Ligand-Binding Characteristics of CXCR4 Incorporated Into Paramagnetic Proteoliposomes

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ABSTRACT

The G protein-coupled receptor CXCR4 is a coreceptor, along with CD4, for the human immunodeficiency virus type 1 (HIV-1) and has been implicated in breast cancer metastasis. We wished to study the binding of the HIV-1 gp120 envelope glycoprotein to CXCR4, but found that the gp120 glycoproteins from CXCR4-using HIV-1 strains bound nonspecifically to several cell lines lacking human CXCR4 expression. Therefore, we constructed paramagnetic proteoliposomes (CXCR4-PMPLs) containing pure, native CXCR4. CXCR4-PMPLs specifically bound the natural ligand, SDF-1α, and the gp120 glycoproteins from CXCR4-using HIV-1 strains. Conformation-dependent anti-CXCR4 antibodies and the CXCR4 antagonist AMD3100 blocked HIV-1 gp120 binding to CXCR4-PMPLs. The gp120-CXCR4 interaction was blocked by anti-gp120 antibodies directed against the third variable (V3) loop and CD4-induced epitopes, structures that have also been implicated in the binding of gp120 to the other HIV-1 coreceptor, CCR5. Compared with the binding of R5 HIV-1 gp120 glycoproteins to CCR5, the gp120-CXCR4 interaction exhibited a lower affinity (K_d=200 nM) and was dependent upon prior CD4 binding, even at low temperature. Thus, although similar regions of X4 and R5 HIV-1 gp120 glycoproteins appear to be involved in binding CXCR4 and CCR5, respectively, differences exist in nonspecific binding to cell surfaces, affinity for the chemokine receptor, and CD4 dependence at low temperature.
INTRODUCTION

Genes encoding seven transmembrane domain, G protein-coupled receptors (GPCRs) account for approximately 1-2% of the human genome (1-3). These proteins are generally expressed at very low levels in the cell (4) and are extremely hydrophobic. Purification of GPCRs from the cell membrane typically results in loss of native conformation (5,6), making functional studies of pure protein essentially impossible. As such, most studies of GPCRs have been limited to cell-based assays. Unfortunately, the heterogeneity of the cell surface can complicate the interpretation of results. These limitations make overexpression, native purification and protein stabilization outside the cell membrane crucial to the understanding of GPCR structure and function.

CXCR4 is a member of the GPCR family of proteins and is the receptor for the chemokine stromal cell-derived factor (SDF-1α) (7,8). CXCR4 has a broad tissue distribution and interaction with SDF-1α leads to chemotaxis (7,9), immunomodulation, as well as other immunoregulatory functions. Disruption of the SDF-1α gene in mice is an embryonic lethal mutation (10), demonstrating the importance of signaling through CXCR4. Recently, it has also been shown that CXCR4 may play a role in breast cancer metastasis (11). These data suggest the importance of CXCR4 in normal human physiology as well as pathogenesis.

CXCR4 is also a relevant molecule in human immunodeficiency virus type 1 (HIV-1) infection. The HIV-1 envelope glycoprotein, gp120, initially binds to the CD4 molecule on the host cell surface (12). This interaction leads to conformational changes
in gp120 that increase the affinity for the HIV-1 coreceptors (13,14), the chemokine receptors CXCR4 (15) and CCR5 (16-20). Association of gp120 with the chemokine receptor is thought to promote conformational changes in the HIV-1 transmembrane envelope glycoprotein, gp41 (21). This culminates in fusion of the virion with the host cell, leading to viral entry. Given the normal physiological role of CXCR4 as well as its role in HIV-1 pathogenesis, biochemical characterization of the interaction of CXCR4 with ligands, including HIV-1 gp120, is of paramount importance.

Paramagnetic proteoliposomes (PMPLs) have been described that allowed the convenient study of a purified GPCR, CCR5, in a native, membrane-associated state (22). Specific detergents were used to solubilize CCR5 in expressing cells in a native conformation (23). Subsequently, the protein was captured on magnetic beads via an affinity tag, and a lipid membrane reconstituted during the removal of detergent. CCR5 in PMPLs bound conformation-dependent antibodies and complexes of HIV-1 gp120 and soluble CD4 (22). These CCR5-PMPLs were stable for up to 2 months and were able to be used in many assays typically reserved for GPCR-expressing cells.

Here we describe overexpression of CXCR4 and subsequent solubilization of CXCR4-expressing cells in a manner that maintains native conformation of the molecule. This was documented by the binding of both conformation-dependent antibodies as well as the binding of SDF-1α to CXCR4 contained in cell lysates. Due to the limited stability of CXCR4 in detergent-containing cell lysates, we reconstituted CXCR4 into paramagnetic proteoliposomes (CXCR4-PMPLs). These CXCR4-PMPLs bound conformation-dependent antibodies, SDF-1α, and the gp120 glycoprotein of a CXCR4-using HIV-1 isolate, HXBe2. All of these interactions were determined to be specific and
were as efficient as those observed with CXCR4 expressed on the cell surface. In fact, CXCR4-PMPLs proved to be a much more efficient tool for studying gp120/CXCR4 interaction by limiting the nonspecific binding that characterized the gp120 interaction with the cell surface. PMPLs represent a valuable tool for the screening and identification of GPCR/ligand interactions and may also be useful in the study of membrane proteins in general.
EXPERIMENTAL PROCEDURES

*Synthesis and Overexpression of Codon-Optimized CXCR4.* Codon-optimized CXCR4 (synCXCR4) was synthesized as previously described for CCR5 (23). CXCR4 was cloned into the PACH vector (a gift from Dr. Velan, Israel Institute for Biological Research) and a sequence encoding the C9 peptide (TETSQVAPA) was introduced immediately 5’ to the natural stop codon for CXCR4. The C9 peptide is a linear epitope recognized by the 1D4 antibody (24).

PACH-SynCXCR4 DNA was transfected into Cf2Th cells using the Geneporter transfection reagent (Gene Therapy Systems) as described by the manufacturer. Stably transfected CXCR4-expressing cells were grown in medium containing 0.5 mg/ml G418 (GibcoBRL). After selection, cells expressing the highest levels of CXCR4, as determined by 12G5 antibody staining, were sorted using FACS.

*Cell Culture.* Cells were grown in DMEM supplemented with 10% fetal bovine serum and 100 IU/ml Penicillin/Streptomycin (Complete DMEM) at 37°C with 5% CO₂. Stable transfectants were grown in complete DMEM containing 0.5 mg/ml G418 (Gibco). Cf2Th-CXCR4 and –CCR5 cells were treated for 24 hrs. with 3 mM sodium butyrate prior to harvest.

*Metabolic Labeling of CXCR4-Expressing Cells.* Cf2Th-CXCR4 cells were grown in 100 mm tissue culture dishes to full confluency. Growth medium was removed and the cells were washed twice with PBS. DMEM lacking cysteine and methionine was added and
the cells were incubated for 1 hr at 37°C. After incubation, 40 µCi/ml each of [35S]-
methionine and -cysteine (NEN) was added and the cells were incubated for 16 to 24 hr.
Following incubation, the medium was removed and the cells harvested with PBS/5 mM
EDTA. Cells were pelleted and snap frozen on dry ice.

*Immunoprecipitation of CXCR4.* Approximately 5x10⁶ metabolically labeled Cf2Th-
CXCR4 cells were suspended in solubilization buffer consisting of 100 mM (NH₄)₂SO₄,
20 mM Tris, pH 7.5, 20% glycerol, 1x Complete Protease Inhibitor Cocktail (Roche), 1%
CHAPSO detergent (Anatrace). After suspension, the solution was incubated for 5
minutes on ice followed by 25 minutes on a rocker at 4°C. The lysate was then cleared by
centrifugation at 14,000xg for 30 minutes at 4°C. CXCR4 in the cleared lysate was
precipitated with either 1D4-Sepharose (23) or Protein A-Sepharose conjugated with one
of the following anti-CXCR4 antibodies: 12G5 (Pharmingen), 44708.111, 44716.111 or
44717.111 (all R&D Systems) for >5 hours at 4°C on a rocker. Following incubation, all
precipitates were washed four times in solubilization buffer and resuspended in an equal
volume of 2xSDS sample buffer. Samples were incubated at 37°C for 45 minutes and run
on 12% SDS-polyacrylamide minigels (BioRad). Gels were washed for 30 minutes in
10% acetic acid/ 20% methanol, 30 minutes in ENHANCE (NEN), and 20 minutes in 2%
glycerol. Washed gels were dried at 80°C under vacuum for 45 minutes and visualized by
autoradiography on Kodak Biomax MR film.

*Coimmunoprecipitation of CXCR4 and SDF1α-Ig.* A plasmid expressing SDF1α-Ig (a
gift of Dr. Timothy Springer, Center for Blood Research, Boston, MA) was transfected
into 293T cells using Geneporter transfection reagent as described by the manufacturer. After 24 hrs, the medium was replaced with either complete DMEM or DMEM without cysteine and methionine supplemented with 10% FCS, 100 IU pen/strep and 200 μCi each of [35S]-cysteine and -methionine. Cells were then incubated for 72 hr at 37°C with 5% CO2. The medium was removed and centrifuged at 2000xg to remove debris. SDF1α-Ig is a secreted protein and as such is found in the growth medium.

Precipitation of metabolically labeled CXCR4 with SDF1α-Ig was performed as follows. Radiolabeled cleared lysate containing CXCR4 was incubated with protein A-Sepharose and 200 μl culture supernatant containing SDF1α-Ig for >5 hr at 4°C on the rocker. The protein A-Sepharose was washed, the protein eluted, and gels run as described above. Precipitation of metabolically labeled SDF1α-Ig with CXCR4 was performed as follows. CXCR4-containing cell lysate was incubated with 200 μl of radiolabeled SDF1α-Ig culture supernatant and 1D4-Sepharose for >5 hr at 4°C on the rocker. The 1D4-Sepharose was washed, the protein eluted and gels run as described above.

Conjugation of 1D4 Antibody to M-280 Dynal Beads. Ten ml of tosylactivated M-280 Dynal beads (2x10⁹ beads/ml) were conjugated with 6 mg 1D4 antibody (National Cell Culture Center) as described by the manufacturer. Conjugated beads were resuspended at a concentration of 2x10⁹ beads /ml in PBS/0.5% BSA/0.02% sodium azide and stored at 4°C.

Preparation of Lipids. All lipids were obtained from Avanti Polar Lipids and were dissolved in chloroform. Six mg of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
(POPC), 3 mg of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), and 1 mg of 1,2-dioleoyl-sn-glycero-3-phosphate (DOPA) were pooled in a cryovial and dried under vacuum. This lipid mix was then resuspended in PBS and sonicated. Sonicated lipid was stored under liquid N₂.

**Synthesis of CXCR4-Containing Paramagnetic Proteoliposomes (PMPLs).**

Approximately 4x10⁸ Cf2Th-CXCR4 cells were resuspended in 4 ml of solubilization buffer. The solution was then incubated on ice for 5 minutes followed by 25 minutes at 4°C on a rocker. CXCR4-containing lysate was then cleared by centrifugation at 14,000xg for 30 minutes at 4°C. Cleared lysate was incubated with 2x10⁹ 1D4-coated M-280 Dynal beads at 4°C for >2.5 hrs on the rocker. Beads were washed with solubilization buffer 3 times and resuspended in 10 ml of buffer containing 1% CHAPSO, 20% glycerol, 100 mM (NH₄)₂SO₄, 20 mM Tris, pH 7.5 and 10 mg of POPC/POPE/DOPA lipid mix. The solution was then dialyzed against 100 mM (NH₄)₂SO₄, 20 mM Tris, pH 7.5, and 20% glycerol for 24 hrs at 4°C using a Slide-A-Lyzer, 10,000 MWCO (Pierce) to remove detergent and allow the formation of PMPLs. PMPLs were then purified using a magnetic field. PMPLs were resuspended in PBS/0.5% BSA/0.02% sodium azide. PMPLs were stable for >2 months at 4°C. CCR5-PMPLs were synthesized as previously described (22) with minor modifications. Specifically, the streptavidin-biotin bridge was excluded and CHAPSO, not Cymal™-5, was used as the solubilizing detergent.
**FACS Analysis.** All cells (1x10^6) and PMPLs (5x10^6) were stained in a final volume of 100 µl PBS/0.5% BSA. For detection of CXCR4 either on cells or PMPLs, anti-CXCR4 antibodies 12G5-PE (Pharminen) and 44717.111-PE (R&D Systems) were used at a final concentration of 10 nM. The isotype-matched control antibody MG2a04 (Pharminen) was used as a negative control for all stainings. Samples were incubated for 30 minutes at 4°C and washed twice in PBS/0.5% BSA. All samples were analyzed with a Becton Dickinson FACScan with CellQuest software.

**SDF-1α Competition Assay.** Competition assays were set up with 1x10^6 Cf2Th-CXCR4 cells (positive control), 1x10^7 CXCR4-PMPLs, or 1x10^7 CCR5-PMPLs (negative control). All reactions were performed in 100 µl of buffer containing 0.1 nM [125I]-SDF-1α (NEN) and 1X Binding Buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 5 mM MgCl2, 1 mM CaCl2, 5% dialyzed FCS). Cold SDF-1α competitor was added to samples in duplicate at concentrations ranging from 1 nM to 1 µM. Reactions were incubated at 37°C for 1 hr and washed 3 times in 1x binding buffer. Samples were subsequently read on a gamma counter and the results plotted.

**Metabolically labeled gp120 Binding to CXCR4-PMPL and Cf2Th-CXCR4 Cells.** The pSVIIIexE7 plasmid encoding HIV-1 (HXBc2) gp120 was transfected into 293T cells using Geneporter reagent as described by the manufacturer. After 24 hours, the transfection medium was replaced with Cys-/Met- DMEM supplemented with 10% FCS, 100 IU pen/strep, 200 µCi [35S]-cysteine and -methionine. Cells were grown for 72 additional hours and supernatants were collected. Approximately 1x10^8 PMPLs or 5x10^6
cells were resuspended in 100 µl gp120-containing culture medium (with 1 µg soluble CD4). Reactions were incubated for 2 hours at 25°C and washed 2 times in complete DMEM. Cells or PMPLs were then solubilized in 0.5 ml buffer containing 0.5% Triton X-100, 100 mM Tris, pH 7.5, 150 mM NaCl for 30 minutes on a rocker at 25°C. The use of Triton X-100 allowed for the denaturation of CXCR4 resulting in the dissociation of gp120 from CXCR4. Stripped Dynal beads were then removed by centrifugation at 14,000 rpm for 1 minute. Cell lysates were cleared by centrifugation at 14,000 rpm for 30 minutes. The radiolabeled gp120 glycoprotein was precipitated from supernatants with protein A-Sepharose and the gp120-specific antibody C11 for 1 hour at 25°C. Sepharose beads were washed twice in solubilization buffer, resuspended in SDS sample buffer, boiled for 5 minutes, and SDS-PAGE was performed. Gels were dried and exposed to film for 12 to 72 hours.

**FACS Assay for gp120 Binding to CXCR4-Expressing Cells and CXCR4 PMPLs.** The pSVIIIexE7 plasmid encoding HIV-1 (HXBc2) gp120 was transfected into 293T cells as described above. Subsequently, gp120 was recovered from supernatants using affinity chromatography. Approximately 5x10^6 CXCR4-PMPLs or 1x10^6 Cf2Th-CXCR4 cells were resuspended in PBS supplemented with 5% FCS. HXBc2 gp120 (120 ng; 10nM final concentration) was incubated with 1 µg of soluble CD4 for 1 hour at 25°C and the complexes added to the PMPLs/cells. Incubation proceeded for 1 additional hour at 25°C. Then, 1µg of the C11 anti-gp120 antibody was added and incubated for 45 minutes at 25°C. Tubes were washed once in PBS supplemented with 5% FCS and PMPLs/cells resuspended in 95 µl of PBS/5% FCS containing 5 µl of goat-α-human IgG-PE (Jackson
ImmunoResearch). Reactions were incubated for 20 minutes at 4°C and washed 2 times in PBS/0.5% BSA. Samples were analyzed using a Becton Dickinson FACScan with CellQuest software.

*Measurement of HXBc2 gp120 affinity for CXCR4.* Purified HXBc2 gp120 was prepared in 293T cells, as described above, and purified using affinity chromatography. The purified gp120 was iodinated to a specific activity of 500 Ci/mmole with Iodobeads (Pierce), as described by the manufacturer. [\(^{125}\text{I}\)]-gp120 and unlabeled gp120 were incubated with a 10-fold molar excess of soluble CD4 (sCD4) for 1 hour at 37°C. Approximately 1x10^7 CXCR4-PMPLs were resuspended in 100 µl PBS/5% FCS containing 0.5 nM [\(^{125}\text{I}\)]-gp120 (complexed with sCD4) and varying amounts of unlabeled gp120 (1 nM to 3 µM) (complexed with sCD4) in duplicate. Samples were incubated for 2 hours at 25°C, washed twice in PBS/5% FCS and read on a gamma counter.
RESULTS

Expression of Codon-Optimized CXCR4 and Purification of Native CXCR4 from Cell Lysates. The CXCR4 gene was codon optimized, cloned into the PACH vector and overexpressed in a Cf2Th cell line in the same manner as previously described for CCR5 (23). The DNA sequence for the bovine rhodopsin C9 peptide, which is recognized by the 1D4 antibody, was introduced immediately 5’ to the natural stop codon of the CXCR4 gene. The expressed CXCR4 thus contained the C9 peptide at the intracellular C-terminus, permitting its purification with the 1D4 antibody (24) regardless of the conformational integrity of CXCR4. The level of expression of CXCR4 by the Cf2Th cells was assayed by FACS using the 12G5 antibody. Cf2Th-CXCR4 cells exhibited an approximately 400-fold shift in mean-fluorescence intensity above that seen when an isotype-matched control antibody was used (data not shown).

We wished to solubilize and purify CXCR4 from the Cf2Th-CXCR4 cell line while maintaining its native conformation. The detergent used for solubilization is a critical parameter in this process and must be determined empirically. A panel of approximately 100 detergents, including maltosides, glucosides, brijs, ethyl ethers, CHAPS/CHAPSO, HEGAs, MEGAs, and Fos-Cholines™ was tested. The standard buffer used for solubilization consisted of 100 mM (NH₄)₂SO₄, 20 mM Tris, pH 7.5, 20% glycerol, 1x Complete Protease Inhibitor cocktail, and 1% of the previously listed detergents. The conformational integrity of CXCR4 in the cell lysates was monitored by precipitation of CXCR4 by the conformation dependent anti–CXCR4 antibody 12G5. The amount of CXCR4 precipitated by 12G5 was then compared to that precipitated by
the 1D4 antibody, which recognizes the linear C9 epitope and is not influenced by CXCR4 conformation. It was determined that CHAPSO was the best detergent for maintaining the native conformation of CXCR4; a high percentage of the CXCR4 protein solubilized in CHAPSO was precipitated by 12G5. Figure 1A shows a representative precipitation of CXCR4 with 12G5 and 1D4, as well as some other conformation-dependent anti-CXCR4 antibodies (44708.111 and 44716.111). All of the antibodies precipitated the 47 kD mature CXCR4 protein and a 34 kD form. A small amount of 24 kD protein was precipitated by all of the antibodies except 1D4; this may represent a CXCR4 variant lacking the C-terminus. These data demonstrate that CXCR4 is maintained in a native conformation in cell lysates containing CHAPSO.

**CXCR4 Binds its Natural Ligand, SDF-1α, in Cell Lysates.** The ability to precipitate CXCR4 with conformation-dependent antibodies from cell lysates prepared in CHAPSO suggested that CXCR4 was maintained in a native conformation. To assay the conformational state of CXCR4 solubilized in CHAPSO detergent further, we asked if CXCR4 in cell lysates could bind its natural ligand, SDF-1α. This was tested using an SDF1α-Ig fusion protein. SDF1α-Ig consists of SDF-1α bound to the Fc domain of human IgG1 through a flexible linker. When expressed in 293T cells, SDF1α-Ig is secreted into the growth medium. The presence of the Fc domain allows for isolation of SDF1α-Ig using protein A.

293T cells were transiently transfected with a plasmid encoding SDF1α-Ig and the cells radiolabeled with [35S]-cysteine and -methionine. Culture supernatants containing labeled SDF1α-Ig were collected and incubated with cell lysates containing
either CXCR4 or CCR5. Samples were precipitated with the 1D4 antibody directed against the C9 peptide tags at the C-termini of CXCR4 and CCR5 (Figure 1B). The 1D4 antibody coprecipitated labeled SDF1α-Ig from lysates containing CXCR4 but not from CCR5-containing lysates. The total amount of SDF1α-Ig in the sample was determined by precipitation with protein A-Sepharose (SDF1α-Ig +C in Figure 1B).

This coprecipitation experiment was also done in reverse. Cf2Th-CXCR4 cells were metabolically labeled, solubilized with CHAPSO-containing buffer and the lysate incubated with unlabeled SDF1α-Ig-containing supernatant and protein A-Sepharose. Figure 1C shows the precipitation of CXCR4 with the 1D4 and 12G5 antibodies and with SDF1α-Ig. An Ig protein (identical with that found in the SDF1α-Ig fusion protein) was also used as negative control. 1D4, 12G5, and SDF1α-Ig all precipitated CXCR4 from cell lysates; however, the control Ig protein did not. Together these data demonstrate that CXCR4 contained in cell lysates is in a native conformation and can bind its natural ligand, SDF-1α.

Construction of CXCR4-PMPLs. The stability of solubilized chemokine receptors is short-lived, limiting utility (22). Thus, CHAPSO-solubilized CXCR4 was incorporated into PMPLs as described in Experimental Procedures. The presence of a lipid bilayer stabilizes the conformation of CXCR4 bound to the Dynabead. CHAPSO was an excellent detergent in this context because of its high critical micelle concentration (8 mM), allowing for rapid diffusion of the detergent and its replacement by lipid.
Conformation-Dependent Antibodies Have a Similar Affinity for CXCR4-PMPLs and Cf2Th-CXCR4 Cells. To determine if CXCR4 was in a native conformation in PMPLs, we stained the CXCR4-PMPLs with two separate PE-conjugated antibodies (12G5 and 44717.111) specific for CXCR4. The PMPLs were analyzed by FACS and the results are shown in Figure 2A. Both antibodies recognized CXCR4 on the PMPLs. Isotype-matched controls were also used and no shift in fluorescence was observed compared with unstained PMPLs (data not shown). Also, CCR5-PMPLs were stained with the anti–CXCR4 antibodies and no specific staining was observed (data not shown).

The affinity of conformation-dependent antibodies for CXCR4-PMPLs was compared with that seen using Cf2Th-CXCR4 cells. Varying concentrations of antibodies were incubated with both Cf2Th-CXCR4 cells and CXCR4-PMPLs, and the fluorescence intensity measured by FACS for each concentration of antibody was plotted (Figure 2B). The binding profiles for both antibodies were essentially equivalent for both Cf2Th-CXCR4 cells and CXCR4-PMPLs. This demonstrates that the conformation of CXCR4 found in PMPLs resembles that of CXCR4 naturally expressed in cell membranes.

SDF-1α Binding to CXCR4-PMPL and Cf2Th-CXCR4 Cells. To examine the binding of SDF-1α to CXCR4-PMPLs and Cf2Th-CXCR4 cells, competition experiments were performed using \[^{125}\text{I}]\)-labeled SDF-1α with varying concentrations of cold SDF-1α competitor. As shown in Figure 3, the competition profiles for SDF-1α on Cf2Th-CXCR4 cells and CXCR4-PMPLs were nearly identical. In contrast, SDF-1α exhibited minimal binding to CCR5-PMPLs. This demonstrates that CXCR4 contained in PMPLs is in a native conformation and can bind its ligand, SDF-1α, with wild-type affinity.
HIV-1 gp120 binds to CXCR4-PMPLs. To study binding of HIV-1 gp120 to CXCR4-PMPLs, soluble gp120 from the X4 HIV-1 strain HXBc2 was expressed transiently in 293T cells. Binding studies were performed using CXCR4- and CCR5-PMPLs and culture medium containing HXBc2 gp120 metabolically radiolabeled with $[35\text{S}]$-cysteine and -methionine. PMPLs were incubated with gp120-containing culture medium in the presence of soluble CD4 (sCD4). PMPLs were then solubilized and the bound gp120 precipitated with the anti–gp120 antibody C11 (Figure 4A, right panel). The gp120 glycoprotein from an R5 HIV-1 strain, ADA, bound CCR5-PMPLs but exhibited minimal binding to CXCR4-PMPLs (data not shown). Interestingly, when this experiment was repeated using CXCR4- and CCR5-expressing cells, a quite different result was seen. HXBc2 gp120 bound equally well to CCR5- and CXCR4-expressing Cf2Th cells (Figure 4A, left panel), yet ADA gp120 exhibited specific binding only to CCR5-expressing cells (data not shown). Additional experiments indicated that HXBc2 gp120 glycoprotein bound Cf2Th cells and several other cell types that do not express human CXCR4 (data not shown). These cell types are not infectible by the HXBc2 HIV-1 strain unless CD4 and human CXCR4 are expressed in them (25). These results indicate that CXCR4-using HIV-1 gp120 can bind specifically to CXCR4-PMPLs. The CXCR4/gp120 interaction is difficult to demonstrate with CXCR4-expressing cells because of the high nonspecific binding of HXBc2 gp120 to cells not expressing human CXCR4.

Similar results were obtained using FACS to detect bound envelope glycoproteins. Briefly, CXCR4-PMPLs were incubated with 10 nM HXBc2 gp120
(complexed with sCD4) in PBS containing 5% FCS. The gp120-specific antibody C11 was then added and the incubation continued. The C11 antibody does not interfere with either gp120/CD4 or gp120/CXCR4 interactions (13,26). An anti-human IgG secondary antibody coupled to PE was then added and FACS performed to measure the relative fluorescence intensity. As shown in Figure 4B (lower panel), HXBc2 gp120 bound CXCR4-PMPLs but not CCR5-PMPLs. However, the fluorescence intensity of HXBc2 gp120 staining of Cf2Th-CXCR4 cells was indistinguishable from that seen with Cf2Th-CCR5 cells (Figure 4B, upper panel). By contrast, the R5 ADA gp120 bound only Cf2Th-CCR5 cells (data not shown). These results indicate that, unlike R5 gp120 glycoproteins, an X4 gp120 glycoprotein exhibits considerable background binding to cells. By using CXCR4-PMPLs, specific binding of HXBc2 gp120 to CXCR4 can be demonstrated.

Inhibition of gp120 Binding to CXCR4-PMPLs by gp120 and CXCR4 Ligands. We studied the sensitivity of the gp120/CXCR4 interaction to ligands specific for CXCR4 or gp120. CXCR4-PMPLs were incubated with 10 nM HXBc2 gp120 glycoprotein (complexed with sCD4) and varying concentrations of competitor (antibody or antagonist). As shown in Figure 5, the anti-gp120 antibodies 17b and 48d (both directed against CD4-induced epitopes) (27) and AG1121 (directed against the third variable (V3) loop) (Immunodiagnostics) all completely blocked binding of gp120 to CXCR4. Also, the anti-CXCR4 antibody 12G5 and the CXCR4 antagonist AMD3100 (28) abolished the interaction with gp120. The anti-gp120 antibody 2G12, which is directed against a carbohydrate-dependent epitope on gp120’s outer domain (29), only partially inhibited
the CXCR4/gp120 interaction. The CD4 binding site antibody IgG1b12 (30) did not affect gp120/CXCR4 binding, but this may reflect the fact that sCD4/gp120 complexes were formed prior to the addition of antibody. Finally, the anti-gp120 antibody A32 (31) enhanced binding of gp120 to CXCR4.

These data suggest that CXCR4 is conformationally intact in the PMPLs and can bind CXCR4 antagonists. This also confirms that the gp120 interaction with CXCR4-PMPLs is specific. As was previously shown for gp120/CCR5 interaction (13,14), binding to CXCR4 can be blocked by antibodies directed against the V3 loop and CD4-induced epitopes.

**Affinity of the HIV-1 gp120/CXCR4 Interaction.** The CXCR4-PMPLs provide a unique way to measure the affinity of gp120 for CXCR4 without the complications of poor specificity seen with CXCR4-expressing cells. We used a competition assay to determine the affinity of HXBc2 gp120 for CXCR4. CXCR4-PMPLs were incubated with 0.5 nM [125I]-labeled HXBc2 gp120 (complexed with an excess of sCD4) and varying concentrations of unlabeled gp120/sCD4 complexes. The results are shown in Figure 6. Scatchard analysis demonstrated that HXBc2 gp120 binding had a dissociation constant of 200 nM. This analysis also revealed that CXCR4-PMPLs have approximately 1.1x10⁴ HIV-1 gp120 binding sites. The experiment was repeated using Cf2Th-CXCR4 cells instead of CXCR4-PMPLs, but increasing concentration of unlabeled gp120 did not result in a diminution of labeled gp120 binding (data not shown). Again, nonspecific binding of HXBc2 gp120 to cell lines prevented an assessment of gp120 binding to
CXCR4. These data allowed the determination of the affinity of gp120 for CXCR4 and support the usefulness of PMPLs as a tool to study membrane protein biochemistry.

CXCR4/gp120 Interaction is Dependent on CD4. CXCR4-PMPLs provide a unique opportunity to study many aspects of CXCR4/HIV-1 gp120 interaction. The gp120 envelope glycoprotein derived from the ADA strain of HIV-1 can bind CCR5 in the absence of CD4 at low temperature (4°C), but at 37°C, binding is dependent on CD4 (32). We questioned whether this was a property of all gp120s or only some CCR5-using envelope glycoproteins. The gp120 glycoproteins of the R5 ADA and YU2 viruses and of the X4 HXBc2 and HXBc2P 3.2 viruses were radiolabeled and incubated with CCR5- and CXCR4-PMPLs, respectively, at varying temperatures (4°C, 25°C, and 37°C). Soluble CD4 was included in some of the assays and gp120 binding was monitored by SDS-PAGE followed by autoradiography. As negative controls for the binding experiments, CXCR4-PMPLs were incubated with CCR5-using envelope glycoproteins, and CCR5-PMPLs with CXCR4-using gp120 glycoproteins. As shown in Figure 7, the ADA and YU2 gp120 glycoproteins bound CCR5-PMPLs at all these temperatures when sCD4 was present in the reaction mix. Association of R5 gp120 glycoproteins with CXCR4-PMPLs was minimal. Binding to CCR5-PMPLs was completely dependent on CD4 only at 37°C. At lower temperatures, the YU2 and ADA gp120 glycoproteins bound to CCR5-PMPLs in the absence of sCD4. This CD4-independent binding was more efficient at 4°C than 22°C. In contrast to the R5 gp120 glycoproteins, both HXBc2 and HXBc2P 3.2 gp120 glycoproteins bound CXCR4 PMPLs only when sCD4 was present, irrespective of temperature. Binding of the X4 gp120 glycoproteins to CXCR4-PMPLs in the absence of sCD4 was equivalent to that seen on the CCR5-PMPL negative control.
These data demonstrate that, although R5 gp120 glycoproteins do not require CD4 for CCR5 interaction at 4°C, this is not true for X4 gp120 glycoproteins.
DISCUSSION

Here we describe human CXCR4 overexpression, native purification, and reconstitution into PMPLs. CXCR4 in cell lysates and PMPLs was able to bind the SDF-1α chemokine and conformation-dependent antibodies. Importantly, the CXCR4-PMPLs could specifically bind the gp120 glycoproteins from CXCR4-using (X4) HIV-1 isolates, but did not bind gp120 from CCR5-using (R5) isolates. X4 gp120 binding to CXCR4-PMPLs could be blocked by AMD3100, a small molecular weight compound that targets CXCR4 (28). Parallel comparisons of the binding of conformation-dependent antibodies and SDF-1α to CXCR4-expressing cells and CXCR4-PMPLs indicate that the affinities of these ligands for CXCR4 in these two contexts are indistinguishable. The efficient binding of four different kinds of ligands to CXCR4-PMPLs strongly suggests that the CXCR4 protein in the PMPL retains a native conformation.

A few modifications of the original approach previously used to create CCR5-PMPLs were introduced into our preparation of CXCR4-PMPLs. First, the Cymal™-5 detergent that allowed native CCR5 solubilization and incorporation into PMPLs apparently denatured CXCR4. CHAPSO was found to retain the native conformation of both CXCR4 and CCR5. Because of its high critical micelle concentration, CHAPSO can be rapidly removed by dialysis; the concomitant decrease in the time that the solubilized protein remains outside the lipid bilayer should reduce the possibility of denaturation. CHAPSO may be a useful detergent for solubilization and/or membrane reconstitution of other GPCRs. Second, the Dynabeads were not conjugated with biotin, and streptavidin-conjugated phosphoethanolamine (PE) was not included in the lipid mix. This step was
included in our original protocol so as to provide additional anchorage of the lipid bilayer to the bead surface. Empirical studies, however, revealed little difference in the behavior of the CCR5-PMPLs and CXCR4-PMPLs prepared with and without this step. Therefore, we created all of the PMPLs reported in this paper without a biotin-streptavidin bridge between bead and lipid bilayer.

We were unable to demonstrate the specific binding of gp120 from X4 HIV-1 strains to cells expressing CXCR4. Using FACS and radiolabeled gp120 binding analyses, we consistently observed moderate, equivalent HXBc2 gp120 binding to both CCR5- and CXCR4-expressing Cf2Th cells, and to other cell types. This binding could not be competed with anti-gp120 or anti-CXCR4 antibodies. In contrast, binding of R5 gp120 glycoproteins to cells has been shown to be highly dependent on the presence of CCR5 on the cell surface (13,14). The reason for the high nonspecific binding of the HXBc2 gp120 glycoprotein to cells is unclear. The strong structural similarity of the gp120 cores of R5 and X4 viruses suggests that such differences in binding cells are probably determined by the composition of the gp120 variable loops. The loops of the X4 virus gp120 glycoproteins have been suggested to possess more basic residues than the corresponding loops of the gp120 glycoproteins from R5 viruses (33). Highly charged and abundant proteoglycans on the cell surface could attract the more basic X4 gp120 molecules. Indeed, peptides corresponding to the V3 loop of the X4 gp120 glycoproteins have been reported to bind mammalian cell surfaces (34). Our observation that using purified CXCR4 in the PMPLs eliminates this background binding indicates that protein or carbohydrate moieties on the cell surface that are not incorporated into PMPLs are likely responsible for the observed binding of X4 gp120 glycoproteins.
The PMPLs proved to be useful in determining the affinity of the HXBc2 gp120/CXCR4 interaction. The calculated dissociation constant of approximately 200 nM is nearly 100 fold lower than previous estimates of R5 HIV-1 gp120 binding to CCR5 (13). Our estimate of the dissociation constant is consistent with an independent approximation of HIV-1 gp120/CXCR4 binding affinity obtained by using virion-like particles that incorporate CXCR4 along with other cell surface entities as targets for gp120 binding (35). Thus, gp120s, even from more efficient X4 HIV-1 isolates, probably bind their coreceptor much less efficiently than do R5 HIV-1 envelope glycoproteins. This could, in part, account for the preference of clinical HIV-1 isolates to use CCR5 as a coreceptor. The ability of X4 HIV-1 to replicate efficiently even with a lower coreceptor binding affinity could be explained by higher surface densities of CXCR4 compared with CCR5 or by auxiliary compensatory factors. Perhaps the nonspecific cell surface binding observed for X4 HIV-1 gp120 glycoproteins, which could assist in the process of HIV-1 attachment, represents an example of the latter.

The binding of HIV-1 envelope glycoproteins to the CCR5 chemokine receptor requires prior CD4 binding at 37°C (32). However, at 4°C, the ADA R5 HIV-1 gp120 was shown to bind CCR5 efficiently in the absence of CD4 (32). Here we show that another R5 HIV-1 gp120 from the YU2 primary isolate can also bind CCR5 efficiently in the absence of sCD4 at 4°C, but not at 37°C. Thus, R5 gp120 glycoproteins may generally be able to achieve a conformation capable of binding CCR5 in the absence of CD4 at 4°C. Our previous studies of CD4-independent ADA viruses suggested that some of the temperature-dependent effects on CCR5 binding were regulated by the presence of the V1/V2 gp120 variable loops (32). This dependence on variable loops may explain
why CXCR4 binding does not become CD4-independent at low temperature. There are likely to be significant differences in loop configuration between R5 and X4 HIV-1 gp120 glycoproteins and therefore differences in loop-dependent phenotypes are not unexpected. The full meaning of these differences to the biology of R5 and X4 viruses requires further investigation.

Our studies also revealed similarities in the gp120 regions involved in binding CCR5 and CXCR4. The binding of the R5 and X4 gp120 glycoproteins to the respective chemokine receptor was blocked by antibodies against the gp120 V3 loop and CD4-induced epitopes. Mutagenic analysis and studies of the binding of sulfated N-terminal CCR5 peptides indicate that a highly conserved region of gp120, overlapping the CD4-induced epitopes, is involved in CCR5 interaction (36). Changes in this region were observed upon the generation of CD4-independence in an X4 HIV-1 isolate (37), so the same region may participate in CXCR4 binding. The V3 loop appears to be a major determinant in the choice of chemokine receptor used by an HIV-1 isolate (13,38,39). The establishment of a robust assay for gp120/CXCR4 binding through the use of CXCR4-PMPLs should allow mutagenic mapping of the precise gp120 residues involved in the interaction.

The IgG1b12 antibody, which recognizes the CD4 binding site on gp120 (30), exhibited little effect on gp120 binding to CXCR4-PMPLs. This lack of effect probably results from the experimental protocol, where gp120/sCD4 complexes were allowed to form prior to the addition of competing antibody. The slightly enhancing effects of the A32 antibody on gp120/sCD4 complex binding to CXCR4-PMPLs are worthy of note. The A32 antibody has been shown to induce exposure of the gp120 epitopes for the CD4-
induced antibodies (31). Thus, A32 binding may exert a similar effect on the exposure of the CXCR4-binding site. These results support the proximity of the CD4-induced gp120 epitopes, one of which has been resolved crystallographically (40,41), and the CXCR4-binding site.

PMPLs containing CCR5 and CXCR4 will no doubt be useful in characterizing the binding characteristics of these key HIV-1 receptors. PMPLs may also facilitate the discovery of new ligands and potential inhibitors of CCR5, CXCR4 and possibly other G protein-coupled receptors.

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Fig. 1. **Retention of CXCR4 in Native Conformation in Cell Lysates.**

**A.** Cf2Th-CXCR4 cells were metabolically labeled for 24 hours, lysed in buffer containing CHAPSO, and precipitated with either 1D4, 12G5, 44708.111 or 44716.111 antibodies. The 1D4 antibody precipitates all CXCR4 in the lysate, whereas the 12G5, 44708.111 and 44716.111 antibodies only precipitate conformationally intact CXCR4. **B.** CXCR4 contained in cell lysates was used to precipitate radiolabeled SDF1α-Ig. Cf2Th-CXCR4 or Cf2Th-CCR5 cells were lysed and radiolabeled SDF1α-Ig fusion protein and 1D4-Sepharose were added to the cell lysates. Labeled SDF1α-Ig was only precipitated in the presence of CXCR4-containing cell lysates. The SDF1α-Ig +C lane represents the total SDF1α-Ig protein used in the assay as determined by precipitation with Protein A-Sepharose. **C.** SDF1α-Ig was used to precipitate radiolabeled CXCR4 from cell lysates. Cf2Th-CXCR4 cells were metabolically labeled for 24 hours and lysed. CXCR4 was precipitated with 1D4, 12G5, or SDF1α-Ig. As a negative control, we used a protein (Ig construct) consisting of the same Fc domain as that found in the SDF1α-Ig fusion protein.
Fig. 2. **Binding of Conformation-Dependent Antibodies to CXCR4-Expressing cells and CXCR4-PMPLs.**

**A.** CXCR4-PMPLs were stained with either 12G5-PE (black peak) or 44717.111-PE (gray peak). Unstained CXCR4-PMPLs are shown as a white peak. The fluorescence intensities for 12G5 and 44717.111 were 100- and 200-fold, respectively, over that of the unstained cells. **B.** Varying concentrations of either 12G5 (upper panel) or 44717.111 (lower panel) were added to either CXCR4-PMPLs (open circles) or Cf2Th-CXCR4 cells (filled diamonds). All points were plotted by normalizing the maximum fluorescence intensity seen to 100%.
Fig.3. Binding of SDF-1α to CXCR4-PMPLs and CF2Th-CXCR4 Cells. [125I]-SDF-1α (0.1 nM) was incubated with CXCR4-PMPLs (white circles), CCR5-PMPLs (gray triangles), or Cf2Th-CXCR4 cells (black diamonds) in the presence of varying amounts of unlabeled SDF-1α for 1 hour at 37°C. Tubes were washed three times and read on a gamma counter. The X-axis represents the concentration of unlabeled SDF-1α added and the Y-axis represents the percent of counts seen as compared to the counts observed for the sample containing no unlabeled SDF-1α.
Fig. 4. Binding of HIV-1 gp120 (HxBc2) to CXCR4-PMPLs and Cf2Th-CXCR4 Cells. A. 293T cell supernatants containing radiolabeled gp120 were harvested after 72 hours of labeling. Cf2Th-CCR5, Cf2Th-CXCR4 cells, CCR5-PMPLs, and CXCR4-PMPLs were resuspended in the gp120-containing culture supernatants and incubated in the presence of 10 µg/ml sCD4 for 2 hours at 37°C. Samples were washed, SDS-PAGE performed, and gels visualized by autoradiography. B. Cf2Th-CXCR4 (black peak) or Cf2Th-CCR5 (gray peak) cells were incubated with 100 nM HxBc2/sCD4 complexes and stained using the anti-gp120 antibody C11 followed by a PE-conjugated anti-human IgG secondary antibody (upper panel). The unstained cells are indicated by the white peak. The same was performed for CXCR4-PMPLs (black peak) and CCR5-PMPLs (gray peak) (lower panel). Unstained PMPLs are shown by the white peak.
Fig. 5. Effects of gp120 and CXCR4 Ligands on gp120 Binding to CXCR4-PMPLs. HXBc2 gp120/sCD4 complexes were incubated with CXCR4-PMPLs in the presence of ligands against gp120 and against CXCR4. The anti-gp120 antibodies (and their gp120 epitopes) were as follows: IgG1b12 (CD4 binding site), 2G12 (complex carbohydrate-dependent epitope), 17b (CD4-induced), 48d (CD4-induced), AG1121 (V3 loop), and A32 (C1-C4). We also used CXCR4-directed ligands: the anti-CXCR4 antibody 12G5, or the small molecule CXCR4 antagonist, AMD3100.
Fig. 6. Measurement of HXBc2 gp120 Affinity for CXCR4-PMPLs. $^{[125]}$I-labeled HXBc2 gp120 (0.5 nM) (complexed with sCD4) was incubated with CXCR4-PMPLs in the presence of varied concentrations of unlabeled gp120 (complexed with sCD4). Samples were analyzed in duplicate, washed, and read using a gamma counter. The X-axis represents the concentration of the unlabeled gp120 competitor and the Y-axis represents the percent of the value obtained for a sample incubated with no added unlabeled gp120 competitor.
Fig. 7. **CD4- and Temperature-Dependence of gp120/CXCR4 Interaction.** The CCR5-using envelope glycoproteins ADA and YU2 as well as the CXCR4-using envelope glycoproteins HXBc2 and HXBc2P 3.2 were metabolically labeled with [³⁵S]-cysteine and -methionine and incubated with either CCR5- or CXCR4-PMPLs, respectively. Incubations were carried out in the presence or absence of sCD4 at varying temperatures. Temperatures (4°C, 22°C, and 37°C) and CD4 addition (either +, sCD4 added, or −, no sCD4 added) are listed at the top of the figure. −C represents CCR5-using envelope glycoprotein added to CXCR4-PMPLs or CXCR4-using gp120 added to CCR5-PMPLs. These controls allow the assessment of the background binding of each particular gp120 to elements of the PMPL other than the specific chemokine receptor used by the virus. The right side depicts the envelope glycoprotein used as well as the type of PMPL the gp120 was incubated with in the experimental samples.
Figure 1

A

[Image of Western blot showing CXCR4 expression across different samples]

B

[Image of Western blot showing SDF1α-Ig expression across different samples]

C

[Image of Western blot showing CXCR4 expression across different samples]
% Maximal Counts Bound

Unlabeled SDF-1α (nM)
Figure 4

A

CCR5 Cells  CXCR4 Cells  CCR5 PMPLs  CXCR4 PMPLs

gp120

B

Cell Lines

PMPLs

Fluorescence Intensity
Figure 6

% Maximal Binding

Unlabeled HXBc2 gp120 (nM)
| Temp  | 4°C | 22°C | 37°C |
|-------|-----|------|------|
| CD4   | +   | -C   | +    |
|       |     | +    | -C   |
|       |     | +    | -C   |
| Envelope | PMPL | ADA | CCR5 |
| YU2   |     | CCR5 |
| HXBc2 |     | CXCR4 |
| HXBc2P 3.2 | | CXCR4 |
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