

Molecular Characterization of the Human Macrophage Mannose Receptor: Demonstration of Multiple Carbohydrate Recognition-like Domains and Phagocytosis of Yeasts in Cos-1 Cells

By R. Alan B. Ezekowitz, Kedarnath Sastry, Pascal Bailly, and Angeline Warner*

*From the Division of Hematology/Oncology, Children's Hospital, Dana-Farber Cancer Institute, Department of Pediatrics, Harvard Medical School; and the *Department of Respiratory Biology, Harvard School of Public Health, Boston, Massachusetts 02115*

Summary

The macrophage mannose receptor is an integral membrane protein expressed on the surface of tissue macrophages. After ligation of mannose-rich glycoconjugates or pathogens, the receptor mediates endocytosis and phagocytosis of the bound ligands by macrophages. The cDNA-derived primary structure of the mannose receptor predicts a cysteine-rich NH₂-terminal domain, followed by a fibronectin type II region. The remainder of the ectodomain is comprised of eight carbohydrate recognition-like domains, followed by a transmembrane region, and a cytoplasmic tail. Transfection of the mannose receptor cDNA into Cos-1 cells is necessary for receptor-mediated endocytosis of mannose-rich glycoconjugate as well as phagocytosis of yeasts. Deletion of the cytoplasmic tail results in a mutant receptor that is able to bind but not ingest the ligated pathogens, suggesting that the signal for phagocytosis is contained in the cytoplasmic tail.

Plasma clearance and cell uptake experiments (1) indicated the existence of a receptor that bound glycoproteins bearing high mannose chains. Receptor activity was especially evident in the liver and spleens, although most other tissues were also positive. Subsequent experiments have established that the mannose receptor is expressed on the cell surface of tissue macrophages which reside in a wide anatomical distribution throughout most organs (2). Although originally defined by its ability to mediate endocytosis of mannosylated or fucosylated glycoproteins, the mannose receptor's predominant role appears to be in host defense. The receptor engages yeasts (3) and parasites (4) directly resulting in the engulfment of these particles and the release of biologically active secretory products like reactive oxygen intermediates (5), arachidonate metabolites (6), neutral proteinases (7), and monokines like IL-1 and tumor necrosis factor (8). As circulating monocytes do not express the mannose receptor on their cell surface (9), the functional role for the mannose receptor appears to be on tissue macrophages, which in addition to their localization in the reticular endothelial system line the alveolus and form a reticular network beneath epithelial surfaces in the skin, the gastrointestinal tract, kidney, and placenta (10). These sites are the portals of maximum antigen load and underscore the role for the mannose receptor in first line host defense.

Recent experiments suggest that a circulating hepatocyte-

derived serum mannose-binding protein is the functional serum equivalent of the tissue macrophage mannose receptor. The human mannose-binding protein (MBP)¹ is an acute-phase reactant (11) that binds certain viruses (12) and other mannose rich pathogens (13). Mannose-binding proteins after engaging organisms mediate attachment, uptake, and killing of opsonized bacteria by circulating phagocytes that do not express the mannose receptor (13). Underlying structural similarity between the MBP and the mannose receptor, thereby explaining their functional equivalence, was suggested by the discovery that heteroantisera raised against human MBP were able to immunoprecipitate a glycoprotein of 170 kD from the surface of macrophages (Ezekowitz, R.A.B., and P. Stahl, unpublished data), this being the molecular weight of the macrophage mannose receptor (14).

Based on these findings, it appeared likely that the mannose receptor is part of the same family of lectin-like proteins as the mannose-binding protein that includes soluble proteins and cell surface receptors, all of which share structural homology in their carbohydrate recognition domains and require calcium for ligand binding (15). Some of the cell surface receptors in this group mediate endocytosis of glycoproteins with specific terminal residues exposed (15). The functions of the lectin domains of more recent additions to

¹ *Abbreviations used in this paper:* CRD, carbohydrate recognition domains; MBP, mannose-binding protein; PGK, phosphoglycerate kinase.

this family which include the lymphocyte homing receptors Mel14/Leu8 (16, 17), GMP140 (18), a granular membrane protein of platelets and endothelium, and ELAM I (19), an inducible endothelial antigen, have yet to be defined. The mannose receptor, like the asialoglycoprotein receptor family, can rapidly recycle to the cell surface (1); however, the mannose receptor is the only member of this group of receptors that can mediate phagocytosis.

The purification of the mannose receptor from human and rabbit macrophages and human placenta, an organ rich in many cell types, including macrophages, yielded a 175-kD glycoprotein that specifically bound a radiolabeled mannose-BSA glycoconjugate (14, 20). Shared peptide information obtained from mannose receptor purified from human placenta (as described in reference 21) enabled the characterization of overlapping cDNAs that represent the human macrophage mannose receptor as described in this study. In addition, we show the macrophage expression of the mannose receptor in Cos-I cells is necessary to mediate binding and phagocytosis of mannose rich yeasts as well as pinocytosis of radiolabeled mannosylated glycoconjugates. The cytoplasmic tail is essential for phagocytosis, as a tailless mutant receptor fails to induce ingestion of bound yeasts. The macrophage mannose receptor was found to be identical to the form from placenta (21), suggesting that the cell of origin in placenta may be the macrophage.

Materials and Methods

Isolation of cDNAs. Sequences of peptides derived from human placenta mannose receptor were shared and are described previously (21). A degenerate oligonucleotide probe was synthesized on a oligonucleotide synthesizer (DuPont Co., Wilmington, DE) purified by gel filtration and labeled with [³²P]ATP and polynucleotide kinase based on the peptide YVSWATGEPNFANED. Radiolabeled probe was used to screen a pCDM8 placental cDNA library (gift of Dr. B. Seed, Harvard Medical School, Boston, MA) by colony hybridization. Four duplicate colonies were purified by two further rounds of screening. Sequence analysis of all clones revealed an exact match of encoded peptide. The insert from the largest clone (3.3 kb) was then radiolabeled as described and used to screen a human macrophage λGT11 cDNA library. This library was prepared from poly(A) mRNA isolated from human monocytes cultivated for 7 d in Teflon beakers in the presence of 10% autologous human serum. These cultivated macrophages express 5×10^5 mannose receptors/cell. The cDNAs were constructed by a modification (22) of the Okayama and Berg (23) method. The cDNA pools were blunt-ended by treatment with T4 DNA polymerase and ligated to EcoRI linkers after protection of internal EcoRI sites with *Escherichia coli* methylase. The linker-ligated cDNAs were then digested with EcoRI and passed down a Sepharose CL4B column and then ligated to phosphatase-treated λGT11 vector DNA. The library contains 2×10^6 recombinants and was plated on *E. coli* strain Y1088. Filters were prehybridized, hybridized, and washed as described (24). 25 positive phages were isolated by two rounds of amplification and analyzed. In addition, the library was screened with a 500-bp cDNA fragment which contained the NH₂-terminal portion of the receptor (a generous gift from Drs. M. Taylor and K. Drickamer, Columbia University, New York, NY) isolated from a placental cDNA library.

Sequential Strategy. The initial placental clone was sequenced

by doubled-stranded sequencing using a modified T7 polymerase. Sequenase was based on the Sanger et al. chain termination method (25). Specific oligonucleotides were synthesized and used as sequencing primers. For phage clones, 2 μl of purified stock was annealed to λGT11 primers from each of the arms, and the Taq polymerase-amplified product obtained after 25 cycles (94°C, 30-s denaturation, 55°C 30-s annealing, and 72°C 3-min extension) on a thermal cycler (26) and the products were gel purified by agarose gel electrophoresis. The purified products were digested with EcoRI and subcloned into pUC-19 vector and the nucleotide sequenase was determined as described above.

Mannose Receptor Expression. A full-length clone was constructed in CDM8 expression vector by a multiple step procedure. First, a PCR product between 100 and 1,900 bp was constructed using a sense 5' primer containing an XhoI site and an antisense 3' primer containing an EagI site using a 5' clone as a template. The product was digested with XhoI and EagI and vectorally cloned into CDM8 digested with XhoI and NotI. Secondly, a 3' 3.3-kb clone was cloned into the EcoRI site of CDM8 and clones of correct orientation were determined by sequence analysis. As the 3' EcoRI/NotI fragment comigrated with the vector, a triple digestion with DraI which digests the vector and the 3' noncoding region of the DNA was performed, and the 2.6-kb EcoRI-DraI fragment was cloned into the 5' CDM8 construction after digestion with EcoRI and NotI. Partial sequence analysis confirmed that construction was as predicted. Cos-I cells were transfected as described (27). Transfection efficiency was 10–30% as assessed by immunofluorescence and FACS analysis with a rabbit anti-human mannose receptor antisera (a gift of Dr. P. Stahl, Washington University, St. Louis, MO). A tailless mutant was prepared by constructing an antisense primer from 4,276 to 4,308 bp containing an Hpa I site and including the first two amino acids of the cytoplasmic tail. The sense primer was prepared from 3,400 bp encompassing the NsiI site to 3,510 bp. The primers were annealed to full-length cloned MR DNA and a 812-bp fragment was amplified using the polymerase chain reaction. The full-length cDNA, MR CD8M8, was then digested with NsiI and HpaI which released a fragment from the NsiI site in the cDNA to a unique flanking HpaI site, thereby removing the cytoplasmic tail and some vector sequence. This was replaced with the 812-bp PCR product, thereby creating a clone confirmed by sequence analysis that contained the entire ectodomain, the transmembrane region, and two lysines of the cytoplasmic tail. This DNA was transfected into Cos-I cells as described above and the surface expression was verified by immunofluorescence and FACS analysis. The FACS analysis was performed on 10^5 cells that were specifically stained with a rabbit anti-human mannose receptor antisera (gift from Dr. P. Stahl) and a FITC-conjugated goat anti-rabbit second antibody (Fisher Scientific Co., Pittsburgh, PA). Mock-transfected Cos cells represented controls for these experiments.

Uptake of ¹²⁵I-Mannose-BSA. Mannose-BSA (EY Laboratories, San Mateo, CA) was radiolabeled as described (28) and uptake of radiolabeled ligand was performed on Cos-I cells transfected with MR CDM8 full-length clone. Cos-I cells transfected with CD64 served as controls and thioglycollate elicited mouse peritoneal macrophages served as a positive control (28).

Phagocytosis Assays. MR CDM8-transfected Cos-I cells and CD64-transfected Cos-I cells were trypsinized for 24 h after transfection and replated on glass coverslips in 24-well tissue culture trays and cultivated for a further 2 d in Dulbecco's modified medium supplemented with 5% FCS. The cells were then incubated with *Candida albicans* strain 9177 (gift of Dr. D. Bonner, Squibb Medical Institute, Princeton, NJ) for 5 and 20 min, respectively, at 37°C. Thereafter the cells were washed several times in HBSS plus 1%

FCS and either wet mounted for Nomarski (differential interference contrast) microscopy or fixed with 1% formaldehyde and viewed by phase-contrast microscopy. For electron microscopy, transfected cells were cultivated on glass coverslips or in 100-mm tissue culture dishes and incubated with *Candida albicans* as described above, for 5 or 20 min at 37°C. As the cells that had ingested yeasts appeared to be less adherent than the nonphagocytic nontransfected cells, the media were aspirated and the remaining cells were treated with trypsin and then pelleted with the cells in the aspirate. Thereafter the cell pellet was fixed in 2% glutaraldehyde in cacodylate buffer, pH 7.4, and 500-nm sections were cut and stained with toluidine blue, which stains the yeasts and the nucleus blue and viewed by light microscopy, or uranyl acetate and lead citrate-stained sections were examined by transmission electron microscopy as described (29). Quantitation of phagocytosis was undertaken by examining fixed whole mount preparations of cells after they had been incubated for 20 min at 37°C with a ratio of 20:1 *Candida albicans*/cells. The preparation was stained with toluidine blue and ingested yeasts were scored by Nomarski microscopy. Cells transfected with the full-length MR cDNA particles that bound, but were not ingested, scored negatively. Positive cells were graded as those having <5 or >5 ingested particles. At least 100 cells were counted in three independent coverslips. For cells transfected with the tail minus construct, no particles were ingested and hence binding of yeasts to the cells was viewed as positive and graded as <5 or >5.

RNA Analysis. RNA was prepared from human alveolar macrophages (gift of Dr. R. Rose, Harvard Medical School), human umbilical vein endothelial cells, Hela cells, and HepG2 cells as described. The RNA was size fractionated on an agarose gel and transferred to Nytran hybrid membrane (Amersham Corp., Arlington Heights, IL) and hybridized with a radiolabeled MR cDNA and cDNA for phosphoglycerate kinase (PGK) (30).

Results

Characteristics of the Macrophage Mannose Receptor

Partial cDNAs obtained from a human placental cDNA library were used as probes to screen a macrophage cDNA library as it was not certain, although likely, that the cell that expressed the mannose receptor in placenta was a macrophage. Analysis of overlapping cDNAs that represent the human macrophage receptor (EMBL accession number X55635) reveal that the macrophage-derived clones are identical to those from placenta (21) except for a C to T polymorphism at nucleotide 2284 that does not alter the encoded amino acid Thr.

The features of the receptor are depicted in a schematic diagram (Fig. 1 *a*) and include (*a*) a typical hydrophobic signal peptide; (*b*) a cysteine-rich NH₂-terminal region; (*c*) a fibronectin type II-like domain; (*d*) eight carbohydrate recognition-like domains; (*e*) a hydrophobic transmembrane region; and (*f*) a cytoplasmic tail.

Domain 1 Cysteine-rich NH₂ Terminals. This region is comprised of 147 amino acids and contains six cysteines. It bears no homology to any other known structure, although a cysteine-rich region has been described in the unpublished COOH-terminal end of the type I macrophage scavenger receptor (31), which has the opposite orientation to the man-

nose receptor. The function of this region in the mannose receptor is not known.

Domain 2 Fibronectin Type II Domain. The next region between amino acids 139 and 192 resembles the type II repeat of fibronectin with identity at 26 out of 62 amino acids (32). Although the precise function of this region is not known, it is likely that it may play a role in interaction with the extracellular matrix and may contribute to the spreading and adhesion of tissue macrophages that express the mannose receptor.

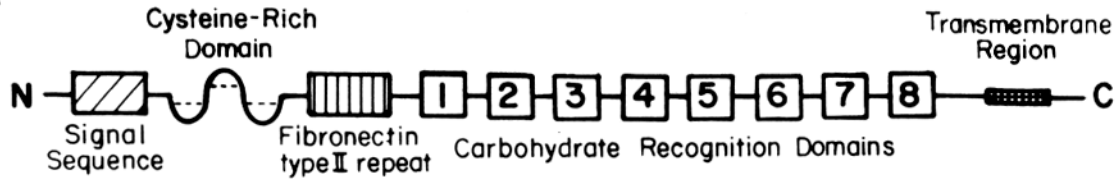
The Carbohydrate Recognition-like Domains. The remainder of the extracellular regions of the receptor are comprised of eight segments which are related to the C-type carbohydrate recognition domains (CRDs) of animal lectins (15). The prototype of this ever growing family of lectin-like receptors is the mammalian asialoglycoprotein receptors, which mediate endocytosis of glycoproteins. However, these receptor-related proteins are type II membrane proteins, have opposite orientation to the mannose receptor and a single CRD, and are not known to be phagocytic receptors.

A detailed analysis of the sequences of the CRDs of the mannose receptor is shown in Fig. 1 *b*. The domains are aligned with the positions of the "invariant" residues of the C-type lectins. The repeats are grouped to emphasize the identity of conserved amino acids and includes short intervening stretches of amino acids that are not homologous to one another. Domains 4 and 5 are the most homologous to one another with 34% identity and 49% similarity. Both of these domains have a characteristic WND motif, which is a feature of all other C-type lectins. These two domains bear the closest homology to other known mannose-binding proteins as shown in Fig. 1 *c*. The alignment of the COOH-terminal 62 amino acids of the mouse MBP-A and C, rat MBP-A and C (Sastri, K., K. Zahedi, J.-M. Lemas, A.S. Whitehead, and R.A.B. Ezekowitz, manuscript submitted for publication), and human MBP (11, 24, 33) is shown with domains 4 and 5 of the mannose receptor. 32 of 62 amino acids from domains 4 and 5 of the receptor are identical to at least one of the mannose-binding proteins (Fig. 1 *c*).

Cytoplasmic Domain. The COOH-terminal hydrophilic cytoplasmic domain of the mannose receptor is of great interest, especially since transfection of the receptor alone in Cos-I cells is sufficient to mediate pinocytosis and more surprisingly phagocytosis (see below). The localization of certain endocytic receptors to coated pits appears to be dependent upon the motif FXNPXY (34). The mannose receptor has a motif FXNNTXY in the cytoplasmic tail positions 1406 to 1411, and it is of note that the proline, which appears to be essential for LDL receptor localization to coated pits, is replaced by a threonine in the mannose receptor. Noteworthy in the analysis of the rest of this sequence is that it reveals no other obvious homology to the cytoplasmic domains of other endocytic receptors; however, there are several serines and threonines that may be possible sites of phosphorylation.

RNA Analysis

Total RNA was isolated from a limited number of cell types and analyzed by Northern analysis for mannose receptor tran-

A**B**

Invariant [C type lectins]

G C

```

CRD 1 (189)          GYCPLKF..EGSESLWNKDP L TSVS...YQINSK SALTWHQARKSCQQQAELL SITEI
CRD 2 (324)      KGNTTLN.SF...VIPSE.SDVPTHC...PSQW WPYAGHCYKI...HRDEKKIQRD..ALTTCRKEGGDLTSIHTI
CRD 3 (470)      MKRSRSGP...EIP EIVEVEK.GCRK...GWKKHHFYCYMI..GHTLSTFAE...ANQTCNNENAYLTTIEDR
CRD 4 (609)      HWAEGVTHPP...KPTTTP E.P.K..CPED..WGASSR TSLCFK...LYAKGKHEKKTWFESRDFCRALGGDLASINN K
CRD 5 (761)      IQKGQTPKP...EPTPAPQDNPPV.TED...GWVIYKDY...QYFYSKEKETMDN.ARAFCKRNFGLVSIQSE
CRD 6 (906)      RHNS SINAT...TVMPTMPSVPS.GCKE...GWNFYSNKCFKIFGF MEE...ERKNWQ EARKACIGFGN L VSIQNE
CRD 7 (1063)     TRSD...PSLTNPPATIQT.D...GFVKYKSS...YSLMRQ.KFQWHEAETYCKLHNSLIASILD P
CRD 8 (1196)     RSDEIPATEPPQL...PGR.CPESDHTAWIPFHGHC...YYIESSYTRNWQASLECLRMGSSLVSIESA

```

S P P C E G W Y C Y W A R C G G L S I

G T D T W P G E C G W N D C C E

```

HEQTYL.TG.LT.S.SLTS.GLWIGLNSLSFN SGW.QWSDRSPFRYL N WLP GSPSAEPG...K.SCVSLNPG...KNAKWENLECVQLGYICK
EELDFI.ISQLGY..EP.NDELWIGLNDIKIQMY.FEWS DGTPTVFTKWL RGEPSHE...NNRQEDCVVMK.G...K DGYWADRGCCEWPLGYICK
YEQAFL.TSFVGL..RP.EKYFWTGLSDIQTKGT.FQW TIEEEVRFTHWNSDMP...GRKPGCVAMRTG..IAGGLWDLVKCDEKAKFVCK
EEQQTIIWRLI.TASGS.YHKLFWLGLTYGSPSEG.FTWS D GSPVSYENWAYGEPN...NYQNVEYCGELK.G..DPTMSWNDINCEHLN N WICQ
SEKKFLWKYVNRN..DAQSAYF.IGL.LISL DKK.FAWMDGSKVDYVSWATGEPN...FANEDENCVTMYS...NSGF WNDINCGYPNAFICQ
KEQAFL.TYHMKD..STFSA..WTGLNDVNSEHT.FLWTDGRGVHYTNWCKGYPGRRSSLSYEDADC VVIIGGASNEAGK WMD DTCDSKRGYICQ
YSNAFAW.LQMET..SN.ERV.WIALNS.NLTDNQYTWDKWRVRYTNWAADEPKLK.SA...CVYL...DL DGYWKT AHCNESFYFLCK
AESSFLS.YRVEPLKSKTN..FWIGLFR.NVEGTW.LWINNSPVSVFNWNTG DPS...GERND CVTLHA...SSGFWSNIHCSSYKGYICK

```

EQ FL S FWIGLN F W DGSPV YTNW GEP CV G G W D C GYICK

C

```

Mouse MBP A  FLGIITDEATEGGMYVTSGR LTRSNWKKDEPNHGSQEDCVTILDNG..LWNDISGQASFKAVG (174-235)
Rat MBP A    FLGIITDEVTEGGMYVTSGR LTRSNWKKDEPNHGSQEDCVTIVDNG..LWNDISGQASHTAVG (173-234)
Human MBP   FLGIITDEKTEGGVVDLTONRLTMTNWNNEGERNNVGSQEDCVTELKNG..QWNDVPGSTSHLAVG (183-244)
Rat MBP C   FLGIITDQRTENVEDLTONRMTNWNNEGERNNVGSQEDCVMLTNG..KWN DVPSSDSFLVVG (179-240)
Mouse MBP C YLGIITDVRVEGSEEDLTONRMTNWNNDGEPNNTGDGECVIVLNG..KWN DVPSSDSFLAIE (179-240)
MRCRD4      WLGLTYGSPSEGFTWS DSSPVS MENWAYGEPN NYQNVEYCGELKCDPTMSWNDINCEHLN N WIC (696-759)
MRCRD5      FLGILLISL DKKFAWMDGSKVDYVSWATGERNFANEDENCVTMYSNS.GFWNDINCGYPNAFIE (843-904)

```

FLG T F G V Y NW GEPNNA DENCVTL GN WNDI C IC

Figure 1. (A) Proposed structural domains of the macrophage mannose receptor. The nucleotide sequence can be obtained via EMBL accession number X55635 and in reference 21. (B) Alignment of eight carbohydrate recognition-like domains with each other with reference to the invariant residues of C-type lectins (15). Not all the "invariant" residues are conserved in each repeat and amino acids present in at least four of eight repeats are shown below as common. Domains 4 and 5 are the most homologous to one another with 34% identity and 49% similarity. (C) Alignment of carbohydrate recognition-like domains 4 and 5 with the COOH-terminal (64 amino acids of the carbohydrate recognition-like domains of MBP from rat, mouse, and human). Amino acids common to one or both mannose receptor carbohydrate recognition-like domains (MR CRD) 4 and 5 that are represented in at least one of the five MBP sequences are boxed and highlighted below.

scripts. A single 5.2-kb mRNA species was identified in human alveolar macrophages, but not in RNA from human umbilical vein endothelial cells, HeLa or HepG2 cells (Fig. 2). In addition, no detectable transcripts were observed in RNA isolated in freshly isolated human monocytes or human myelomonocytic cell lines U937, HL60, or THP cells, all of which do not express mannose receptor activity (results not shown). Southern blot analysis of human genomic DNA digested with different restriction enzymes revealed that a full-length MR cDNA hybridized to a limited number of fragments (not shown). The relatively simple hybridization pattern suggests that a single gene encodes the mannose receptor.

Mannose Receptor Expression and Endocytosis

Glycoconjugates prepared by derivatizing BSA with mannose have proven to be invaluable as high-affinity ligands for a variety of receptors, including the mannose receptor, particularly when the molar ratio of sugar to protein is 20>1 (35). As ¹²⁵I-mannose-BSA has been used to quantify receptor number and activity on macrophages isolated from rodents and humans, we used this glycoconjugate to assess receptor activity on populations of Cos-I cells that had been transfected with MR CDM8, an expression vector containing the full-length mannose receptor or Cos-I cells transfected with the high affinity Fc receptor CD64 to serve as a control. The transfection efficiency varied between 10 and 30% of cells as assessed by FACS analysis (Fig. 3 A) and immunofluorescence (not shown). Fig. 3 B shows mannose-specific uptake occurred only in the Cos-I cells transfected with the mannose receptor and not in cells transfected with CD64. The uptake of the radiolabeled ligand was specifically prevented by the mannose-rich yeast mannan which serves as a competitive inhibitor.

Mannose Receptor Phagocytosis

The predominant physiological role of the mannose receptor on tissue macrophages is the engagement microorganisms that have mannose-rich cell walls. *Candida albicans* are examples of such microorganisms and were used to assess whether the mannose receptor alone was sufficient to mediate binding and uptake of these microorganisms in the absence of other macrophage receptors in heterologous cells. Populations of Cos-I cells transfected with MR CDM8 or the control plasmid CD64 CDM8 expressed the surface receptor as determined by the typical pattern of membrane staining and by FACS analysis. *Candida albicans* were incubated with populations containing MR⁺ Cos-I cells for 5 or 20 min at 37°C. MR⁺ Cos-I cells had many adherent yeasts at 5 min and formed membrane extensions around the particles (Fig. 3 c), which are reminiscent of the early phagocytic events observed in macrophages (36). After 20 min of incubation the yeasts had been internalized by the mannose receptor-transfected Cos cells, as evidenced by the presence of yeast within a phagocytic vacuole (Fig. 3 D). To quantitate the phagocytosis, transfected Cos cells were incubated with *Candida albicans* for 20 min at 37°C. Thereafter cell monolayers were fixed and stained

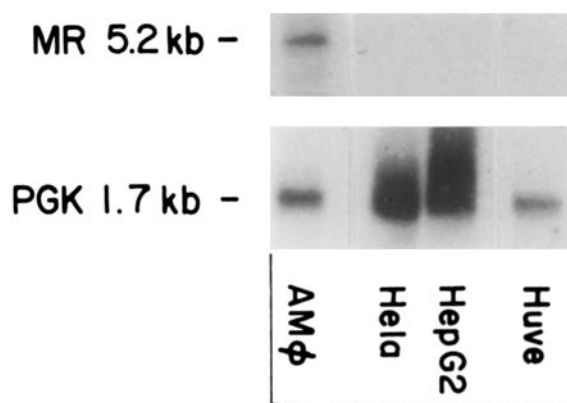


Figure 2. Northern blot analysis of macrophage mannose receptor. Total RNA from human alveolar macrophages, HepG2 cells, HeLa cells, and human umbilical vein endothelial cells was size fractionated on an agarose gel and transferred to a nylon membrane and hybridized with radiolabeled cDNA representing the mannose receptor and a phosphoglycerate kinase cDNA as a control (1.7-kb transcript present in all cells) (30). Mannose receptor transcripts of 5.2 kb were observed only in human alveolar macrophages as expected.

into toluidine blue, which stains yeasts and nuclei. 100 cells from three independent coverslips were scored by Nomarski microscopy. 10% of the cells had internalized 1–5 yeast particles and 7% internalized >5. The cells that had ingested many particles became less adherent, and in this way are similar to post-phagocytic macrophages. It also appeared that some of the ingested yeasts were degraded and had begun to coalesce, as seen on this unstained Nomarski light photomicrograph (Fig. 3 e). It is likely, although not formally proven by these studies, that these events are occurring in phagolysosomes. Mock-transfected Cos-I cells did not bind or ingest yeast (Fig. 3 f), nor did CD64-transfected cells (not shown).

Tail Minus Receptor Is Internalization Defective

We surmised that the signal for phagocytosis most likely resides in the 45 amino acids that comprise the cytoplasmic tail. To test this hypothesis we constructed a mutant receptor that had all but two lysines of the cytoplasmic tail deleted. 15–20% of the transfected Cos-I cells expressed the mutant receptor on the cell surface as detected by FACS analysis (Fig. 4 A). The level of expression of the mutant receptor on the cell surface appeared comparable to the surface expression of the entire receptor (compare Fig. 4 A to Fig. 3 A). The analyses of tails plus and tails minus populations were performed as part of the same experiment in which identical reagents and machine parameters were used. Cell populations transfected with the tail minus receptor had greatly reduced uptake of radiolabeled mannose-BSA that was almost entirely mannan inhibitable (Fig. 4 B).

We next incubated the tail minus transfected cells with yeasts for up to 45 min at 37°C. The particles bound to the cells, as shown by electron microscopy (Fig. 4 C), and did not appear to form membrane extensions around the yeast observed in the cells expressing the entire receptor (Fig. 3 C). Light microscopy was performed on 500-nm sections, in which hundreds of cells can be easily examined that were

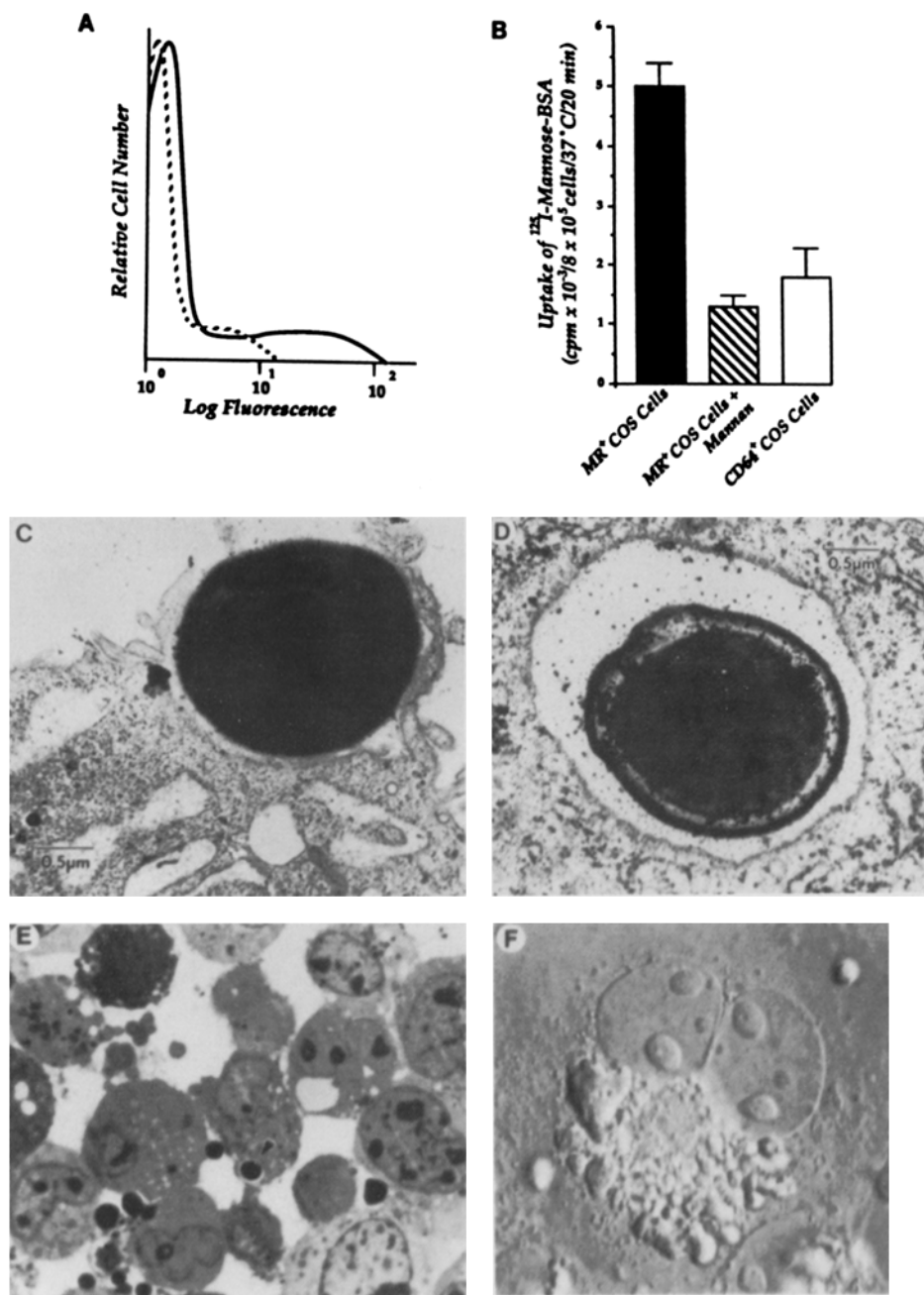


Figure 3. Mannose receptor expression and endocytosis in transfected Cos-I cells. (A) FACS analysis of transfected Cos cells stained with a rabbit anti-human mannose receptor antisera (solid line). Relative cell number was 10,000 cells, of which 20% were positive. The control represents an equal number of mock-transfected cells. The experiment represents three independent experiments. (B) 8×10^5 MR cDNA-transfected Cos-I cells or CD64-transfected Cos-I control cells were incubated with ^{125}I -mannose-BSA (specific activity 2×10^6 cpm/ μg) in the presence or absence of 1.25 mg/ml of yeast mannan for 20 min at 37°C . Results are a mean \pm SD of quadruplicates and are representative of two independent experiments. (C) Electron micrograph of a phagosome forming around a yeast that had been incubated for 5 min at 37°C . (D) Electron micrograph of a yeast within a phagocytic vacuole after 20 min of incubation at 37°C . (E) Light micrograph of 500-nm section showing a yeast within a vacuole as well as some bound yeast and an occasional free particle. Sections were stained with toluidine blue, which stains the yeast and the cell nuclei. (F) A Nomarski photomicrograph of a binucleate cell in which numerous yeasts are seen in the perinuclear region, many of which have lost their typical morphology and therefore appear to be degraded. The incubation period was 20 min at 37°C .

stained with toluidine blue, which clearly stains the yeasts. Only bound, but not internalized particles were observed (Fig. 4 D), thereby supporting the contention that the receptor tail is essential for endocytosis. Quantitation analysis similar to that described for the tail plus transfectants was performed by scoring cells that had specifically bound yeasts. This was done on whole mount preparations stained with toluidine blue and viewed by Nomarski light microscopy in order to distinguish bound versus internal particles. No internal yeasts were observed; however, 11% of the cells bound 1–5 yeasts and 6% bound >5. An example of a cell that bound many yeasts is shown in Fig. 4 E. The cells expressing the tail minus receptors formed rosettes of many bound yeasts, which when

viewed by Nomarski optics appeared adherent, but not internal to the cell (Fig. 4 E). No specific binding was observed in mock transfected cells (Fig. 4 F), although the occasional adherent yeast was seen.

Discussion

Recognition and ingestion of microorganisms are hallmarks of the macrophage as an effector cell in host defense. Macrophage receptors for different isotypes of Ig (FcR) (reviewed in 37, 38) and third component of complement (CRI, CR3) that mediate opsonin-dependent and -independent clearance of pathogens have been well characterized (reviewed in 39,

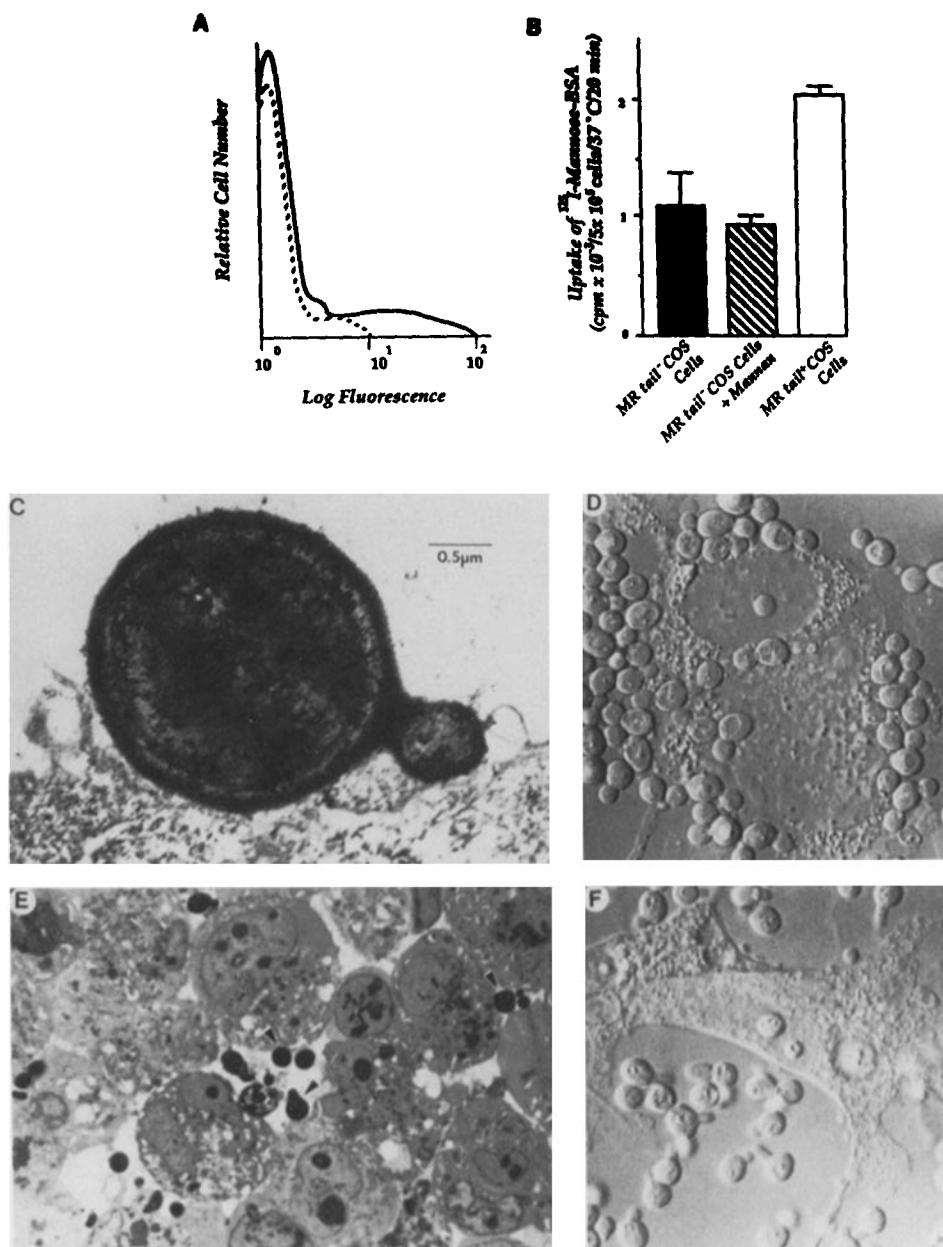


Figure 4. Surface expression and binding of yeast to cells transfected with tail minus mutant receptor. Cos-1 were transiently transfected with a mannose receptor construction that had all but the first two amino acids of the cytoplasmic tail deleted. (A) FACS analysis of transfected Cos-1 cells. The relative cell number (10,000) and parameters of analysis were identical to that described in Fig. 3 A. The intensity of fluorescence and percent of positive cells were very similar to those for the entire receptor shown in Fig. 3 A. This experiment is representative of two independent experiments in which triplicate samples were examined. (B) 5×10^5 MR tail minus and MR tail plus transfected cells were incubated with ^{125}I mannose-BSA (0.6×10^6 cpm/ μg) for 20 min at 37°C . Results are the mean \pm SD of triplicate samples and are representative of two independent experiments. Also shown is inhibition uptake by 1.25 mg/ml of yeast mannan. (C) Electron micrograph of yeast adherent to a cell after incubation of 37°C for 45 min. (D) Nomarski photomicrograph of yeasts rosetting to, but not being internalized by, MR tail minus transfected cells despite incubation for 45 min at 37°C . (E) Light micrograph of 500-nm section stained with toluidine blue shows several yeasts bound but not internalized by the tail minus transfected cells. (F) Nomarski photomicrograph of mock-transfected Cos-1 cells incubated with yeasts for 45 min at 37°C . The yeasts are not specifically bound or ingested.

40). Phagocyte lectin-like receptors that recognized specific configurations of carbohydrates in target organisms have been described (2, 41). The 170-kD mannose receptor, which is expressed on tissue macrophages and not on circulating phagocytes is able to engage complex ligands directly (reviewed in reference 2). The binding of pathogenic microorganisms that expresses high mannose glycans results in engulfment by the macrophage as well as the release of an array of biologically active mediators, which in large part regulate inflammatory responses. In this study, we describe the structure of the human macrophage mannose receptor which is identical to the human placental receptor (21). Several structural features of the mannose receptor provide insights into its function as a phagocytic receptor.

Notable among the features of the mannose receptor's ectodomain is the presence of eight carbohydrate recognition-like domains. The inclusion of each domain is based on the presence of invariant residues that have been described for C-type lectins, a family of soluble and extracellular matrix and membrane proteins (15) that now includes the mannose receptor. The mannose receptor is the only member of this family that can mediate phagocytosis and the presence of multiple CRDs may in part explain how this receptor recognizes multivalent complex mannose glycans. Although the relative contribution of each of the CRDs to ligand binding and specificity is yet to be determined, inspection of the sequence provides some potential insights. In particular, domains four and five are the most homologous to one another,

with 34% identity and 49% similarity. In addition, they both contain the motif WND which is invariant in all C-type lectins. These two domains bear closest homology to the MBPs, the proteins in this family that have the closest binding specificity to the mannose receptor (42). Comparison of the mannose receptor CRDs 4 and 5 to five MBPs, two each from rats (43) and mice (Sastry, K., K. Zahedi, J.-M. Lelas, A. S. Whitehead, and R. A. B. Ezekowitz, manuscript submitted for publication) and one from human (11, 24, 33), reveals that 32 amino acids out of 62 amino acids are represented in at least once in the last half of the CRDs of the MBPs. Mutational analysis may allow some estimates of the relative contributions of these conserved amino acids to ligand binding specificity. The structural similarities between the hepatocyte-derived soluble MBPs and the membrane mannose receptor in part explain their functional equivalence. The mannose receptor is not expressed on circulating granulocytes or monocytes, thereby conferring the ability to recognize and clear circulating mannose-rich ligands on the hepatocyte-derived MBP. Conversely, at uninflamed tissue sites where mannose receptor-positive macrophages abound, the MBP is absent, thereby conferring recognition of specific mannose-rich pathogens to the mannose receptor.

The cysteine-rich NH₂-terminal region bears no homology to any other known sequence and its significance is not known. It is possible that it may play a role in stabilizing multimerization of receptors in the membrane, which would then increase the valency and affinity of the mannose receptor. Similarly, the function of the type II fibronectin-like repeat is uncertain, but it may be important in the interaction of tissue macrophages with the intracellular matrix, thereby promoting adhesion and spreading at tissue sites.

Transient expression of the mannose receptor on the surface of Cos-I cells results in uptake of mannose rich glycoconjugates as expected. However, ability of these transfected cells to bind and phagocytose yeast particles was surprising. Receptor ligand interactions appeared to occur sequentially around the full circumference of the target (Fig. 3 C) like a zipper, with eventual fusion around the particle, consistent with the model of phagocytosis proposed by Silverstein et al. (36). According to this scheme, local segments of plasma membrane mediate binding of the particle, but ingestion results from involvement of the cytoskeletal apparatus (36). The precise role of the contractile proteins in mannose receptor-dependent phagocytosis in Cos-I cells has yet to be determined; however, it would appear that the critical signal that mediates phagocytosis resides in the 45 amino acids of the cytoplasmic tail. Mutant receptors lacking the mannose receptor tail are expressed on the cell surface, but are unable to ingest bound particles. The absence of the receptor tail appears to interrupt membrane flow around the particle (Fig. 4 c) and subsequent phagocytosis is impeded. These experiments imply that

ligation of the ectodomain of the mannose receptor by a multivalent ligand is able to signal phagocytosis and what appears to be correct routing to phagolysosomes even in a heterologous cell. Receptor-mediated endocytosis was also greatly reduced by removal of the cytoplasmic tail, although still detectable. The mannose receptor is distinct from other human macrophage phagocytic receptors, in particular, different isoforms of Fc receptors, which in macrophages are constitutively phagocytic, yet when expressed in heterologous cells mediates binding and not internalization of antibody-coated erythrocytes (44, 45; Ezekowitz, R. A. B., unpublished). The endocytic properties of mouse macrophage Fc receptor FcR_{II}-B2 have been extensively studied (46) by transfection into FcR⁻ cells and these studies revealed that FcR_{II}-B2 was able to accumulate in coated pits and mediate fluid phase pinocytosis of bound ligand. Recent experiments have shown that these FcR_{II}-B2 Chinese hamster ovary cell transfectants phagocytose opsonized heat-killed toxoplasma (47), thereby providing an example of another macrophage phagocytic receptor that is able to function in the heterologous cell. Inspection of the 45 amino acids in the cytoplasmic tail of the mannose receptor fails to reveal any obvious homology with FcR_{II}-B2 isoform or with other receptors, except for the presence of the motif FXN₁TX₂Y, which is almost identical to the motif FXNPXY that appears to be required for localization of certain receptors to coated pits (34). Although the proline appears central for receptor localization to coated pits, the mannose receptor does localize to coated pits and has a threonine at this position. This difference implies that the precise conservation of the motif is not essential or that in the mannose receptor sequences other than this motif account for its localization to coated pits. Clearly, a more detailed analysis is necessary to determine which residues in the receptor tail are critical for coated pit-dependent and -independent endocytosis and phagocytosis. Open to question is whether cells other than Cos-I cells can be induced to mediate mannose receptor-dependent phagocytosis, or whether this is a feature of Cos cells that express accessory molecule(s) that are required to complement the receptor in the phagocytic event.

Mannose receptors on macrophages are responsible for the signaling of the secretion of a number of potent inflammatory mediators like arachidonate metabolites, reactive oxygen intermediates, monokines, and neutral proteases. The mechanisms by which these receptors transmit transmembrane signals have yet to be determined and the cytoplasmic domains required for these functions remain to be determined. Further studies are required to assess whether the mannose receptor can signal secretion as well as phagocytosis in heterologous cells or whether this triggering event is restricted to macrophages.

We are indebted to Drs. Philips Stahl and Kurt Drickamer for providing critical reagents and communicating their results prior to publication. We thank Drs. S. Lux, B. Seed, and F. McKeon for advice and members

of our Division for stimulating discussion. We thank Eileen Deignan, Grace Chang, and Carol Mathew for excellent technical assistance and Marsha Kartzman for preparation of the manuscript. We gratefully acknowledge the help of Katherine John and Sabra Goff in preparing synthetic oligonucleotides.

This work is supported by National Institutes of Health grants R01-23786, P01-HL43510-01, and a grant from the Cancer Research Institute (R. A. B. Ezekowitz).

Address correspondence to Dr. R. Alan B. Ezekowitz, Harvard Medical School, The Children's Hospital, Division of Hematology-Oncology, Enders Building, 7th Floor, 300 Longwood Ave., Boston, MA 02115.

Received for publication 16 August 1990 and in revised form 18 September 1990.

References

1. Stahl, P., P.H. Schlesinger, E. Sigardson, J.S. Rodman, and Y.S. Lee. 1980. Receptor-mediated pinocytosis of mannose glycoconjugates by macrophages. Characterization and evidence for receptor recycling. *Cell*. 19:207.
2. Ezekowitz, R.A.B., and P.D. Stahl. 1988. The structure and function of vertebrate mannose lectin-like proteins. *J. Cell Sci. Suppl.* 9:121.
3. Sung, S.J., R.S. Nelson, and S.C. Silverstein. 1983. Yeast mannan inhibit binding and phagocytosis of zymosan by mouse peritoneal macrophages. *J. Cell Biol.* 96:160.
4. Blackwell, J.M., R.A.B. Ezekowitz, M.B. Roberts, J.Y. Channon, R.B. Sim, and S. Gordon. 1985. Macrophage complement and lectin-like receptors bind Leishmania in absence of serum. *J. Exp. Med.* 162:324.
5. Berton, G., and S. Gordon. 1983. Modulation of macrophage mannosyl-specific receptors by cultivation on immunobilised zymosan. Effects on superoxide-anion release and phagocytosis. *Immunology*. 49:705.
6. Rouzer, C.A., W.A. Scott, A.L. Hamill, and Z.A. Cohn. 1982. Synthesis of leukotriene C and other arachidonic acid metabolites by mouse pulmonary macrophages. *J. Exp. Med.* 155:720.
7. Gordon, S., and R.A.B. Ezekowitz. 1981. Macrophage neutral proteinases: nature, regulation, and role. In *The Reticuloendothelial System*. Vol. III. Plenum Press, New York. 95-115.
8. Nathan, C. 1987. Macrophages secretion. *J. Clin. Invest.* 79:319.
9. Ezekowitz, R.A.B., R. Sim, M. Hill, and S. Gordon. 1983. Local opsonization by secreted macrophage components. Role of receptors for complement in uptake of zymosan. *J. Exp. Med.* 159:244.
10. Hume, D.A., and S. Gordon. 1985. The mononuclear phagocyte system of the mouse defined immunohistochemical localization of the antigen F4/80. In *Mononuclear Phagocytes: Characteristics, Physiology and Function*. R. vanFurth, editor. Martinus-Nijhoff, Dordrecht. 9-15.
11. Ezekowitz, R.A.B., L. Day, and G. Herman. 1988. A human mannose-binding protein is an acute phase reactant that shares sequence homology with other vertebrate lectins. *J. Exp. Med.* 167:1034.
12. Ezekowitz, R.A.B., M. Kuhlman, J. Groopman, and R. Byrn. 1989. A human serum mannose-binding protein inhibits in vitro infection by the human immunodeficiency virus. *J. Exp. Med.* 169:185.
13. Kuhlman, M., K. Joiner, and R.A.B. Ezekowitz. 1989. The human mannose-binding protein functions as an opsonin. *J. Exp. Med.* 169:1733.
14. Wileman, T., M. Lennartz, and P. Stahl. 1986. Identification of the macrophage mannose receptor as a 175 Kd membrane protein. *Proc. Natl. Acad. Sci. USA.* 83:2501.
15. Drickamer, K. 1988. Two distinct classes of carbohydrate-recognition domains in animal lectins. *J. Biol. Chem.* 263:9557.
16. Lasky, L.A., M.S. Singer, T.A. Yednock, D. Dowbenko, C. Fennie, H. Rodriguez, T. Nguyen, S. Stachel, and S.D. Rosen. 1989. Cloning of a lymphocyte homing receptor reveals a lectin domain. *Cell*. 56:1045.
17. Camarini, D., S.P. James, I. Stamenkovic, and B. Seed. 1989. Leu8/TaqI is human equivalent of Mel14 lymph node homing receptor. *Nature (Lond.)*. 342:78.
18. Johnston, G.I., R.G. Cook, and R.P. McEver. 1989. Cloning of GMP-140, a granule membrane protein of platelets and endothelium: sequence similarity to proteins involved in cell adhesion and inflammation. *Cell*. 56:1033.
19. Bevilacqua, M.P., S. Stengelin, Jr., M.A. Gimbrone, and B. Seed. 1989. Endothelial leukocyte adhesion molecule 1; an inducible receptor for neutrophils related to complement regulatory proteins and lectins. *Science (Wash. DC)*. 243:1160.
20. Lennartz, M.R., F.S. Cole, V. Shepherd, T.E. Wileman, and P.E. Stahl. 1987. Isolation and characterization of a mannose specific endocytosis receptor from human placenta. *J. Biol. Chem.* 262:9942.
21. Taylor, M.E., J.T. Conary, M.R. Lennartz, P.D. Stahl, and K. Drickamer. 1990. Primary structure of the mannose receptor contains multiple motifs resembling carbohydrate recognition domains. *J. Biol. Chem.* 265:12156.
22. Grubler, U., and B.J. Hoffman. 1983. A simple and very efficient method for generating cDNA libraries. *Gene (Amst.)*. 25:263.
23. Okayama, H., and P. Berg. 1982. High efficiency cloning of full-length cDNA. *Mol. Cell. Biol.* 2:161.
24. Sastry, K., G. Herman, L. Day, E. Deignan, G. Burns, C. Morton, and R.A.B. Ezekowitz. 1989. The human mannose-binding protein gene. Exon structure reveals its evolutionary relationship to a human pulmonary surfactant gene and its localization to chromosome 10. *J. Exp. Med.* 170:1175.
25. Sanger, F., A.S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463.
26. Dorfman, D.M., L.I. Zon, and S.H. Orkin. 1989. Rapid amplification of lambdaGT11 bacteriophage library reaction. *Biotechniques*. 7:568.
27. Aruffo, A., and B. Seed. 1987. Molecular cloning of a CD28 cDNA by a high efficiency COS cell expression system. *Proc. Natl. Acad. Sci. USA.* 84:8573.
28. Ezekowitz, R.A.B., J. Austyn, P.D. Stahl, and S. Gordon. 1981. Surface properties of Bacillus Calmette-Guerin activated mouse macrophages: reduced expression of mannose-specific endocytosis, Fc receptors and antigen F4/80 accompanies induction of Ia. *J. Exp. Med.* 154:60.

29. Ezekowitz, R.A.B., R.B. Sim, G.G. MacPherson, and S. Gordon. 1985. Interaction of human monocytes, macrophages, and polymorphonuclear leukocytes with zymosan in vitro: role of type 3 complement receptors and macrophages-derived complement. *J. Clin. Invest.* 76:2368.
30. Michaelson, A.M., A.F. Markham, and S.H. Orkin. 1983. Isolation and DNA sequence of a full-length cDNA clone for human X-chromosome encoded phosphoglycerate kinase. *Proc. Natl. Acad. Sci. USA.* 80:472.
31. Kodama, T., M. Freeman, L. Roher, J. Zabrecky, P. Matsudaira, and M. Krieger. 1990. Type-I macrophage receptor contains alpha-helical and collagen-like coiled coils. *Nature (Lond.)* 343:531.
32. Kornblitt, A.R., K. Umezawa, K. Vibe-Pedersen, and F.E. Baralle. 1985. Primary structure of human fibronectin: differential splicing may generate at least 10 polypeptides from a single gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1755.
33. Taylor, M.E., P.M. Brickell, R.K. Craig, and J.A. Summerfield. 1989. The structure and evolutionary origin of the gene encoding a human mannose-binding protein. *Biochem. J.* 262:763.
34. Chen, W.J., J.L. Goldstein, and M.S. Brown. 1990. NPXY, a sequence often found in cytoplasmic tails, is required for coated pit-mediated internalization of low density lipoprotein receptor. *J. Biol. Chem.* 265:3116.
35. Lee, Y.C., C.P. Stowell, and M.J. Krantz. 1976. 2-imino-2-methoxyethyl-1-thioglycosides. New reagents for attaching sugar to proteins. *Biochemistry.* 15:3956.
36. Silverstein, S.C., R.M. Steinman, and Z.A. Cohn. 1977. Endocytosis. *Annu. Rev. Biochem.* 46:669.
37. Mellman, I. 1989. Relationships between structure and function in the Fc receptor family. *Curr. Opin. Immunol.* 1:16.
38. Ravetch, J.V., and C.L. Anderson. 1988. Fc receptors and the action of antibodies. *Annu. Rev. Immunol.* 6:251.
39. Arnout, A.M. 1990. Structure and function of the leukocyte adhesion molecules CD11/CD18. *Blood.* 75:1037.
40. Klickstein, L.B., T.J. Bartow, V. Miletic, L.D. Rabson, J.A. Smith, and D.T. Fearon. 1988. Identification of distinct C3b and C4b recognition sites in the human C36/C46 receptor (CRI CD35) by deletion mutagenesis. *J. Exp. Med.* 168:1699.
41. Czop, J.K., and K.F. Austen. 1985. A glucan-inhibitable receptor on human monocytes: its identity with the phagocytic receptor for particulate activators of the alternative complement pathway. *J. Immunol.* 134:2588.
42. Childs, R.A., K. Drickamer, T. Kawasaki, S. Thiel, T. Mizuochi, and T. Feizi. 1989. Neoglycolipids as probes of oligosaccharide recognition by recombinant and natural mannose-binding proteins of the rat and man. *Biochem. J.* 262:131.
43. Drickamer, K., M.S. Dordal, and L. Reynolds. 1986. Mannose-binding proteins isolated from rat liver contain carbohydrate-recognition domains linked to collagenous tail. *J. Biol. Chem.* 261:6878.
44. Allen, J.M., and B. Seed. 1989. Isolation and expression of functional high affinity Fc receptor complementary DNAs. *J. Exp. Med.* 243:378.
45. Brooks, D.G., W.Q. Qui, A.D. Luster, and J.V. Ravetch. 1985. Structure and expression of human IgGFcRII (CD32). *J. Exp. Med.* 170:1369.
46. Miettinen, H.M., J.K. Rose, and I. Mellman. 1989. Fc receptor isoforms exhibit distinct abilities for coated pit localization, a result of cytoplasmic tail heterogeneity. *Cell.* 58:317.
47. Joiner, K.A., S.A. Fuhrman, H.M. Miettinen, L.H. Kasper, and I. Mellman. 1990. *Toxoplasma gondii*: Fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts. *Science (Wash. DC).* 249:641.