Delayed Activation of the Mannose Receptor following Synthesis

REQUIREMENT FOR EXIT FROM THE ENDOPLASMIC RETICULUM*

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The macrophage mannose receptor specifically recognizes proteins and particles bearing mannose terminal oligosaccharide chains. In the present study, we examined the ability of newly synthesized receptor to bind ligand. Human monocyte-derived macrophages were pulse-labeled with [35S]Met and prepared for affinity chromatography on mannose-Sepharose. Mannose receptor in the flow-through and eluted fractions was detected by fluorography following immunoprecipitation and gel electrophoresis. Labeled mannose receptor was found exclusively in the nonbinding fraction until 10 min of chase. Following a 60-min chase, 67–86% of newly synthesized receptor was precipitated from the bound column fraction. The half-time for development of receptor binding activity was determined to be 35–40 min compared with a 45-min half-time for development of endoglycosidase H resistance. Mannose receptor synthesized by cells incubated in brefeldin A required more than 120 min to acquire endoglycosidase H resistance and maximal binding activity. Inhibitors of N-linked oligosaccharide processing or of O-glycosylation had no effect on the development of mannose receptor binding activity. Monensin prevented terminal sialylation of oligosaccharide side chains but did not inhibit receptor activation. Inclusion of aluminum fluoride in the chase medium reversibly inhibited development of endoglycosidase H resistance. Mannose receptor synthesized by cells incubated in brefeldin A required more than 120 min to acquire endoglycosidase H resistance and maximal binding activity. Inhibitors of N-linked oligosaccharide processing or of O-glycosylation had no effect on the development of mannose receptor binding activity. Monensin prevented terminal sialylation of oligosaccharide side chains but did not inhibit receptor activation. Inclusion of aluminum fluoride in the chase medium reversibly inhibited development of endoglycosidase H resistance and mannose-binding activity. We conclude that the mannose receptor undergoes delayed activation following synthesis and suggest that the activating event(s) occur following exit of the receptor from the endoplasmic reticulum and prior to its entry into the trans-Golgi.

Macrophages recognize and internalize mannose-terminating glycoconjugates through the surface expression of a membrane glycoprotein, the mannose receptor (MR)1 (1, 2). Like other calcium-dependent animal lectins (3), the MR binds ligand through the cooperative interaction of multiple CRDs, homologous sequences containing invariant residues and structural features that mediate interactions with sugar and calcium (4). The eight CRDs of the mannose receptor are arranged linearly along a single polypeptide backbone that crosses the membrane once to form a short C-terminal cytoplasmic tail (5–6). Capping the exoplasmic N terminus are two domains of unknown function; truncated forms of the MR lacking these regions bind and internalize ligand normally when transfected into cells (7).

Binding characteristics of MR CRDs have been thoroughly investigated through binding assays utilizing CRDs generated by in vitro bacterial and insect translation systems (8). Expressed alone, only CRD 4 of the MR exhibits binding activity, recognizing monomeric sugars for which the receptor has affinity. Sugar binding by CRD 4 requires two calcium ions in a pH-dependent interaction that leads to calcium and ligand dissociation under acidic conditions (9). High affinity binding of naturally occurring proteins bearing high-mannose structures requires the expression of CRDs 4 and 5 together, while binding of extended polymeric ligands is dependent upon CRDs 4–8 (8–9).

The biosynthesis of the mannose receptor has been characterized in human macrophages (10). The initial translation product exhibits an average Mr of 154 kDa on SDS-polyacrylamide gels and is sensitive to Endo H digestion. The receptor matures to its 162-kDa, Endo H-resistant form with a half-time of about 45 min. The observed shift in electrophoretic mobility following synthesis reflects mainly addition of negatively charged sialic acid residues to O-linked sugars, with the maturation of N-linked chains to hybrid and/or complex forms contributing only slightly to the change in Mr.

Carbohydrate recognition recently has been found to play important roles in maintaining the integrity of the secretory pathway and the protein products generated within. Calnexin and calreticulin are resident ER lectins that bind and retain monoglucosylated oligosaccharides for presentation to a glucosyltransferase which detects improperly folded proteins before they can exit the ER (11). A resident protein of the intermediate compartment, ERGIC53, binds mannose and may facilitate transport of glycoproteins between the ER and Golgi (12, 13). The ER, intermediate, and early Golgi compartments are rich in potential MR ligands (i.e. proteins with high-mannose chains), which could act as retention or retrieval factors.

In addition, the environment of the early secretory pathway, with a neutral pH and abundant calcium (14), should favor MR ligation. However, the half-time for development of Endo H resistance reported for the MR suggests that the receptor is not retained in or returned to the ER following synthesis (10). Empirically, the MR is too restricted in its cellular distribution to function intracellularly as a molecular chaperon. Further, data from experiments with oligosaccharide-processing inhibitors suggest that engagement by endogenous membrane proteins may target the MR for early degradation (15). Therefore, we hypothesized that the binding activity of the MR must be
regulated in the biosynthetic pathway so as to avoid intracellular ligation.

In this study we have examined the ability of the mannose receptor to bind ligand while it is localized to early secretory compartments. Utilizing metabolic pulse labeling, affinity chromatography, and a battery of inhibitors of post-translational transport and processing, we demonstrate that the mannose receptor is synthesized as an inactive precursor requiring transport from the ER for acquisition of binding activity. We present data suggesting that MR activation is an enzyme-mediated event, and possible mechanisms are discussed.

MATERIALS AND METHODS

Materials—Concentrated human lymphocytes from single donors were purchased from the Barnes Hospital Blood Bank, St. Louis, MO. Ficoll-Hypaque was purchased from Pharmacia Biotech Inc. Human AB serum was purchased from North American Biologicals, Inc., Miami, FL. Tissue culture reagents were supplied by the Tissue Culture Support Center, Washington University School of Medicine, St. Louis, MO. Endo H was purchased from Genzyme Corp., Boston, MA. Tran35S-labeled (a mixture of 35S-labeled Met and Cys) was purchased from ICN Biochemicals, Cleveland, OH. Brefeldin A was obtained from Epitrend Technologies Corp. All other reagents were purchased from Sigma unless otherwise specified.

Antibodies—Antibodies against the isolated human placental MR were generated in New Zealand White rabbits by intradermal injection. Collection of antiserum and characterization of antibody specificity were described previously (10).

Cells—Human peripheral blood monocytes were obtained by counter-current elutriation. Briefly, concentrated lymphocytes were centrifuged over Ficoll-Hypaque and interfaces (buffy coats) were collected. Leukocytes were washed twice in Hank’s balanced salt solution containing 2 mM EDTA to remove platelets. Monocytes were isolated in a J2–21 centrifuge (Beckman Instruments) using a JE-6 elutriator rotor with two Sanderson separation chambers as described (16). Purity of monocytes was ascertained by sizing in a Coulter counter (Coulter Corp., Hialeah, FL). Monocytes were plated at a density of 2–3 × 106 cells/ml in RPMI 1640 medium containing 2% human AB serum (heat-inactivated) for 1 h and then maintained in 12.5% human AB serum for up to 6 days as reported previously (10). All experiments were performed 4 or 5 days post-plating.

Pulse-Chase Protocol—Cells were incubated for 30 min in Cys- and Met-free RPMI 1640 medium supplemented with 10% diazyl fetal calf serum. For the pulse, the starvation medium was replaced with the same medium containing 0.1–0.3 mCi/ml Tran35S-labeled. Cells were washed twice in Hanks’ balanced salt solution containing 2 mM EDTA to remove platelets. Monocytes were collected in a J2–21 centrifuge (Beckman Instruments) using a JE-6 elutriator rotor with two Sanderson separation chambers as described (16). Purity of monocytes was ascertained by sizing in a Coulter counter (Coulter Corp., Hialeah, FL). Monocytes were plated at a density of 2–3 × 106 cells/ml in RPMI 1640 medium containing 2% human AB serum (heat-inactivated) for 1 h and then maintained in 12.5% human AB serum for up to 6 days as reported previously (10). All experiments were performed 4 or 5 days post-plating.

Membrane Preparation—The following steps were performed on ice. Cells were harvested by scraping in 5 mM Tris and 10 mM EGTA, pH 7.4, and were homogenized by 20 strokes in a Dounce homogenizer. Membranes were pelleted by ultracentrifugation for 20 min at 100,000 × g in a TL-100 ultracentrifuge (Beckman Instruments). Membrane pellets were solubilized by resuspension in loading buffer (1.25 mM NaCl, 10 mM Tris, 15 mM Ca2+, 1% Triton X-100, pH 7.4).

Affinity Chromatography—Solubilized membranes were applied to mannose-Sepharose columns (17) pre-equilibrated in loading buffer and rocked overnight at 4 °C. The flow-through fraction was collected and reserved for immunoprecipitation. The columns were washed extensively with loading buffer and eluted with 4 bed-volumes of loading buffer containing 0.2 M D-mannose.

Immunoprecipitation—Fractions to be immunoprecipitated were precleared by incubation with protein A-Sepharose for 1 h at 4 °C. Beads were pelleted by centrifugation and supernatants were incubated overnight with the indicated polyclonal rabbit antiserum. Protein A-Sepharose was added and samples were incubated for an additional hour. Beads were washed three times by suspension in 0.1% Triton X-100 and centrifugation. For Endo H digestion, samples were incubated with 0.2 units/ml Endo H for 17 h at 37 °C. Beads were then boiled for 5 min in buffer containing SDS and β-mercaptoethanol. Immunoprecipitated proteins were separated by electrophoresis on 7.5% SDS-polyacrylamide gels. Following electrophoresis, gels were fixed and impregnated with Enlightening (DuPont NEN) according to the manufacturer’s instructions. Gels were dried onto filter paper and exposed to film at ~80 °C for 1–5 days.

RESULTS

Kinetics of MR Transport through the Golgi—MR was immunoprecipitated from metabolically labeled human monocyte-derived macrophages. Precipitated material was treated with or without Endo H and subjected to SDS-PAGE. A representative autoradiograph is shown in Fig. 1. Initially, MR was completely sensitive to Endo H digestion, shifting approximately 4 kDa in apparent molecular mass (lanes 1 and 2). By 30 min of chase, a portion of the receptor’s N-linked oligosaccharides resisted removal (lanes 3 and 4). More than 95% of the labeled MR exhibited Endo H resistance 60 min after the pulse (lanes 7 and 8). Densitometric analysis (Fig. 2B) confirmed the reported half-time of 45 min for maturation of MR N-linked oligosaccharides. A protein band with an Mr of approximately 200 kDa was nonspecifically precipitated by all antisera, including normal rabbit serum (lane 9).

Acquisition of Receptor Binding Activity—Macrophages were pulsed for 5 min with Tran35S-label and chased prior to affinity chromatography on mannose-Sepharose. Following elution of the columns with mannose, both the flow-through and eluted fractions were immunoprecipitated with anti-MR antiserum. Immediately after the pulse, all labeled MR was immunoprecipitated from the flow-through or nonbinding fraction (Fig. 2A, lanes 1 and 2). The failure of MR to bind mannose-Sepharose at this time point was apparent in each of nine experiments, even after prolonged exposure of the gels to film (not shown). By 20 min of chase (lanes 5 and 6), about 10% of the receptor bound to the column. The ratio of bound to nonbinding receptor increased with chase time (lanes 7–12). After a 90-min chase, >95% of the labeled receptor was bound and specifically eluted (lanes 11 and 12).

The ability of the higher-mobility, Endo H-sensitive form of the MR to bind mannose residues is evident in Fig. 2A (lanes 5, 7, and 9). However, nearly all of the lower-mobility, mature form of the receptor is localized to the bound column fraction at each time point following its initial appearance. This observation suggests that the shift in apparent molecular weight occurs in temporal and spatial proximity to the activating event(s). Fig. 2B illustrates the kinetic relationship between the maturation of oligosaccharide side chains and development of binding activity. Densitometric analysis of autoradiographs from several experiments revealed a half-time of 35–40 min for MR activation compared to a 40–45-min half-time determined for maturation of N-linked carbohydrates.

Fig. 1. Development of Endo H resistance occurs with a half-time of 45 min. Monocyte-derived macrophages were pulsed for 15 min with Tran35S-label and chased for the indicated times. MR was immunoprecipitated, treated with (2, 4, 6, 8) or without (lanes 1, 3, 5, 7) Endo H, and subjected to SDS-PAGE and fluorography as described under “Materials and Methods.” The sample in lane 9 was immunoprecipitated with normal (nonimmune) rabbit serum. The arrow indicates position of mature MR in the gel.
Brefeldin A Alters MR Maturation Kinetics—The effect of BFA on MR maturation can be seen in Fig. 3. MR synthesized in the presence of BFA exhibits an Mr of about 154 kDa rather than the 162 kDa of control cells for up to 3 h post-translation (Fig. 3A, lane 7). The failure of the MR to undergo its characteristic shift in electrophoretic mobility presumably reflects the inaccessibility of the MR to trans-Golgi compartments containing sialyltransferase in BFA-treated cells (18). The processing of N-linked sugars occurs in BFA-treated macrophages as assessed by development of Endo H resistance but is slowed approximately 2-fold compared to controls. Approximately 120 min of chase was required before complete resistance to Endo H digestion was developed (Fig. 3A, lanes 5–8).

Affinity chromatography of newly synthesized MR from BFA-treated cells revealed that activation was retarded but not diminished by the drug pretreatment. Fig. 3B shows the amount of MR precipitated from the bound column fraction of control and BFA-treated cell lysates. After 90 min of chase, the amount of MR from control cells that was bound to the column was maximal (lanes 1 and 3), while only about half of the labeled MR from BFA-treated cells was retained (lane 2). The half-time for development of MR binding activity was approximately 85 min as assessed by densitometric analysis of several autoradiographs. By 180 min of chase, nearly all of the labeled MR from treated cells was specifically eluted from the mannos-Sepharose (lane 4). The slowed kinetics of activation in BFA-treated cells corresponds to the delayed development of Endo H resistance (Fig. 3A) and probably reflects the disorganization of post-translational processing in these cells.

Role of Oligosaccharide Side-chain Processing—Cells were treated with agents which disrupt the processing of N-linked oligosaccharides prior to metabolic labeling and affinity chromatography. A representative autoradiograph of MR immunoprecipitated from the bound column fractions is shown in Fig. 4. Pretreatment with castanospermine, deoxynojirimycin, deoxymannojirimycin, or swainsonine failed to prevent activation of the MR by 60 min of chase. These drugs inhibit the activities of α-glucosidase, α-glucosidase I and II, α-mannosidase I, and α-mannosidase II, respectively, and prevent maturation of N-linked oligosaccharides to complex forms (19). Despite the similar electrophoretic mobilities of the control and experimental receptor bands, Endo H sensitivity was confirmed for all receptor protein made in the presence of the inhibitors for each experiment (data not shown). Deoxynojirimycin and, to a lesser extent, deoxymannojirimycin had a toxic effect on the cells, which displayed a lower rate of protein synthesis.

Experiments conducted in the presence of tunicamycin,
Maturation of Mannose Receptor Binding Activity

FIG. 4. Development of MR binding activity is independent of N-linked oligosaccharide processing. Macrophages were preincu-
bated with castanospermine (CS, 0.1 mg/ml), deoxyxynojirimycin (dNM, 5 mM), or deoxymannojirimycin (dMM, 5 mM) for 3 h or with swa-
sosamine (SWS, 10 μg/ml) for 30 min prior to a 30-min pulse and 60-min chase. The autoradiograph shows MR immunoprecipitated from bound man-
nose-Sepharose column fractions.

FIG. 5. The MR develops binding activity independently of O-glycosylation and terminal sialylation. Macrophages were preincu-
bated with monensin (10 μg/ml, 1 h) or phenyl-GalNAc (2 mM, 3 h) and then pulsed with Tran35S-label for 30 min and chased for 60 min. Total amount of labeled MR loaded onto the columns was the same for all samples. Following affinity chromatography, bound fractions were immunoprecipitated with anti-MR serum and prepared for SDS-PAGE and autoradiography.

FIG. 6. Inhibition of processing and activation by AlF4-. Human macrophages were pulse-labeled for 2 min and chased in the presence or absence of AlF4 (60 min). AlF4 recovery followed an additional 60-min chase in control media. A, immunoprecipitated MR was subjected to Endo H digestion and SDS-PAGE/autoradiography. B, cell membranes were chromatographed on mannose-Sepharose. Nonbinding (NB) and bound (B) fractions were collected and immunoprecipitated. Arrows indicate position of mature MR on the autoradiographs.

Reversible Inhibition of Processing and Activation by Aluminum Fluoride—To block ER-to-Golgi transport (22, 23), cells were incubated with AlF prior to and throughout the chase period following a 2-min pulse with Tran35S-label. The presence of AlF inhibited MR maturation, preventing development of Endo H resistance (Fig. 6A, lanes 3 and 4) normally observed after the chase (lanes 1 and 2). The effect of AlF on MR transport was reversed by an additional 60-min chase in media lacking AlF, but reversal was dependent on extensive washing of cells prior to the second chase period. Recovery from the transport block was characterized by maturation of N-linked side chains, which became resistant to Endo H within the 60-min chase period (Fig. 6A, lanes 5 and 6).

Acquisition of MR binding activity was also inhibited by the presence of AlF (Fig. 6B, lanes 1–4), with all labeled MR from treated cells immunoprecipitated from the nonbinding fraction after affinity chromatography. Removal of AlF from the chase media resulted in the shift of labeled MR from the nonbinding to the bound column fraction, indicating that resumption of biosynthetic transport allowed activation of the MR for ligand binding.

DISCUSSION

We have examined the activity of newly synthesized man-
nose receptor in human monocyte-derived macrophages. We observed that the human MR undergoes a lag period ($t_1/2 = 35–45$ min) between synthesis and detectable binding activity. We believe this delay represents a distinct change from an inactive to an active binding state that occurs as the receptor is being transported through the biosynthetic pathway. In support of this hypothesis, maturation of MR activity can be ar-
rested by blocking transport to the Golgi. Aluminum fluoride, an activator of heterotrimeric G-proteins (24), prevents disso-
ciation of β-COP from vesicles mediating anterograde trans-
port from the ER (25). Although the exact mechanism remains unclear (22, 23, 26), AlF mimics the effect of GTPγS in cur-
tailing transport to the cis-Golgi cisternae and clearly prevents processing of the MR by Golgi enzymes.

The kinetics with which the receptor becomes capable of binding ligand suggest an early Golgi localization for the activat-
ing event. The MR develops Endo H resistance with a half-
time of approximately 40–45 min via the processing of N-
linked oligosaccharides to hybrid and complex forms in the medial Golgi area (27). While these modifications are tempo-
ral and perhaps spatially related to maturation of receptor activity, processing of N-linked sugars does not influence de-
velopment of MR activity; Endo H-sensitive MR can clearly bind ligand. The initial step in addition of O-linked sugars is thought to occur in the intermediate compartment between the ER and Golgi (28). One function of O-GalNAc-linked sugars is to cause a rodlike extension of polypeptides in space (29). The determination of MR primary structure has revealed that po-
tential sites for O-glycosylation all lie between presumed sugar binding domains (5), which could form a basis for development of MR activity through induction of a conformational change leaving critical CRDs accessible for binding. However, incuba-
tion of macrophages with an inhibitor of O-glycosylation, phen-
yl-GalNAc (20), affected only the resulting $M_0$ of the receptor, not its activity.

Further evidence for the early Golgi stacks as the site of MR
activation is derived from our results using BFA-treated cells. BFA has been shown to disrupt the transport of newly synthesized proteins beyond the medial or trans-Golgi cisternae by blocking anterograde transport from the ER and causing fusion of the early Golgi stacks with the ER (30–32). Newly synthesized MR from BFA-treated macrophages becomes ER-resistant with slowed kinetics when compared to controls but fails to attain its mature $M_r$, presumably because terminal glycosylation of both N- and O-linked sugars is prevented. Development of MR activity is also slowed but not inhibited by BFA treatment. This result suggests that activation of newly synthesized MR may be an enzyme-mediated event, which is slowed by disruption of the sequential processing reactions normally occurring in the Golgi. The kinetics with which MR bound affinity columns following synthesis in the presence of monensin were similar to controls and support the idea that activation occurs prior to transport into trans-Golgi cisternae. Although transport to the trans-Golgi is not inhibited by monensin, the pH of this compartment is neutralized, which affects the activity of enzymes localized there and prevents the processing and sorting events that normally occur (21). These processing steps are not prerequisite to MR activation.

If the MR becomes active prior to entering the trans-Golgi cisterna, the question of why it does not become bound to other newly synthesized and resident glycoproteins remains. One possibility is that the microenvironment does not favor MR binding. A pH gradient exists across the Golgi stacks that is most acidic in the later Golgi region. Since MR binding has a neutral pH optimum (33), certain Golgi compartments may not promote ligation of the MR. Insufficient calcium would also preclude MR ligation intracellularly (34).

We currently favor the hypothesis that an enzyme activity triggers a change in MR conformation that allows exposure of critical binding domains in proper configuration for ligand binding. This suggestion is based on the AlF$_4^-$ and BFA experiments presented here and on the ability of CRDs to bind ligand following translation in vitro. The type of enzyme activity responsible for MR activation requires definition, although several possibilities are evident. The existence of a binding-site antagonist that may be removed by proteolysis or other event has not been ruled out, although no co-precipitating bands have been visualized in fluorographs of MR immunoprecipitated soon after synthesis. It is more likely that the outermost N-terminal regions of the MR form a block to ligand binding that is removed by the action of our undefined enzyme. One well-characterized enzyme responsible for conformational changes in newly synthesized proteins is protein disulfide isomerase I, which is known to reside mainly in the ER and intermediate compartment (35, 36). Both the N-terminal domain and the CRDs of the MR are rich in cysteine residues, which presumably play a role in forming and maintaining MR structure. It is improbable that protein disulfide isomerase mediates the activation of the MR due to our localization of initial binding activity to the Golgi stacks. However, a similar activity with effects on protein conformation may localize to later secretory compartments. Efforts are underway to clarify the activating event in intact and semipermeabilized macrophages.

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