Oligomerization of the Macrophage Mannose Receptor Enhances gp120-mediated Binding of HIV-1

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C-type lectin receptors expressed on the surface of dendritic cells and macrophages are able to bind glycoproteins of microbial pathogens via mannose, fucose, and N-acetylgalactosamine. Langerin on Langerhans cells, dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin on dendritic cells, and mannose receptor (MR) on dendritic cells and macrophages bind the human immunodeficiency virus (HIV) envelope protein gp120 principally via high mannose oligosaccharides. These C-type lectin receptors can also oligomerize to facilitate enhanced ligand binding. This study examined the effect of oligomerization of MR on its ability to bind to mannan, monomeric gp120, native trimeric gp140, and HIV type 1 BaL. Mass spectrometry analysis of cross-linked MR showed homodimerization on the surface of primary monocyte-derived dendritic cells and macrophages. Both monomeric and dimeric MR were precipitated by mannan, but only the dimeric form was co-immunoprecipitated by gp120. These results were confirmed independently by flow cytometry analysis of soluble monomeric and trimeric HIV envelope and a cellular HIV virion capture assay. As expected, mannan bound to the carbohydrate recognition domains of MR dimers mostly in a calcium-dependent fashion. Unexpectedly, gp120-mediated binding of HIV to dimers on MR-transfected Rat-6 cells and macrophages was not calcium-dependent, was only partially blocked by mannan, and was also partially inhibited by N-acetylgalactosamine 4-sulfate. Thus gp120-mediated HIV binding occurs via the calcium-dependent, non-calcium-dependent carbohydrate recognition domains and the cysteine-rich domain at the C-terminus of MR dimers, presenting a much broader target for potential inhibitors of gp120-MR binding.

The mannose receptor (MR) is a C-type lectin receptor that is expressed on the surface of a variety of cells, including immature monocyte-derived dendritic cells (MDDC), dermal dendritic cells, macrophages, and hepatic endothelial cells. It is a multifunctional protein, involved in antigen recognition and internalization during the early stages of the innate immune response (1) as well as physiological clearance of the endogenous pituitary hormones lutropin and thyrotropin (2, 3). Recognition of foreign antigens occurs via mannan, fucose, and GlcNAc residues (4, 5), which are generally not found as terminal residues on mammalian glycoproteins but are highly abundant on surface proteins of pathogens such as the HIV-1 envelope gp120 (6, 7). Once bound, pathogens can be internalized by endocytosis or phagocytosis, where they are targeted to lysosomes for proteolytic degradation and presentation on major histocompatibility complex class II (8). In immature DCs, soluble recombinant HIV envelope proteins are processed by this pathway, initially binding to both dendritic cell-specific intracellular adhesion molecule 3 grabbing non-integrin (DC-SIGN) and MR and ultimately co-localizing with MR but not DC-SIGN in lysosomes (9). Furthermore, in immature DCs and to a greater extent mature DCs, a proportion of intact HIV-1 enters a unique vesicular compartment that co-localizes with tetratraspin proteins such as CD81 (10, 11). Recently, this compartment has been shown to be continuous with the plasma membrane (11) and does not represent a continuation of the endolysosomal network. Interestingly, this compartment can translocate virus from DCs to CD4 T cells, upon the formation of a virological synapse (10–12). Although viral uptake can occur in DCs independent of HIV env (2), the efficiency of HIV binding and uptake is greatly enhanced by the presence of C-type lectin-env interactions. At least initial binding to DC-SIGN (and most likely also MR) is required for T cell trans-infection (13).

Structurally, the extracellular domain of MR consists of an N-terminal cysteine-rich domain (Cys-RD), followed by a fibronectin type II domain and eight carbohydrate recognition domains (CRD) on a single polypeptide backbone (1). Of the eight CRDs, CRD 4–8 have been shown to be required for high affinity binding of ligands containing terminal mannan/fucose/GlcNAc residues, with CRD 4 having demonstrable monosaccharide binding in isolation (14). Binding and release of ligand within the low pH environment of the endolysosomal compartment are also Ca²⁺-dependent. Acid-induced removal of Ca²⁺ binding in CRD 4 and 5 was shown to cause a conformational rearrangement of the domain, resulting in a loss of carbohydrate binding activity (15). In contrast, binding of sulfated carbohydrates to the Cys-RD appears to be Ca²⁺-independent as no Ca²⁺-binding sites were observed in its crystal structure (2, 16).
Oligomerization of CLRs such as DC-SIGN (17), Langerin (18), and mannose-binding protein (19) has been reported to be essential for binding of oligosaccharide-bearing ligands. Early studies on MR suggested that it exists solely as a monomeric molecule and that clustering of multiple CRDs within the single polypeptide backbone was necessary for high affinity binding of oligosaccharide moieties (20). However, more recent studies have shown that dimerization is possible in the presence of Ca\(^{2+}\) (21) and that an equilibrium may exist between monomeric and dimeric forms on the cell surface (22). It is currently unclear what effect dimerization has on ligand binding to the meric and dimeric forms on the cell surface (22). It is currently unclear what effect dimerization has on ligand binding to the meric and dimeric forms on the cell surface (22).

To date, studies on the oligomerization and ligand binding activity of MR have used solubilized protein from cell lysates (20) or purified recombinant fragments (21). Because the membrane microenvironment can influence protein associations, soluble forms of MR may not necessarily be a true model of the quaternary structure and function of the native protein. Here, we used a well established method of cross-linking (23) on MDDCs, monocye-derived macrophages (MDMs), and MR-transfected Rat-6 cells to preserve lateral protein-protein interactions between MR on the cell surface prior to solubilization. Mass spectrometry analysis of affinity-purified complexes showed they were homo-oligomers, and further resolution of the complex on a low percentage polyacrylamide gel by SDS-PAGE strongly indicates that they are dimers. Dimerization of MR was also found to be essential for binding mannann, monomeric gp120, native trimeric gp140, and HIV-1 viral particles. Persistence of monomeric gp120 and trimeric gp140 binding to dimeric MR in the presence of EGTA and various CRD and other inhibitors, however, suggested that gp120-mediated HIV-1 binding is not Ca\(^{2+}\)-dependent and that at least binding probably occurs to both Ca\(^{2+}\)-dependent and -independent CRDs and also the Cys-RD.

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—All reagents, unless otherwise stated, were purchased from Sigma. Monocytes were positively selected from whole peripheral blood mononuclear cells by magnetic cell sorting using CD14 microbeads (Miltenyi Biotec, Auburn, CA). These cells were used to generate either immature MDDCs or MDMs. Immature MDDCs were prepared as described previously (24), whereas MDMs were generated by culture in RPMI (Invitrogen) supplemented with 10% human AB serum for 5 days (25). The MR-transfected Rat-6 fibroblast cell line has been described previously (20) and were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS). Disuccinimidyl suberate (DSS) was purchased from Pierce; protein G-Sepharose was from GE Healthcare; cyanoide-activated Sepharose was from Sigma; and streptavidin phycoerythrin (SA-PE) was from BD Biosciences. Antibodies used for Western blotting were mouse monoclonal α-MR (Pharmingen) and horseradish peroxidase-conjugated rabbit α-mouse IgG (Silenus, Melbourne, Australia). gp120 inhibitors used were α-CD4 antibody (Q4120), shikimic acid, mannan, EGTA, N-acetylgalactosamine 4-sulfate (all obtained from Sigma), and quinic acid (Supelco, Bellefonte, PA). The following reagents were obtained through the AIDS Research and Reference Reagents Program (Division of AIDS, NIAID, National Institutes of Health): R5 tropic monomeric and trimeric Ug037 HIV gp120 from Dr. James Arthos and anti-HIV-1 gp120 monoclonal antibody (2G12) from Dr. Hermann Katinger. Monomeric AD8 gp120 and trimeric gp140 were kind gifts from Dr. Robert Centre (University of Melbourne).

**Cross-linking and Cell Lysis**—Chemical cross-linking of cell surface molecules and preparation of cell lysates for immuno-preparation have been described previously (17). Briefly, harvested cells were washed twice with cross-link buffer (CLB) (10 mM HEPES, pH 8.0, 140 mM NaCl, 1 mM MgCl\(_2\), 0.1 mM EGTA, 0.02% (w/v) Na\(_2\)SO\(_4\)) and resuspended at 5 \times 10\(^6\) cells/ml. DSS (25 mM) dissolved in DMSO was then added to the cell suspension (20 µl per 1 ml of suspension). For the preparation of mock lysates DMSO without DSS was added to the cells. Cross-linking was carried out for 30 min at room temperature followed by quenching of the cross-linker with Tris-buffered saline (25 mM, pH 7.5) for 15 min. Cells were then pelleted and resuspended at 10 \times 10\(^6\) cells/ml in lysis buffer (10 mM HEPES, 150 mM NaCl, 1% (v/v) Triton X-100, 10 mM CaCl\(_2\), pH 7.5) for 1 h at 4 °C. Insoluble debris was then removed by centrifugation of the lysate at 14,000 rpm for 10 min at 4 °C.

**Affinity Purification of MR Complexes**—Affinity beads were generated by coupling mouse IgG1 and monoclonal α-MR to cyanogen bromide-activated Sepharose according to the manufacturer’s protocol. Nonspecifically, binding proteins were absorbed by initially running the lysate from ~9 \times 10\(^7\) DSS-treated MDDCs over IgG1-Sepharose (100 µl, containing 100 – 150 µg immobilized antibody). The lysate was subsequently run over 100 µl of α-MR beads. The beads were washed with 10 mM Tris-buffered saline, pH 8.0, containing 0.1% Triton X-100. MR complexes were eluted from the beads by boiling for 5 min in SDS sample buffer.

**SDS-PAGE and Protein Identification Using Mass Spectrometry**—The eluate (50 µl) containing MR complexes was loaded onto a 8–16% gradient gel (Gradipore, Frenchs Forest, New South Wales, Australia), and proteins were separated with a constant current of 25 mA per gel. Proteins were stained with Coomassie Brilliant Blue as described previously (23). Protein bands were excised using a scalpel, washed, and subjected to in-gel tryptic digest, and tryptic peptides were purified for mass spectrometry as described previously (23).

Electrospray ionization mass spectrometry analyses were carried out on a Q-ToF\(^{TM}\) mass spectrometer (Waters) using a nanoelectrospray interface. Typically 5–8 µl of the extracted peptides were loaded into a coated glass capillary (Protaga, Odense, Denmark). The mass spectrometry data acquisition was performed similarly as described in our previous studies (23). Briefly, an MS survey scan was acquired from m/z 50 to 2000. Multiple charged ions that could be clearly distinguished from the background were then subjected to MS/MS analysis using collision energies of 25–35 eV. Peptide and protein identification was performed using the Mascot\(^{TM}\) search engine (version 2.2.1). Briefly, each MS/MS
spectrum was subjected to smoothing using the Savitzky-Golay algorithm with a window of three channels and a total of two smoothing iterations using the MassLynx™ software version 4.0 (Waters). Subsequently, the spectrum was made centroid using a minimum peak width of three channels and an 80% centroid top, and a peak list (.dta file) was exported. This file was submitted to a Mascot™ Search against the SwissProt data base (version uniprot_V53.1; 270,778 entries) with the following search parameters: enzyme, semi-trypsin; missed cleavages, two; fixed modifications, none; variable modifications, oxidized methionine; peptide tolerance, 0.8 Da; MS/MS tolerance, 0.6 Da. Peptide matches were verified manually using MassLynx to display the MS/MS spectrum and ProteinProspector to calculate the fragment ions according to published guidelines (26).

**Immunoprecipitation and Co-immunoprecipitation**—All incubations for precipitation experiments were performed at 4 °C. To precipitate MR with mannan-agarose beads (Sigma), lysates were incubated with 50 µl of a 50% slurry of mannan beads for 1 h. The beads were washed twice with lysis buffer before the incubation. For co-immunoprecipitations, monomeric Ug037 gp120 was added to the lysate at a concentration of 25 µg/ml and incubated overnight at 4 °C. The sample was then incubated with 1 µg of α-gp120 (2G12) antibody for 2 h followed by an incubation with 30 µl of a 50% slurry of protein G-Sepharose beads for 2 h. After binding, the beads were washed with lysis buffer five times and resuspended in a nonreducing SDS sample buffer. Proteins were eluted by boiling the beads for 5 min and separated on a 7.5% polyacrylamide gel (Bio-Rad), and MR was detected with a monoclonal α-MR antibody by Western blotting.

**Ligand Binding and Inhibition on Cross-linked MR-transfected Rat-6 Cells and MDMs**—All incubations in this assay occurred at 4 °C. To prevent proteolytic degradation of cell surface MR, the adherent MR Rat-6 cells and macrophages were lifted using Hanks’-based dissociation buffer (Invitrogen). Cells were cross-linked as described above and resuspended in binding buffer (RPMI, 1% bovine serum albumin, 10 mM HEPES, pH 7.5) at 1 × 10^6 cells per 50 µl. For inhibition of gp120 binding, 5 mg/ml mannan, 1 mg/ml α(+)-mannose, 1 mg/ml quinic acid, 1 mg/ml shikimic acid, 10 mg/ml GalNAc-4-SO₄, or 20 mM EGTA were added to the cell suspension and incubated for 30 min. Levels of inhibitors were initially determined using a broad range of concentrations to assess the maximal level of gp120 binding. To block CD4 on MDMs, 20 µM 1,4-bis(guanadino)cyclohexane (B-g120) to each sample for 30 min. 1 µg/ml of biotinylated mannan-agarose beads (Sigma) was similarly blocked with 5 mM EGTA. Cells were then washed with phosphate-buffered saline, and bound ligands were labeled by incubating the cells with 1 µg/ml SA-PE for 30 min for detection by flow cytometry. Differences between treatments were analyzed by Student’s t test, modified for unequal variance.

**Generating High Titer Purified HIV-1 BaL Stocks**—To increase infectivity of initial seed stocks, vesicular stomatitis virus g protein (VSVg) envelope pseudotyped HIV-1 BaL was generated by co-transfecting HEK 293T (Invitrogen) with full-length infectious molecular clone pBaL and the VSVg env expressing construct pHEF-VSVg (both obtained from the AIDS Reagents Repository, National Institutes of Health) using Exgen 500 transfection reagent (Fermentas, Burlington, Canada). Viral supernatants were collected 48 h later and centrifuged at 3,000 rpm for 20 min to remove cellular debris. Using the TZM-Bl indicator cell line (AIDS Reagents Repository, National Institutes of Health), which enables quantitative analysis of HIV using LTR-β-galactosidase as a reporter, the titer of the VSVg pseudotyped HIV BaL stock was 1 × 10^5 TCID₅₀/ml as determined by the Reed and Muench formula (see Ref. 27). VSVg-pseudotyped HIV BaL was subsequently used to seed 20 × 10^6 Supt-1-CCR5 cells (courtesy of Prof. James Hoxie) at a multiplicity of infection of 0.05, and after culturing for 2 h at 37 °C, fresh media were added for a final concentration of 1 × 10^6 cells/ml. After overnight culture, cells were washed thoroughly and cultured for 4 days (after cell entry VSVg envelope is lost and subsequent progeny virions will express only HIV BaL-
TABLE 1

| seen in band | precursor m/z | calci-lated mass | Δm | sequence | Mascot score | expectation value | protein name | accession no. | position |
|--------------|---------------|------------------|-----|----------|--------------|------------------|--------------|--------------|----------|
| Monomer 479.2 | 2 | 956.41 | -0.03 | K.FAMB*DGSK.V | 22 | 34 | Macrophage mannose receptor 1 | P22897 | 872–879 |
| Monomer 531.3 | 2 | 1060.51 | 0.07 | R.GVYTNNGK.G | 28 | 37 | Macrophage mannose receptor 1 | P22897 | 1027–1035 |
| Monomer 535.3 | 2 | 1068.55 | 0.03 | K.SALTHQAR.A | 41 | 1.6 | Macrophage mannose receptor 1 | P22897 | 236–244 |
| Monomer 538.8 | 2 | 1075.57 | 0.01 | R.LTATGSSYR.K | 25 | 36 | Macrophage mannose receptor 1 | P22897 | 702–711 |
| Monomer 545.3 | 2 | 1088.53 | 0.05 | K.ERQVTWLR.K | 60 | 0.024 | Macrophage mannose receptor 1 | P22897 | 294–701 |
| Monomer 557.3 | 2 | 1112.48 | 0.1 | K.DQYYFSK.E | 23 | 89 | Macrophage mannose receptor 1 | P22897 | 809–816 |
| Monomer 572.8 | 2 | 1143.35 | 0.08 | K.EKGNFYSNK.C | 41 | 0.79 | Macrophage mannose receptor 1 | P22897 | 947–955 |
| Monomer 577.3 | 2 | 1152.61 | -0.03 | K.TNFWIQLFR.N | 39 | 1.1 | Macrophage mannose receptor 1 | P22897 | 1293–1301 |
| Monomer 579.3 | 2 | 1156.52 | 0.06 | K.FGQFNEER.K | 35 | 7.3 | Macrophage mannose receptor 1 | P22897 | 959–967 |
| Monomer 586.8 | 2 | 1171.62 | 0.04 | R.ALOGGLASINK.E | 48 | 0.18 | Macrophage mannose receptor 1 | P22897 | 682–693 |
| Monomer 597.8 | 2 | 1193.54 | 0.04 | R.YTHWAADKEP.L | 23 | 52 | Macrophage mannose receptor 1 | P22897 | 1176–1185 |
| Monomer 598.8 | 2 | 1195.55 | 0.03 | K.FEROSSELANK.D | 21 | 71 | Macrophage mannose receptor 1 | P22897 | 213–222 |
| Monomer 607.8 | 2 | 1213.62 | -0.04 | R.SQPQPEIVEK.G | 51 | 0.077 | Macrophage mannose receptor 1 | P22897 | 492–502 |
| Monomer 642.3 | 2 | 1282.6 | -0.02 | K.EQAFYTIHPK.G | 22 | 65 | Macrophage mannose receptor 1 | P22897 | 992–1001 |
| Monomer 804.9 | 2 | 1679.84 | -0.06 | R.NPDDLVSISQSESEK.K | 53 | 0.054 | Macrophage mannose receptor 1 | P22897 | 831–845 |
| Oligomer 729.8 | 2 | 1457.72 | -0.14 | K.YPRWODSDIQTK.G | 45 | 0.38 | Macrophage mannose receptor 1 | P22897 | 562–573 |
| Oligomer 764.4 | 2 | 1526.74 | 0.04 | R.MVESIGIM*SUAFLK.L | 60 | 0.0092 | Macrophage mannose receptor 1 | P22897 | 60–72 |
| Oligomer 666.3 | 2 | 1330.59 | -0.01 | K.GEFLFNYGRR.Q | 40 | 1 | Macrophage mannose receptor 1 | P22897 | 112–122 |
| Oligomer 615.4 | 2 | 1228.68 | 0.1 | R.TGIAQGGLNDVLC.C | 59 | 0.018 | Macrophage mannose receptor 1 | P22897 | 605–616 |
| Oligomer 783.9 | 2 | 1565.77 | -0.01 | K.DLVGHIQHNHSVPI.L | 27 | 19 | Macrophage mannose receptor 1 | P22897 | 1443–1456 |

MR Forms Homodimers—To identify whether proteins other than MR participate in the complexes detected after cross-linking, MR was affinity-purified from 10⁸ MDDCs that had been subjected to cross-linking, and purified proteins were resolved via SDS-PAGE and stained with Coomassie Blue on a 8–16% polyacrylamide gel. Fig. 1A shows the protein bands that were detected as follows: a band corresponding to the molecular weight of monomeric MR (175 kDa) and a high molecular weight oligomeric band. The band corresponding to the monomeric MR and the gel area containing the oligomeric band were excised and subjected to tryptic digest and mass spectrometry analysis to determine their protein composition. Table 1 lists the peptides detected in gel slices corresponding to monomeric and oligomeric MR. A total of 15 MR-derived peptides were identified in the monomeric band and 5 in the oligomeric band confirming the presence of MR in both gel slices. Several additional ions corresponding to tryptic peptides of the MR were detected in both bands but not selected for fragmentation. To identify any other proteins that may be part of the MR oligomers, all detected multiply charged ions not corresponding to tryptic MR peptides were selected for further MS/MS analysis; however, no such proteins were identified. This suggests that the purified band at 175 kDa is monomeric MR and that the bands of high molecular weight are oligomers of MR.

To determine the oligomerization state of MR with greater accuracy, cleared lysates from cross-linked immature MDDCs were run on a 5% polyacrylamide gel, which provides better resolution in the high molecular weight range. The high molecular weight complex seen in Fig. 1A resolved to a dimeric band with a molecular mass of 350 kDa (Fig. 1B). It can also be seen that the amount of monomeric MR gradually decreases, whereas dimeric MR appears with increasing concentrations of the cross-linker. Preservation of lateral associations between

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monomeric MR molecules by DSS therefore suggests that
dimerization of MR occurs on the surface of MDDCs. Detection of two high molecular weight bands just below the dimeric band in all lanes could be due to nonspecific detection with the primary α-MR antibody or perhaps due to detection of differ-
tentially glycosylated or different isoforms of MR.

**Dimeric MR Binds Mannan, gp120, gp140, and HIV-1 BaL**

**Particles with Higher Affinity than Monomeric MR—**We have previously shown that tetramerization of DC-SIGN enhances
ligand binding (17). Here, to examine if MR oligomerization is
similarly required for ligand binding, we performed precipita-
tion and co-immunoprecipitation experiments with mannan
and HIV-1 monomeric Ug037 gp120, respectively. Macrophages rather than MDDCs were used here to avoid simultane-
ous ligand binding to DC-SIGN, which is expressed at high
levels on MDDCs but not on MDMs (30, 31). Cross-linked MR
was found to bind with increased affinity to mannan beads
compared with the monomer (Fig. 2A, 3rd and 4th lanes). As
seen in the cross-linked cell lysate control (Fig. 2, lane 2), a
relatively large amount of monomeric MR was available for pre-
cipitation. However, the amount of monomeric MR that was
precipitated in the DSS (+) sample was low (Fig. 2, lane 3)
compared with the amount of precipitated dimer (Fig. 2, lane 4).
Similarly, in the DSS (−) samples, a relatively low amount of
monomeric MR was precipitated from the total pool found in
the cell lysate control. This demonstrates the relatively higher
specificity and affinity of dimeric MR toward mannan.

Monomeric Ug037 gp120 displayed a similar interaction
with dimeric MR as a dimer band was co-immunoprecipitated
with gp120 only after cross-linking (Fig. 2B, 4th lane). As no
dimer bands were detected in the absence of cross-linking or
after cross-linking but without gp120, the interaction between
gp120 and dimeric MR is highly specific. However, the presence
of monomeric bands in all lanes except for the Sepharose bead
control was most likely the result of nonspecific binding
between monomeric MR and the α-gp120 (2G12) antibody that
was used to co-immunoprecipitate the MR-gp120 complex.

This was demonstrated upon addition of gp120 to both DSS (−)
and DSS (+) samples (Fig. 2B, 2nd and 4th lanes, respectively),
where fading of the monomeric band occurred because of com-
petition with gp120. This provided a paradoxical pattern where
more monomeric MR was precipitated by 2G12 in the absence
than presence of gp120. Interestingly, only the monomer
displayed nonspecific binding, suggesting that 2G12 binds to an
area of MR that becomes occluded upon dimerization. As 2G12
recognizes N-glycans on gp120 (32), it is possible that recogni-
tion of post-translationally glycosylated MR occurred in a sim-
ilar way.

These results demonstrate that dimerization of MR is impor-
tant for facilitating ligand binding. The requirement for MR
dimerization to enhance both strains of gp120, trimeric gp140,
and HIV-1 BaL (and mannan) binding was further confirmed
by flow cytometry analysis and HIV p24 ELISA on cross-linked
MR-transfected Rat-6 cells. These cells were MR-positive, DC-
SIGN-negative, and CD4-negative by phenotyping (data not
shown) and have been previously described as showing func-
tional similarity to MR expressed on cultured monocytes, in
particular regarding protein expression, binding, and internal-
ization of glycoprotein ligands (33). Following cross-linking,
a similar significant fold increase for both strains of gp120 and
gp140 (Fig. 3, A–E) and HIV-1 virion binding (Fig. 3F) was
observed, whereas no such increase was seen in gp120/gp140
binding to the untransfected Rat-6 cells (Fig. 3E). Differences
in the degree of binding between the two gp120/gp140 strains
were also observed with Ug037 envelopes showing higher bind-
ing than the AD8 envelopes overall. Furthermore, compared
with the DSS (−) Rat-6 cells, DSS (−) MR Rat-6 cells displayed
increased gp120 binding, which is likely because of the presence
of naturally dimerized MR on the cell surface. This provides

![FIGURE 2. A, precipitation of monomeric and dimeric MR with mannan beads from MDM lysates. In the DSS (−) sample, only monomeric MR was precipitated (3rd lane), whereas in the DSS (+) sample, predominantly dimeric MR was precipitated (4th lane). Cross-linked cell lysate (2nd lane) showed MR expressed on MDMs was capable of dimerization without mannan binding, and a proportion of either intracellular MR or cell surface monomeric MR co-existed in parallel with the dimer. A small proportion of dimer in the mock-treated cell lysate (1st lane) was also detected. This is likely to be dimeric MR that has resisted dissociation under the relatively weak lysis conditions. No MR was precipitated with Sepharose beads alone. This result is representative of three independent experiments. B, co-immunoprecipitation of dimeric MR with gp120. Cross-linked MDMs were lysed, and where indicated, the lysate was incubated with Ug037 monomeric gp120, and MR-gp120 complexes were immunoprecipitated with α-gp120 (2G12)-Sepharose beads. Only dimeric MR was shown to specifically co-immunoprecipitate with gp120 after the cells were treated with DSS (4th lane). Detection of a 175-kDa band in all co-immunoprecipitations with the α-gp120 beads, even in the absence of gp120, suggests these are the result of nonspecific binding between mono-
meric MR and the α-gp120 beads. No binding was observed with Sepharose beads alone. This result is representative of two independent experiments.](Image 366x410 to 504x488)
further evidence that cell surface dimeric MR facilitates enhanced gp120 binding. Similarly, mannan binding to DSS (+) MR Rat-6 cells was enhanced compared with DSS (−) MR Rat-6 cells (data not shown).

**gp120 Partially Binds to Dimeric MR via the Ca^{2+}-dependent CRDs—**To determine whether gp120 binding takes place via the Ca^{2+}-dependent CRDs (4 and 5), a number of known and potential CRD inhibitors were used to block gp120 binding to cross-linked and mock-treated MR Rat-6 cells. These included mannan, D(+)-mannose, quinic acid, and shikimic acid. DSS (−) and DSS (+) MR Rat-6 cells were preincubated with saturating concentrations of each inhibitor before monomeric gp120 or trimeric gp140 was added. Although no inhibition of gp120/gp140 binding was observed with D(+)-mannose, quinic acid, and shikimic acid (data not shown), a partial block with mannan was shown for both DSS (−) and DSS (+) cells. A and B of Fig. 4, respectively, show a 15–20% drop in AD8 and Ug037 monomer and trimer binding after the addition of mannan (also shown in Fig. 4D, panels a–j), and Fig. 4E shows a similar trend where virion binding was also partially blocked with mannan.

As a positive control for mannan inhibition, MDDCs were similarly preincubated with mannan, and up to 80% inhibition and 90% inhibition for both forms of AD8 and Ug037 gp120, respectively, was achieved (Fig. 4, C and D, panels k–o). This high level of inhibition is likely to be mediated by DC-SIGN which is the dominant CLR involved with HIV binding on these cells (24) and to a lesser extent by MR.

Another approach to determine whether gp120 binds to certain CRDs is to deplete Ca^{2+} through chelation with EGTA. Because CRDs 4 and 5 are involved in Ca^{2+}-dependent recognition of terminal mannose, fucose, and GlcNAc (14, 20), removal of Ca^{2+} should inhibit
gp120 binding. DSS (−) and DSS (+) MR Rat-6 cells and similarly treated MDMs were preincubated with EGTA before the addition of gp120. CD4 was expressed on MDMs at moderate levels, with an average of 54% of cells positive (data not shown). As CD4 is a major gp120-binding protein, it was crucial to block CD4-mediated binding so that any binding observed would be primarily due to MR. CD4 was blocked by the neutralizing α-CD4 (Q4120) antibody, which was incubated with the cells together with EGTA before gp120 was added. As a control, CRD-mediated binding of mannan to both DSS (−) and DSS (+) MR Rat-6 cells (Fig. 5A) and MDMs (Fig. 5B) was blocked using EGTA.

In both cell types, mannan binding to both DSS (−) samples was significantly decreased with EGTA compared with negative controls. Significant reductions in binding were also observed in DSS (+) samples. In contrast, binding of Ug037 gp120 was not significantly inhibited by EGTA treatment in both DSS (−) and DSS (+) MR Rat-6 cells and MDMs. In MDMs, the relatively high binding of gp120 observed after EGTA treatment in DSS (−) cells was most likely due to CD4 as gp120 binding was blocked significantly after treatment with α-CD4. Co-incubation with both α-CD4 and EGTA did not significantly change gp120-binding levels compared with only α-CD4 antibody, indicating that binding is not Ca2+-dependent. This indicates that whereas mannan binding is mediated by the Ca2+-dependent CRDs, gp120 binding to MR is not Ca2+-dependent.

**gp120 Binds to the Cysteine-rich Domain**—Another possible binding site on MR is the Cys-RD. Binding of ligands bearing oligosaccharides terminating in GalNAc-4-SO4 moieties has been shown to be dependent on MR dimerization. Specifically, the apposition of two Cys-RDs was required to allow binding of GalNAc-4-SO4 (22). To determine whether gp120 binds to the Cys-RD, as well as whether dimerization enhances this binding, DSS (−) and DSS (+) MR Rat-6 cells were preincubated with GalNAc-4-SO4 before the addition of gp120. No block of gp120 binding was observed with the DSS (−) cells, whereas a mean inhibition of 20% occurred on cross-linked cells (Fig. 6) (p < 0.02). This suggests that some gp120 binds to the Cys-RD and that this is facilitated by dimerization. Interestingly, co-incubation of GalNAc-4-SO4 (~20% inhibition) and mannan (~30% inhibition) resulted in an additive inhibition of gp120 binding on DSS (+) cells (~50% inhibition). Overall, these results suggest a complex mode of binding for gp120 on dimerized MR, involving different domains.

**DISCUSSION**

In this study we have defined the quaternary structure of native MR as homodimers on the surface of a variety of cells, including MDDCs, MDMs, and MR-transfected Rat-6 cells. Early studies on the oligomerization of MR suggested that it existed solely as a monomer by equilibrium density gradient centrifugation and affinity chromatography with mannose-Sepharose (20). These techniques, as with many others used for analyzing protein-protein interactions, often require detergent-based solubilization of the proteins of interest. Although strong interactions such as those between antibodies and their antigens (K_d, values within nanomolar range) are more likely to resist dissociation, weak or transient interactions such as self-associated MR would most likely dissociate into their monomeric components (22). As a result, any complexes of MR would not be detected. To overcome this problem, we have used an amine-reactive, homo-bifunctional cross-linking agent (DSS) to covalently preserve any lateral protein-protein interactions on the cell surface before solubilization. In addition, little nonspecific cross-linking has been observed with this procedure (23), most likely due to the short range of DSS (11.4 Å). With this technique, we were able to determine by affinity purification and mass spectrometry that MR forms dimers on the cell surface. Without cross-linking, as shown in Fig. 1B, MR was detected only as a monomer, illustrating that the interactions between MR molecules are usually weak or transient. Although dimerization has recently been shown to occur with soluble recombinant extracellular fragments of MR (21, 34) and with MR obtained from rat liver (22), this study is the first to demonstrate that dimerization occurs on the surface of human cells, in particular on MDMs and MDDCs.

Currently, little is known of how MR oligomerization is mediated. Oligomerization of polypeptides containing a single CRD is a common feature of many CLR, including DC-SIGN and Langerin. Both have neck regions consisting of heptad repeats that mediate oligomerization through the formation of α-helical coiled-coils (35, 36). Unlike DC-SIGN and Langerin, however, members of the mannan receptor family have no such neck domain and consist of multiple CRDs. Therefore, another mechanism of oligomerization is most likely to exist for MR. As MR is glycosylated, one possibility could be that terminal mannose residues on one receptor binds to the CRDs of another receptor in a Ca2+-dependent manner (21). Another possibility could be that variations in glycosylation patterns determine whether or not oligomerization occurs. A study by Su et al. (37) suggested that sialylation of MR may be important in regulating MR oligomerization and function by demonstrat-

**FIGURE 3.** Monomeric AD8 and Ug037 gp120, trimeric AD8 and Ug037 gp140, and HIV-1 BaL bind with higher affinity to dimeric MR on MR-transfected Rat-6 cells. b-gp120/gp140 binding to DSS (−) and DSS (+)-treated Rat-6 and MR Rat-6 cells were compared using flow cytometry, and HIV BaL binding to similarly treated cells was detected by p24 ELISA. Histogram representation shows a positive shift in the following: A, monomeric AD8; B, trimeric AD8; C, monomeric Ug037; and D, trimeric Ug037 b-gp120/gp140 binding after cross-linking MR Rat-6 cells. Compared with the control DSS (+)-treated MR cells, increased binding of b-gp120/gp140 by DSS (−) and to a greater degree by DSS (+) MR Rat-6 was also observed. These results are representative of three independent experiments. E, approximate 2-fold increase in monomeric and trimeric AD8 and Ug037 gp120/gp140 occurred only after cross-linking MR on MR Rat-6 cells. No such increase was observed for the cross-linked untransfected Rat-6 cells. F, similar 2-fold increase in HIV binding was detected after cross-linking by p24 ELISA for input HIV p24 concentrations ranging between 1 and 68 μg/ml. Values for fluorescence-activated cell sorter data are normalized to the fluorescence values obtained from mock-treated Rat-6 cells and are represented as the fold change in geometric mean of PE fluorescence of three experiments plus standard deviation (p < 0.01). Values for p24 ELISA are represented as the mean captured p24 antigen from bound virions plus standard deviation (n = 3, p < 0.01 for all input HIV p24 concentrations).
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A

B

C

D

E

MR Rat-6

MR Rat-6

MDDC

MR Rat-6
Blocking AD8 and Ug037 gp120/gp140, Bal gp120, and HIV-1 BalL virion binding to cross-linked MR Rat-6 cells with mannan. A, DSS (−) and DSS (+)-treated cells were incubated with 5 mg/ml of mannan for 30 min before the addition of AD8 gp120/gp140. SA-PE was added for detection of bound b-gp120/gp140 by flow cytometry. B, DSS (−) and DSS (+)-treated cells were similarly pretreated with mannan before the addition of monomeric or trimeric Ug037 gp120/gp140. Values are represented as percent binding of gp120/gp140, normalized to the respective untreated DSS (−) or DSS (+) control. Cell viability was maintained in all samples treated with mannan based on forward and side scatter analysis (data not shown). Values are represented as the geometric mean of PE fluorescence in three experiments plus standard deviation (*, **, and ***; p < 0.01).

FIGURE 5. Inhibition of mannan but not gp120 binding to cross-linked MR Rat-6 cells and monocyte-derived macrophages with EGTA. A, DSS (−) and DSS (+)-treated MR Rat-6 cells were incubated with 20 mM EGTA for 30 min before addition of mannan or gp120. SA-PE was added for detection of bound gp120/gp140 to block parallel gp120 binding to CD4. Values are represented as percent binding of mannan or gp120, normalized to their respective untreated controls. Values are represented as the geometric mean of PE fluorescence in three experiments plus standard deviation (*, **, and ***; p < 0.01).

A MR Rat-6

B Monocyte-derived macrophages

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FIGURE 6. Partial additive blocking of gp120 binding to cross-linked MR Rat-6 cells with a cysteine-rich domain inhibitor and mannan. DSS (−) and DSS (+)-treated cells were incubated with mannan or GalNAc-4-SO4 and/or mannan before Ug037 gp120 was added. Values are represented as percent binding of gp120, normalized to the respective untreated DSS (−) or DSS (+) control in three experiments plus standard deviation (p < 0.02).
of a variety of pathogens (19). In these cases, clustering of CLR does not appear to bind to both monomeric and dimeric forms of MR, depicted in a bent conformation (34), via at least CRD 4 and 5 in a Ca\(^{2+}\)-dependent manner. Binding of multiple gp120 (monomer and trimer) molecules only occurs after dimerization of MR and may occur via all CRDs in a non-Ca\(^{2+}\)-dependent manner as EGTA was unable to inhibit gp120 binding. Close inter-molecular proximity of two CRDs may allow stable complexes to form with gp120. Ca\(^{2+}\)-dependent binding by mannan to CRD 4 and 5 may block gp120 binding to these two domains by steric hindrance, resulting in an ~20–30% inhibition of total gp120 binding. Parallel binding of GalNAc-4-SO\(_4\) to two closely apposed cysteine-rich domains in the dimeric molecule also blocks gp120 binding to this site, reducing total gp120 binding by a further 20%, resulting in an additive block between mannan and GalNAc-4-SO\(_4\) by up to 50%.

In contrast, gp120 binding was shown to bind only to dimeric MR by co-immunoprecipitation. This dimeric binding was confirmed by flow cytometry and HIV p24 ELISA as binding of three different strains of monomeric gp120 and two native trimeric gp140 to DSS (+) MR Rat-6 cells was ~2-fold higher compared with that in DSS (−) MR Rat-6 cells. Furthermore, infectious HIV BaL virions bound at higher levels to MR dimers than monomers. The strong correlation observed between envelope and virion binding (and blocking) in cross-linked cells suggests that the interaction of HIV with MR dimers is mediated by gp120.

Being a heavily glycosylated protein, it was expected that, like mannan, gp120 would bind to the CRDs in a Ca\(^{2+}\)-dependent manner. However, EGTA did not inhibit gp120 binding to both DSS (−) and DSS (+) MR Rat-6 cells and MDMs, suggesting that gp120 binding is not Ca\(^{2+}\)-dependent. Although mannan inhibited monomeric/trimeric gp120 and BaL virion binding to MR dimers by up to 30%, presumably via the Ca\(^{2+}\)-dependent CRDs (4 and 5), it may be doing so as a result of steric hindrance rather than through a Ca\(^{2+}\)-dependent mechanism. In addition, a wide range of other potential CRD blockers, including d(+)mannose, quinic acid, and shikimic acid (39), did not inhibit gp120 binding. Thus, gp120 does not appear to bind to MR dimers via the conventional Ca\(^{2+}\)-dependent CRDs. Although monosaccharide ligand binding to individual and linked Ca\(^{2+}\)-dependent CRDs on monomeric soluble recombinant MR fragments is weak (20), it is possible that dimerization of cell surface MR may enhance Ca\(^{2+}\)-independent gp120 binding to either CRDs 4 and 5, one or more Ca\(^{2+}\)-independent CRDs (e.g. CRDs 1–3 and 6–8), or both. This dual binding ability is not unique to MR as a Ca\(^{2+}\)-independent binding site with weak maltose binding affinity has also been identified on Langerin CRD in addition to the Ca\(^{2+}\)-dependent site (40). It is also possible that MR oligomerization may be a Ca\(^{2+}\)-dependent event and under normal circumstances the presence of Ca\(^{2+}\) would allow oligomerization to occur and hence facilitate gp120 binding. After cross-linking, Ca\(^{2+}\) may no longer be required for oligomerization, and therefore its importance in gp120 binding is diminished.

Another likely binding site for gp120 is the Cys-RD. So far, only endogenously expressed sulfated proteins, such as the pituitary hormones lutropin and thyrotropin, have been shown to bind to the Cys-RD (41). Here, two closely associating Cys-RDs resulting from MR dimerization were required for binding of terminal GalNAc-4-SO\(_4\) moieties on lutropin. Although GalNAc has been identified on gp120 (42), it is not known whether it is sulfated at the C-4 position, which is required for high affinity binding (41). Our results indicate that, like lutropin, gp120 binds with higher affinity and specificity to dimeric MR, suggesting that gp120 may also be binding to two Cys-RD. Blocking of the Cys-RD with GalNAc-4-SO\(_4\) also resulted in only 20% inhibition of gp120 binding. Although this is only a partial inhibition, it was consistent and significant and demonstrated that the Cys-RD probably plays a role in some gp120 binding.

The additive effect of inhibiting gp120 binding to dimeric MR with both mannan and GalNAc-4-SO\(_4\) demonstrated here suggests a model whereby multiple gp120 molecules may be oriented at the surface of pathogens. This ability also confers a large degree of selectivity toward foreign antigens as mannone residues are less abundantly expressed on mammalian cells (19). The MR, however, not only consists of eight CRDs to mediate binding of mannone, fucose, and GlcNAc residues but also consists of a Cys-RD for binding GalNAc-4-SO\(_4\) expressing ligands. Therefore, MR oligomerization may influence these dual binding functions and consequently be important in determining binding affinity to different ligands.

Although strong binding to multivalent ligands such as mannan has been demonstrated for monomeric MR via CRDs 4–8 (14, 21, 22, 34), we have shown that dimeric MR (after cross-linking) also binds agarose-bound mannan with higher affinity. Another likely binding site for gp120 is the Cys-RD. So far, only endogenously expressed sulfated proteins, such as the pituitary hormones lutropin and thyrotropin, have been shown to bind to the Cys-RD (41). Here, two closely associating Cys-RDs resulting from MR dimerization were required for binding of terminal GalNAc-4-SO\(_4\) moieties on lutropin. Although GalNAc has been identified on gp120 (42), it is not known whether it is sulfated at the C-4 position, which is required for high affinity binding (41). Our results indicate that, like lutropin, gp120 binds with higher affinity and specificity to dimeric MR, suggesting that gp120 may also be binding to two Cys-RD. Blocking of the Cys-RD with GalNAc-4-SO\(_4\) also resulted in only 20% inhibition of gp120 binding. Although this is only a partial inhibition, it was consistent and significant and demonstrated that the Cys-RD probably plays a role in some gp120 binding.

The additive effect of inhibiting gp120 binding to dimeric MR with both mannan and GalNAc-4-SO\(_4\) demonstrated here suggests a model whereby multiple gp120 molecules may be...
binding to several different sites on a MR dimer at any one time (Fig. 7). It is likely that Ca\(^{2+}\)-independent mechanisms of binding by the CRDs may be playing a large role in gp120 binding, given that Ca\(^{2+}\) depletion with EGTA had no effect on binding. Currently, little is known about any mutable ligands for the Ca\(^{2+}\)-independent CRDs and with no known inhibitors for any of these domains, the ability to fully block gp120 binding to MR will be a future challenge.

The fate of HIV-1 upon binding to MR, as for other foreign antigens, is internalization and entry into the endocytic pathway of a variety of antigen-presenting cells as shown in immature MDDCs (43), dermal dendritic cells (24), MDMs (31, 44), and astrocytes (45). Where DC-SIGN and MR are co-expressed on dermal DCs, HIV binding and entry probably occurs primarily via DC-SIGN. However some dermal DCs and most MDMs express MR alone, and HIV trans-infection of CD4 T cells by MDMs at least have been shown to be mediated by MR (31). gp120 and intact HIV are processed differently in the endocytic pathway. Soluble monomeric gp120 accompanied by MR is transported to the lysosome and completely digested, whereas intact HIV is only transported to the late endosome (46). Thus, although significant amounts of virus are degraded within 24 h, a small proportion of infectious virus can persist in immature DCs within a CD81-positive vesicular compartment up to this 24-h time point (10, 11). Within 24 h, if the DC contacts a T cell, HIV is transported to the “viral synapse” between DCs and T cells where transfer can occur (9, 47). This trans-infection is thought to represent one mode of CD4 T lymphocyte infection, yet it must be noted that DC infection levels of less than 1% have been shown to result in an explosive HIV-1 infection in CD4 lymphocytes (48), and thus this latter transfer must also be taken into account (49). Similarly, transfer of HIV from infected macrophages to CD4 T cells via MR has been observed (31). In addition, productive infection of macrophages results in the establishment of viral reservoirs within tissues in which they reside such as the brain, lung, or liver.

In conclusion, the data presented here demonstrate that dimerization of MR on the surface of MR Rat-6 cells, MDMs, and MDDCs is important for enhancing gp120-mediated HIV binding to these receptors. This is consistent with gp120 binding to another CLR, DC-SIGN, where oligomerization of these proteins facilitates high affinity binding of oligosaccharide-expressing ligands (17). This is also the first study showing that a foreign (pathogen) glycoprotein is able to bind at least in part to the Cys-RD and probably CRDs of the MR in a Ca\(^{2+}\)-independent manner, as opposed to the conventional Ca\(^{2+}\)-dependent CRD-mediated binding that occurs with many pathogen-derived glycoproteins. In light of this, any attempts to design molecules to completely block MR and inhibit HIV-1 binding via this receptor may need to take into consideration binding to both the Cys-RD and multiple CRDs on the MR dimer.

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