocal microscopy, the presence of an intracellular D-mannose-specific receptor in cells belonging to the monocyte lineage. By affinity chromatography on immobilized D-mannose, we isolated a new mammalian D-mannose receptor from the human promyelocytic leukemia cell line HL60, which was identified by electrophoresis as a 60 000-M_r protein.

EXPERIMENTAL

Materials. — Materials were obtained as follows: Bovine serum albumin (BSA) from IBF Biotechnics (Villeneuve la Garenne, France); Monensin from Calbiochem (San Diego, USA) [a stock solution in ethanol (25mM, 17.5 mg/mL) was prepared before use]; the protease inhibitors, aprotinin, antipain, leupeptin, and pepstatin, from Sigma Chemical Co. (St. Louis, MO, USA); α-toluenesulfonyl fluoride was obtained from Serva (Heidelberg, FRG); peroxide-free Triton X-100 from Pierce (Oud-Beijerland, The Netherlands); Iodogen from Bio-Rad Laboratories (Richmond, CA, USA); $Na^{125}I$ (559 MBq/μg of I) and X-ray film (hyperfilm-MP) from Amersham Corp. (Buckinghamshire, England).

Cells. — Thioglycolate-elicited peritoneal macrophages were harvested from hybrid mice (C57BL/6 × Balb/c)F1 injected i.p. four days earlier with thioglycolate medium (2 mL) (Institut Pasteur Production, Paris) by peritoneal lavage with RPMI 1640 medium (Gibco). Cells were washed and resuspended in phosphate-buffered saline solution (PBS), pH 7.4, containing 1% BSA, supplemented with mM Ca^{2+} and 0.5mM Mg^{2+} (complete PBS).

HL60 promyelocytic cells (kindly given by Dr. Le Floch, Rhone–Poulenc Recherches, Vitry-sur-Seine, France) and U937 promonocytic cells (kindly given by Dr. Turtz, Villejuif, France) were grown in RPMI 1640 medium, supplemented with 10% fetal calf serum (Gibco), 2mM L-glutamine (Merck), 100 units/mL of penicillin, and 0.1 mg/mL of streptomycin (Eurobio) (complete RPMI) in a humidified atmosphere (5% CO_2–95% air).

Monocytes were collected from whole blood of healthy donors (Centre de Transfusion Sanguine, Orléans, France). After centrifugation at 300*g* for 15 min to remove the platelet-rich plasma, mononuclear leukocytes were isolated by centrifugation on Ficoll Paque (Pharmacia, France)[22]. Cells (5–7 × 10^6/mL) in RPMI 1640 medium containing 5% fetal bovine serum were plated on Petri dishes (10 mL/dish), precoated with fetal bovine serum. After a 60-min incubation at 37° in a humidified atmosphere (5% CO_2–95% air), nonadherent cells were removed by three vigorous washes with RPMI 1640 medium and adherent cells were incubated at 37° in complete RPMI medium until use. Monocytes were harvested by incubation for 10 min at 4° in PBS containing 0.02% of ethylenediaminetetraacetic acid (EDTA).

Preparation of fluorescein-labeled neoglycoproteins. — Neoglycoproteins were prepared by treating the 4-isothiocyanatophenyl glycoside with BSA[23]. Neutral sugar content was determined by the resorcinol–H_2SO_4 micromethod[24] and hexosamine content after acid hydrolysis (5.6M HCl for 24 h at 105°) with an amino acid autoanalyzer (LC 6000 E Biotronik, Semsa, Boulogne, France). Neoglycoproteins were fluoresceinylated with fluoresceinyl isothiocyanate isomer I as previously described[23,25]. The number of bound-fluoresceinyl residues per protein molecule was determined from the absorbance at 495 nm after proteolytic digestion with Pronase[26]. The fluorescein-labeled neoglycoproteins used in this study (F-Man-BSA or F-GlcNAc-BSA) were substituted with three fluoresceinyl and 20 or 26 glycosyl residues, respectively.

Flow-cytometry analysis. — Cell-surface binding, uptake, and intracellular binding of fluoresceinylneoglycoproteins by macrophages, U937, or HL60 cells were studied by flow cytometry using a FACS analyzer (Becton Dickinson Immunocy, Sunnyvale, CA, USA). Volume and fluorescence intensity of each cell were simultaneously recorded at a rate of 200 cells/s (excitation wavelength, 485 ± 10 nm; emission wavelength, 530 ± 15 nm). Data were analyzed with the Consort 30 software.

To measure endocytosis, cells were incubated at 37° with fluoresceinylated ligands. As ligands are internalized in acidic compartments (endosomes and lysosomes) where proteolysis occurs and fluorescence of fluorescein decreases at low pH, a neutral pH was restored before flow-cytometry analysis[12,23,26]. Monensin, a proton sodium ionophore, was used to equilibrate the pH between organelles and cytosol. Cell-fluorescence intensities of each experiment were corrected with regard to fluorescent beads so that experiments done at different times were comparable[23].

Binding and endocytosis of fluoresceinylated ligands. — Macrophages, U937, or HL60 cells were incubated in suspension (2 × 10^5 cells/200 μL) with fluoresceinyl-neoglycoproteins (100 μg/mL) in complete PBS, either at 4° for 1 h to study binding at the cell surface, or at 37° for 2 h to study the uptake of the ligands. Cells were then washed with cold sheath fluid [134mM NaCl, 3.75mM KCl, 1.9mM KH_2PO_4, 16.53mM Na_2HPO_4, 15.25mM NaF, and 0.2% (v/v) 2-phenoxyethanol] to remove unbound ligands and resuspended in sheath fluid (0.5 mL). Cell-fluorescence intensities were measured by flow cytometry before and after a 30-min postincubation at 4° in the presence of 50μM monensin[26].

Intracellular binding of fluoresceinylated ligands in permeablized cells. — The concentration of saponin used to permeabilize cells was optimized to give both optimal cell recovery and permeabilization. Best results were obtained when paraformaldehyde was used to fix the cells during the permeabilization step. Cells (5×10^6/mL) were permeabilized with 0.1% saponin (Merck) in PBS in the presence of 2% paraformaldehyde (Merck) for 5 min at 4°, washed with complete PBS, and incubated in suspension (2×10^5/200 μL) with fluorescein-labeled neoglycoproteins (100 μg/mL) for 30 min at 4°. After the cells had been washed with cold sheath fluid, the cell-fluorescence intensity was analyzed by flow cytometry. The integrity of the cells during these experiments was monitored by electron microscopy.

Microscopy analysis. — Fluorescence was localized with a confocal fluorescence microscope (Lasersharp MRC-500, Bio-Rad, Oxfordshire, England) operating with a 488-nm Ar laser excitation wavelength.

Preparation of affinity column. — An affinity column was prepared by coupling 4-isothiocyanatophenyl α-D-mannopyranoside with activated agarose beads bearing amino terminal groups (Affi-102, Bio-Rad, Richmond, CA, USA). Packed beads (2 mL, 30 μmol of amino groups) were extensively washed with 0.3M NaCl, 0.1M $Na_2CO_3NaHCO_3$, pH 9.5 (carbonate buffer); 4-isothiocyanatophenyl α-D-mannopyranoside (11.3 mg, 1.2 equiv.; 36 μmol) in carbonate buffer (1.5 mL) was added and mixed overnight at room temperature with the Affi-gel. Beads were then washed with carbonate buffer (ten times each 10 mL). Washing efficiency was monitored by measuring the absorbance at 280 nm or the amount of 4-isothiocyanatophenyl α-D-mannopyranoside of the washing supernatant solution with the resorcinol–H_2SO_4 micromethod[24]. It was found that all the amino groups were substituted with mannosyl groups and that 9.4 mg of 4-isothiocyanatophenyl α-D-mannopyranoside were coupled to 2 mL of Affi-gel 102 (Affi 102-Man).

Preparation of HL60 and macrophages membranes. — HL60 cells (10^9) collected in exponential-growth phase were carefully washed in order to avoid cell rupture and to be free of culture-medium contaminants. Cells were washed twice with a phosphate-buffered saline solution (PBS), supplemented with mM $CaCl_2$ and 0.5mM $MgCl_2$ (400 mL), and then with 25mM KCl, 2mM $CaCl_2$, mM $MgCl_2$, and 10mM Tris·HCl, pH 7.4 (buffer A; 400 mL). Cells were resuspended in buffer A (2×10^7/mL) containing 0.34 TIU/mL of aprotinin and mM α-toluenesulfonyl fluoride and ruptured with a Potter homogenizer. Cell lysis was monitored by fluorescence microscopy using Acridine Orange in order to verify that nuclei were kept intact. Lysates were centrifuged at 800*g* for 10 min, and the postnuclear supernatant was collected and adjusted to 15mM $CaCl_2$, 0.5% saponin, and 0.2M D-mannose. After an incubation for 30 min at 4°, the membranes were centrifuged off at 100 000 *g* for 1 h in a Beckman L8-70 ultracentrifuge using a 70.1 rotor.

Macrophages (10^9), harvested three days after thioglycolate injection, were treated as described for the HL60 cells.

Purification of the D-mannose receptor from HL60 cells and macrophages. — The membrane pellet of HL60 cells was resuspended in the following solubilization buffer

(buffer B; 10 mL): 10mM Tris·HCl, pH 7.4, containing 150mM NaCl, 15mM $CaCl_2$, mM $MgCl_2$, protease inhibitors (10 μg/mL of antipain, leupeptin, and pepstatin, and 0.17 TIU/mL of aprotinin), and 1% Triton X-100. A peroxide-free Triton was used to avoid protein modifications[27]. The membranes were solubilized for 18 h at 4° under agitation. After centrifugation at 100 000*g* for 30 min, the solubilized membrane components were recovered in the supernatant. In order to monitor the purification of the receptor, an aliquot (2 mL) of solubilized membrane corresponding to $\sim 2 \times 10^8$ cells were iodinated by the Iodogen method[28]. Polypropylene test tubes (17 × 100 mm) were coated with Iodogen (200 μg), dissolved in chloroform (300 μL), and dried under a stream of dry N_2. The protein solution (1 mL) and $Na^{125}I$ (3.7×10^7 Bq; specific activity, 0.56 GBq/μg of I) were added in tubes plated with Iodogen and then incubated for 30 min at 4° under gentle stirring. The reaction was stopped by addition of 0.25M KI and removal of the protein solution from the coated tube. The labeled membrane extract was dialyzed against 10mM Tris·HCl, pH 7.4, containing 150mM NaCl, 15mM $CaCl_2$, mM $MgCl_2$, and 0.15% Triton X-100 (buffer C). The dialyzate was adjusted to 40mM $CaCl_2$ and incubated overnight at 4° with Affi 102-Man packed beads (2 mL). Beads were loaded into a column and washed with buffer C (300 mL) containing 40mM $CaCl_2$ and then with buffer C alone (15 mM $CaCl_2$; 10 vols.). The D-mannose-specific receptor was eluted with buffer C (20 mL) containing 0.2M D-mannose. A second elution was performed with buffer C (20 mL) lacking divalent cations but containing 10mM EDTA. Fractions (0.5 mL) were collected and analyzed for radioactivity (Compugamma 1282, LKB). The labeled fractions were pooled and proteins were precipitated with 40% (v/v) trichloroacetic acid. After centrifugation, the pellet was dissolved in the sample buffer (100 μL) and the solution subjected to sodium dodecyl sulfate–poly(acrylamide)gel electrophoresis (SDS–PAGE) under reducing conditions according to Laemmli[29]. 0.1M Dithiothreitol was used as reducing agent in the sample buffer instead of mercaptoethanol[30]. Proteins were detected by Coomassie Blue staining and the dried gel was then autoradiographed.

To evaluate the dependency on divalent cations for the binding of the receptor to its ligands, the purification was repeated under the same conditions, except that the first elution was performed with Ca- and Mg-free buffer C containing 10mM EDTA. The labeled fractions were pooled, precipitated, and subjected to SDS–PAGE as described above.

Purification of the macrophage D-mannose receptor was achieved as described above; the elution fractions (20 mL) were collected, concentrated on a Centricon microconcentrator C10 (Amicon Corp., Danvers, MA, USA) to 300 μL or by trichloroacetic acid precipitation and analyzed by SDS–PAGE. Electrophoretic bands were revealed by Coomassie Blue staining and autoradiography.

RESULTS

Cell-surface labeling, uptake, and intracellular binding of F-Man-BSA by macrophages, monocytes, and HL60 and U937 cells. — Cells were incubated with F-Man-BSA

TABLE I

Binding and uptake of mannosylated neoglycoprotein by cells of the monocytic lineage[a]

Cells	*Relative cell-fluorescence intensity at*								
	4°			*37°*			*37° + monensin*		
	F-BSA	*F-Man-BSA*	*Diff.*[b]	*F-BSA*	*F-Man-BSA*	*Diff.*[b]	*F-BSA*	*F-Man-BSA*	*Diff.*[b]
HL60	2	3	1	6	9	3	6	8	2
U937	2	3	1	2	4	2	3	6	3
Monocytes	3	6	3	16	38	22	16	43	27
Macrophages	3	19	16	20	350	330	28	600	572

[a] Thioglycolate-elicited mouse peritoneal macrophages, monocytes, and HL60 and U937 cells were incubated for 1 h at 4° or for 2 h at 37° in complete PBS in the presence of fluoresceinylated BSA substituted (F-Man-BSA) or not (F-BSA) with D-mannosyl groups (100 μg/mL), and then the cells were washed and resuspended in cold sheath fluid and the cell-fluorescence intensity was measured by flow cytometry before and after a postincubation with 50μM monensin. [b] Specific F-Man-BSA labeling: Difference between cell-associated fluorescence of cells incubated with F-Man-BSA and with F-BSA used as control. All data were standardized with reference to the fluorescence of calibration beads.

or F-BSA (100 μg/mL) at 4° to measure nonspecific binding and then analyzed by flow cytometry. There was no specific cell-surface labeling of HL60 and U937 cells, or monocytes by F-Man-BSA. A slight labeling of mouse peritoneal macrophages was observed (Table I), which was in agreement with the presence of the D-mannose-specific receptor described by Stahl *et al.*[1]. Since the macrophage D-mannose receptor is known to internalize its ligands[1,9,31], the four cell types were submitted to an incubation step at 37° in the presence of fluoresceinylneoglycoproteins to allow the endocytosis process. The enhancement of cell fluorescence of macrophages incubated at 37° with F-Man-BSA, as compared to the cell fluorescence of macrophages incubated at 4°, could be due either to a better affinity of the ligands to the cell surface receptor or to an internalization process. Cell-fluorescence intensity was analyzed by flow cytometry after a 4° postincubation in the presence or absence of monensin. The increase of fluorescence intensity associated with macrophages after monensin posttreatment (Table I) is in agreement with an internalization process. Indeed, endocytosis of F-Man-BSA in acidic endosomes and lysosomes leads to a decrease of fluorescence emission, which can be restored by a postincubation with monensin[32]. The very slight fluorescence associated with monocytes incubated at 37° did not increase after monensin posttreatment and so does not reflect a receptor-mediated endocytosis. The fluorescence of HL60 and U937 cells did not significantly change after incubation with high concentration of F-Man-BSA (100 μg/mL), thus revealing the absence of cell surface receptors. To determine whether the D-mannose receptor was present inside these cells, incubation with fluoresceinyl neoglycoproteins was performed after permeabilization of the cells with 0.1% saponin. Fixation with 2% paraformaldehyde was necessary to avoid destruction of cell structures. The enhancement of fluorescence intensity, associated with the cells incubated with F-Man-BSA after permeabilization, was very high in each one of the cell types tested,

TABLE II

Binding of mannosylated neoglycoprotein to permeabilized cells[a]

Cells	*Relative cell-fluorescence intensity*		
	F-BSA	*F-Man-BSA*	*Difference*[b]
HL60	4	134	130
U937	6	250	244
Monocytes	4	94	90
Macrophages	5	161	156

[a] The cells were preincubated with 0.1% saponin in the presence of 2% paraformaldehyde for 5 min at 4°, and then washed and incubated with the fluoresceinylated derivatives (100 μg/mL) for 1 h at 4°. After a second washing step, the cells were resuspended in cold sheath fluid and the cell-fluorescence intensity was measured by flow cytometry. [b] Specific F-Man-BSA labeling: Difference between cell-associated fluorescence intensity of cells incubated with F-Man-BSA and with F-BSA used as a control.

indicating the presence of intracellular receptors. BSA, used as a control, did not bind to permeabilized cells (Table II).

The presence of HL60 mannose receptors was assessed by cell incubation with increasing concentration of F-Man-BSA (Fig. 1). F-GlcNAc-BSA, which gave the lowest binding to permeabilized HL60 cells (data not shown), was used as a control instead of unglycosylated F-BSA which lacks phenylthiocarbamyl groups. The cell-associated fluorescence of permeabilized HL60 cells incubated with F-GlcNAc-BSA was subtracted to estimate the amount of specific labeling by F-Man-BSA. As shown in

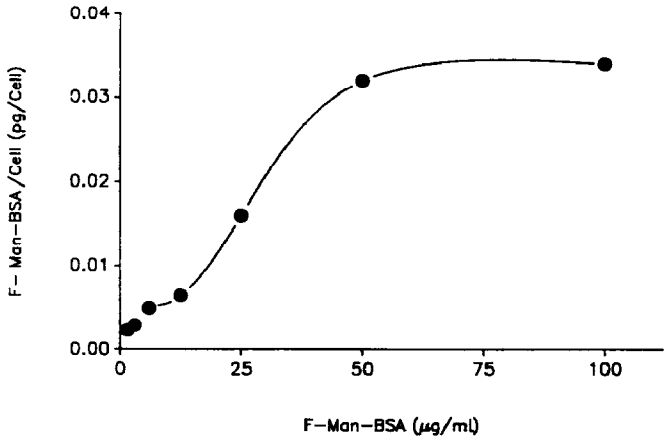


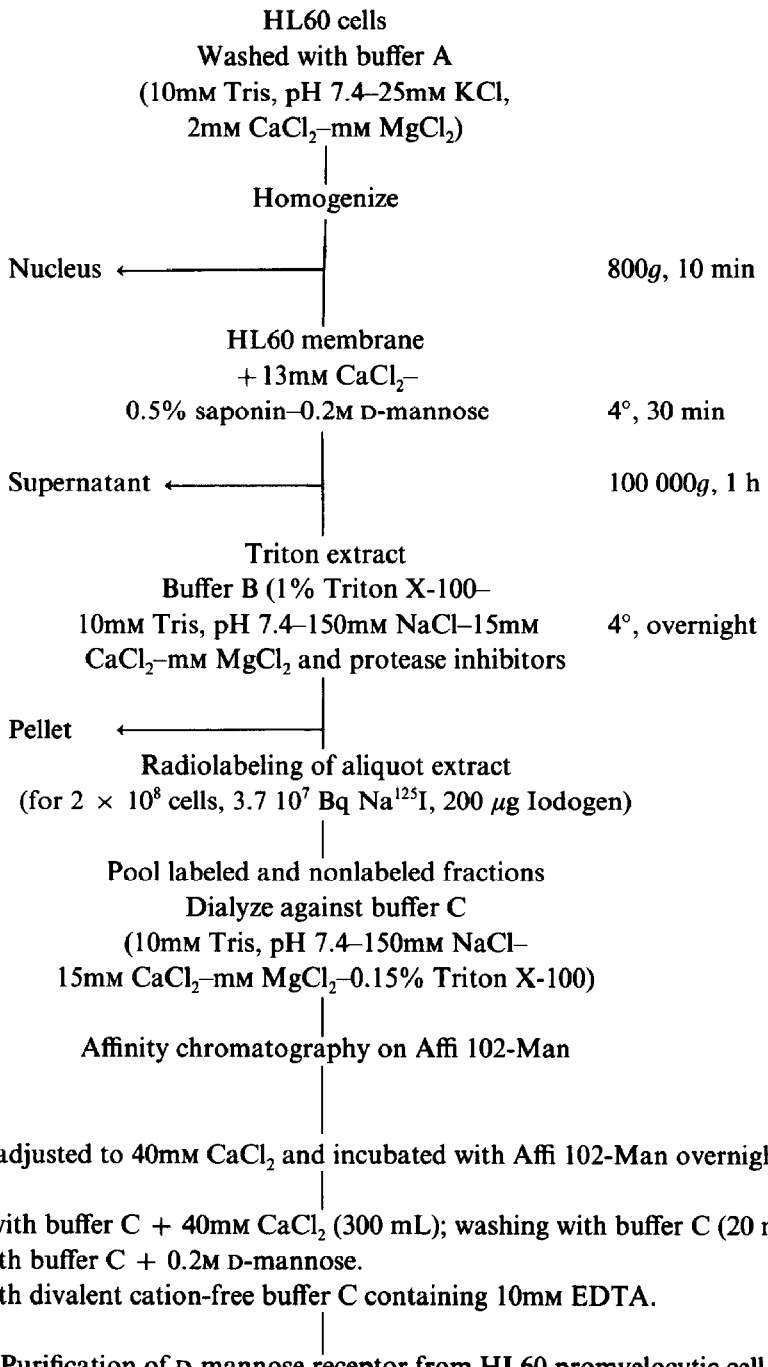
Fig. 1. Binding of F-Man-BSA to permeabilized HL60 cells. The cells were first permeabilized with 0.1% saponin in the presence of 2% paraformaldehyde for 5 min at 4°. After being washed with PBS, the cells were resuspended in complete PBS (2×10^5/200 μL) and incubated with various concentrations of either F-Man-BSA or F-GlcNac-BSA for 1 h at 4°. The cells were then washed and resuspended in cold sheath fluid. The cell-fluorescence intensity was measured by flow cytometry. The specific cell-fluorescence intensity of cells incubated with F-Man-BSA minus the fluorescence intensity of cells incubated with F-GlcNAc-BSA was used to calculate the amount of bound F-Man-BSA per cell according to Midoux *et al.*[26].

Fig. 2. Localization of the receptor specific for D-mannose into permeabilized HL60 cells. The cells were permeabilized with 0.1% saponin in the presence of 2% paraformaldehyde for 5 min at 4°, and then washed, resuspended in complete PBS (10^6/200 μL), and incubated with F-Man-BSA (100 μg/mL) for 1 h at 4°. The cells were washed, centrifuged off onto microscope slides, and observed with a confocal microscope: Eight sections from the top to the bottom of one cell are presented. The dark part of the cell represents the nucleus.

the graph, it is clear that the binding is concentration dependent and saturable, as expected for a receptor-specific labeling. Furthermore, the graph may suggest that there is one receptor in a low concentration with high affinity, and a second receptor with a lower affinity but in a higher concentration.

Localization of the D-*mannose receptor in HL60 cell line.* — It was of interest to localize precisely the D-mannose receptor evidenced by flow cytometry. Hence, cells were permeabilized, fixed, and incubated with F-Man-BSA as described above, and then they were observed with a confocal fluorescence microscope which allowed the observation of different sections of permeabilized HL60 cells (Fig. 2). The experimental conditions used prevented permeabilization of the nuclear membrane; the dark part represents the nucleus and the fluorescence observed was localized in the cytoplasm. This observation demonstrated that fluorescence measured by flow cytometry corresponds to the binding of F-Man-BSA onto intracytoplasmic receptors and not onto intranuclear receptors.

Purification of the D-*mannose receptors.* — Macrophages of various origin specifically bind and internalize mannosylated glycoproteins through a receptor which was identified and isolated as a 175 000-M_r membrane glycoprotein[3-7]. Since flow cytometry showed the presence of intracellular receptors in cell types that lack receptors at the cell surface (HL60 and U937 cells, monocytes), it seemed interesting to know whether the two D-mannose receptors were different proteins or not. D-Mannose-specific lectins were purified from both macrophages and HL60 cells by use of the same affinity column. The receptors were isolated according to the procedure described in the Experimental section and summarized in Scheme 1. Specifically-eluted fractions were concentrated or precipitated before analysis by SDS–gel electrophoresis under reducing conditions.



(1) Dialyzate adjusted to 40mM $CaCl_2$ and incubated with Affi 102-Man overnight at 4° (2 mL).
(2) Washing with buffer C + 40mM $CaCl_2$ (300 mL); washing with buffer C (20 mL).
(3) Elution with buffer C + 0.2M D-mannose.
(4) Elution with divalent cation-free buffer C containing 10mM EDTA.

Scheme 1. Purification of D-mannose receptor from HL60 promyelocytic cell line.

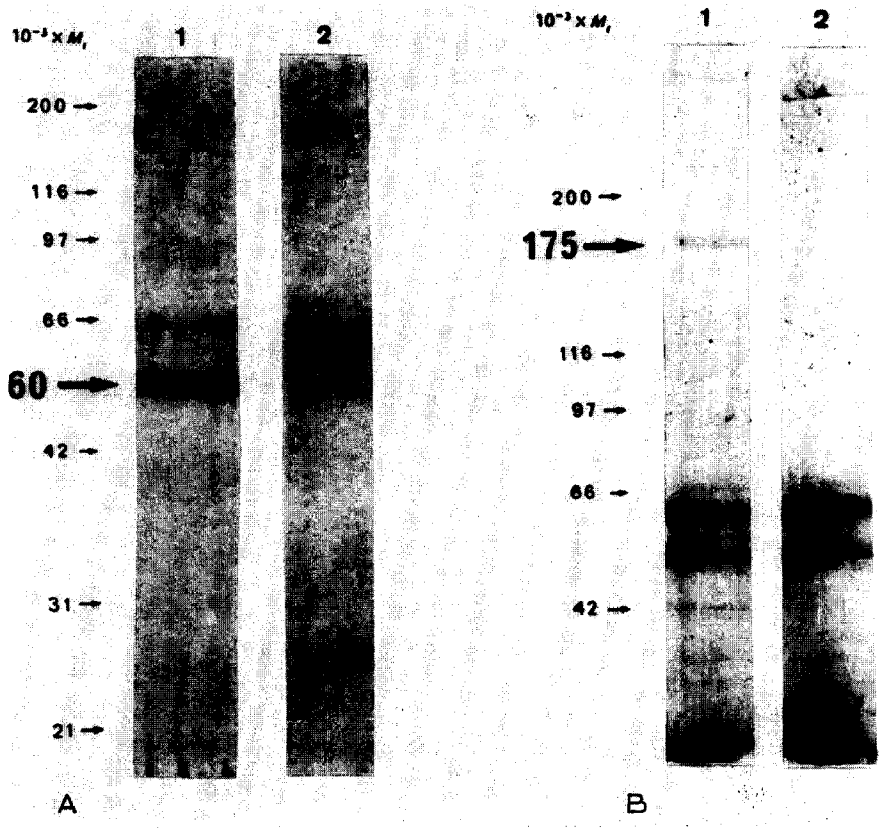


Fig. 3. Electrophoresis of HL60 cell- and macrophage-membrane proteins isolated by affinity chromatography. The membrane extracts of 10^9 HL60 cells (A) or macrophages (B) were chromatographed on Affi-102-Man affinity column. After extensive washing, the column were eluted first with buffer C containing 0.2M D-mannose (lane 1) and then with divalent cation-free buffer containing 10mM EDTA (lane 2). Selected fractions were pooled and analyzed by SDS–PAGE under reducing conditions in 7.5–15% poly(acrylamide) after trichloroacetic acid precipitation (A) or in 10% poly(acrylamide) after concentration (B). Proteins were stained by Coomassie Blue. Standard protein markers (arrows) were: Myosin heavy chain (M_r 200 000), D-galactosidase (116 000), phosphorylase B (97 000), BSA (66 000), ovalbumin (42 000), carbonic anhydrase (31 000), and soybean trypsin inhibitor (21 000).

After Coomassie Blue staining (Fig. 3), the 175 000-M_r protein known to be the macrophage D-mannose-specific receptor was shown to be present in mouse-peritoneal-macrophage membrane preparation. The detection method used also revealed artifactual proteins having M_r values between 50 000 and 68 000, which probably represent the keratins as described by Ochs[33]. No more specific D-mannose-binding proteins were detected in the fraction eluted with EDTA. The intracellular receptor, purified from HL60 cells on Affi 102-Man, had an approximate mol. wt. of 60 000 and no protein was detected having a mol. wt. around 175 000 (Fig. 3). EDTA-containing buffer did not release specific D-mannose-binding proteins when used as the second eluent. The dried gel, analyzed by autoradiography, confirmed the presence of a single 60 000-M_r protein

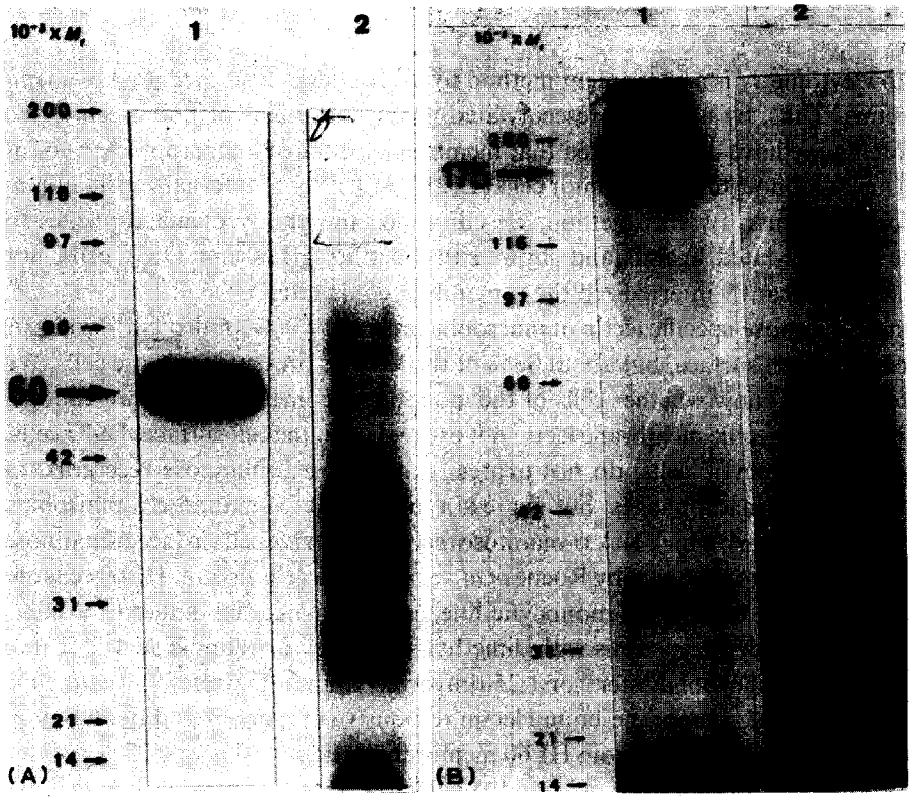


Fig. 4. Autoradiogram of HL60 cell- (A) and macrophage-membrane (B) proteins after purification by affinity chromatography. HL60 cell- and macrophage-membrane proteins, eluted from Affi-102-Man were precipitated and subjected to SDS–PAGE on a 7.5–15% poly(acrylamide) gradient (Fig. 3), and the electrophoresis was followed by autoradiography at −70° for 24 h: Lane 1, elution with buffer C containing 0.2M D-mannose. Lane 2: elution with divalent cation-free buffer C containing 10mM EDTA.

(Fig. 4). Autoradiography of radiolabeled-macrophage proteins specifically eluted from Affi 102-Man showed the 175 000-M_r but no 60 000-M_r protein (Fig. 4). Elution with EDTA led to few labeled bands corresponding to proteins nonspecifically bound to the affinity column. To determine the requirement of divalent cations for the binding of D-mannose to this new receptor, the purification was repeated with 2×10^8 labeled HL60 cells using the same procedure, but the first elution was achieved with divalent cation-free buffer containing EDTA and the second one with buffer containing D-mannose. Electrophoresis, followed by autoradiography, showed that a 60 000-M_r protein was eluted with EDTA at the same time as many other proteins; no more protein was eluted with the second D-mannose–buffer elution (data not shown). Therefore, the binding of the mannosylated ligands to the intracellular receptor is cation dependent.

DISCUSSION

Flow cytometry is a convenient method to study endocytosis mediated by membrane lectins. Thus, by using fluoresceinylated neoglycoproteins or fluoresceinylated glycoproteins, we have demonstrated that membrane lectins of a macrophage hybridoma cell line[34], monocytes[12], and tumor cells (L1210, 3LL)[23,25,26,35] internalized ligands in acidic compartments. By this method, we confirmed the ability of macrophages to endocyte mannosylated ligands and showed that neither HL60 nor U937 cells, nor monocytes significantly internalized the D-mannose conjugate.

The D-mannose-specific lectin of macrophages is a 175 000-M_r protein[3–7] which is expressed at the cell surface, but about 80% of the lectin is present inside the cells. We were interested to know whether cells of the mononocytic lineage possess an endogenous D-mannose-specific membrane lectin not expressed on the cell surface. U937 cells, which are promonocytic cells, do not express cell surface D-mannose receptors, in contrast to mature macrophages, but express a low level of D-mannose 6-phosphate receptors[21]. Monocytes, which lack D-mannose receptors[11], have cell surface D-mannose 6-phosphate receptors as shown by Roche *et al.*[12], but HL60 cells do not. HL60 cells are very early immature cells in the monocytic lineage as they may be induced to differentiate into granulocytic type cells with dimethyl sulfoxide[36] or retinoic acid[37], or into macrophages with phorbol diester[38] or 1,25-dihydroxyvitamin D_3 (ref. 39) treatments. Recently, a 17 000-M_r membrane-bound lectin responsive to monocytic differentiation was characterized and isolated from HL60 membrane extracts[19]. The specificity of this lectin is restricted to both sialic acid and D-galactose units-terminated biantennary oligosaccharides. It was of interest to ascertain the presence of an intracellular D-mannose receptor in these cells that are macrophage precursors. To allow access of ligands to intracellular-binding sites, cells were permeabilized with saponin[18] or digitonin[40] used as detergents. Saponin treatment increased the D-mannose-specific labeling of ^{125}I-labeled Man-BSA in alveolar macrophages since only 20% of the receptors are expressed at the cell surface and the majority are intracellular[18]. For HL60 cells, the best results were obtained by use of a low concentration of saponin (0.1%) during paraformaldehyde fixation. Indeed, HL60 cells are very sensitive to environmental changes, and incubation of permeabilized cells with F-ligands induced dramatic morphological damage when the incubation was performed without cell fixation. After fixation and permeabilization, HL60 cells were strongly labeled with F-Man-BSA, and confocal microscopy analysis showed that the fluorescent labeling was restricted to internal membranes; no nuclear localization was detected. Nuclear-lectin activity has recently been described[41] but labeling of nuclear material required a stronger permeabilization. Although internal D-mannose receptors seemed more important in U937 than in HL60 cells, we chose to isolate the D-mannose receptor from the most immature monocyte precursor cells (HL60) to compare it with the D-mannose receptor of the more mature cells, peritoneal macrophages. Two different D-mannose membrane receptors were characterized from HL60 cells and macrophages by affinity chromatography on a gel substituted with D-mannosyl residues, followed by SDS–gel electrophoresis analysis

under denaturing conditions. As expected, a 175 000-M_r protein was selectively eluted by D-mannose-containing buffer from mouse macrophage membrane extracts; the protein corresponded to the well-known macrophage D-mannose receptor isolated from rabbit, human, and rat macrophages[3–7]. D-Mannose-specific elution of HL60 membrane extracts, chromatographed on the same type of column (Affi 102-Man), released a lower-molecular-mass protein (M_r 60 000), as shown by SDS–electrophoresis and autoradiography. This D-mannose-specific endogenous lectin requires cations because it was eluted by removing the divalent cations. Higher-molecular-mass proteins were not detected in HL60 cells. The intracellular D-mannose receptor of HL60 cells differs from that of macrophage by its molecular mass and, moreover, by its sugar specificity. Whereas the 175 000-M_r D-mannose receptor is known to have a high affinity for *N*-acetyl-D-glucosamine[1,9] and L-fucose[2], the 60 000-M_r D-mannose receptor did not recognize neoglycoproteins bearing terminal *N*-acetylglucosaminyl groups. Furthermore, this HL60 D-mannose lectin differs from the 32 000-M_r D-mannose-binding protein shown to be present in liver and serum of various species (for a review, see Ezekowitz and Stahl[42]) and also detected by Ii *et al.*[43] in rat peritoneal macrophages. The HL60 D-mannose receptor probably does not belong to the family of the liver D-mannose-binding protein. The preparation of monoclonal antibodies against this HL60 D-mannose lectin would allow the characterization of the D-mannose receptor in other cells and the study of biogenesis of the lectin. So far, there is no evidence that this new D-mannose receptor is related to the 175 000-M_r macrophage D-mannose receptor.

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