Paramagnetic proteoliposomes containing a pure, native, and oriented seven-transmembrane segment protein, CCR5

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Seven-transmembrane segment, G protein-coupled receptors play central roles in a wide range of biological processes, but their characterization has been hindered by the difficulty of obtaining homogeneous preparations of native protein. We have created paramagnetic proteoliposomes containing pure and oriented CCR5, a seven-transmembrane segment protein that serves as the principal coreceptor for human immunodeficiency virus (HIV-1). The CCR5 proteoliposomes bind the HIV-1 gp120 envelope glycoprotein and conformation-dependent antibodies against CCR5. The binding of gp120 was enhanced by a soluble form of the other HIV-1 receptor, CD4, but did not require additional cellular proteins. Paramagnetic proteoliposomes are uniform in size, stable in a broad range of salt concentrations and pH, and can be used in FACS and competition assays typically applied to cells. Integral membrane proteins can be inserted in either orientation into the liposomal membrane. The magnetic properties of these proteoliposomes facilitate rapid buffer exchange useful in multiple applications. As an example, the CCR5-proteoliposomes were used to select CCR5-specific antibodies from a recombinant phage display library. Thus, paramagnetic proteoliposomes should be useful tools in the analysis of membrane protein interactions with extracellular and intracellular ligands, particularly in establishing screens for inhibitors.

Keywords: seven-transmembrane protein, G protein-coupled receptor, CCR5, proteoliposomes, HIV-1 gp120, magnetic bead, antibody, screening assays

Seven-transmembrane segment, G protein-coupled receptors (GPCRs) represent 1–2% of the total proteins encoded by the human genome[1–3] and are important targets for pharmaceutical intervention[4–7]. Generally low levels of expression[8] and the dependence of the native conformation of GPCRs on the hydrophobic, intramembrane environment[9,10] have complicated the study of these proteins. Analysis of ligand interactions with GPCRs and screening for inhibitors of such interaction are commonly conducted using live cells or intact cell membranes[11,12]. Interpretation of these studies may be complicated by the presence of numerous cell surface proteins, many of which are expressed at much higher levels than the GPCR of interest.

The entry of human immunodeficiency virus (HIV-1) into host cells typically requires the sequential interaction of the gp120 exterior envelope glycoprotein with the CD4 glycoprotein and a GPCR of the chemokine receptor family on the cell membrane[13,14]. CD4 binding induces conformational changes in gp120 that allow high-affinity binding to the chemokine receptor[15,16]. The β-chemokine receptor CCR5 is the principal HIV-1 coreceptor used during natural infection and transmission[17–21]. Individuals with homozygous defects in CCR5 are healthy but relatively resistant to HIV-1 infection[22,23]. Thus, inhibiting the gp120–CCR5 interaction might be a useful therapeutic or prophylactic approach to HIV-1 infection.

When solubilized using specific detergent and salt conditions, human CCR5 can retain its ability to bind HIV-1 gp120–CD4 complexes and conformation-dependent monoclonal antibodies[24]. However, the limited stability of detergent-solubilized, native CCR5 renders its use in screening assays impractical. To address this issue, we devised a method whereby homogeneous, native CCR5 is affixed to the surface of a paramagnetic bead in an oriented manner. Reconstitution into a lipid bilayer allows the long-term maintenance of native CCR5 conformation.

Results

Formation of CCR5-proteoliposomes. We wished to create paramagnetic, nonporous beads surrounded by a lipid membrane containing human CCR5 in a native conformation (Fig. 1). The human CCR5 protein, which contains a C-terminal nonapeptide (C9) tag that is recognized by the 1D4 monoclonal antibody, was expressed in Cf2Th canine thymocytes[24]. Paramagnetic beads were conjugated with both the 1D4 antibody and streptavidin. The 1D4 antibody allowed simple purification and concentration of CCR5 from Cf2Th/synCCR5 cell lysates containing detergents previously shown to retain CCR5 in a native conformation[24]. The streptavidin allowed stable and saturating membrane reconstitution around the bead by the addition of detergent-solubilized lipids containing 0.1–1% Biotinyl–DOPE (see Experimental Protocol) and subsequent dialysis. A 10:1 molar ratio of 1D4 antibody to streptavidin was found to be optimal with respect to the highest density of reconstituted CCR5 and the completeness of the membrane in the paramagnetic proteoliposomes (data not shown).

Protein composition of CCR5-proteoliposomes. To examine the cellular proteins incorporated into the proteoliposomes, Cf2Th/synCCR5 cells expressing human CCR5[24] were metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine and used for proteoliposome formation. The proteoliposomes were incubated in

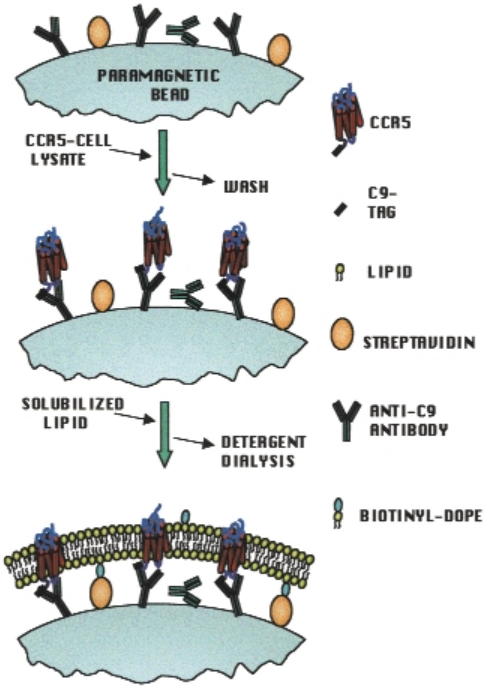

Figure 1. Schematic representation of the formation of paramagnetic CCR5 proteoliposomes. The surface of nonporous paramagnetic beads was covalently conjugated with streptavidin and an antibody that recognizes the C-terminal C9 tag on CCR5. The conjugated beads were used to capture the C9-tagged CCR5 from the cell lysate. After extensive washing, the beads were mixed with detergent-solubilized lipid containing 0.1–1% of Biotinyl-DOPE. During the removal of detergent by dialysis, the lipid bilayer membrane self-assembles around the beads and CCR5 is returned to its native environment.

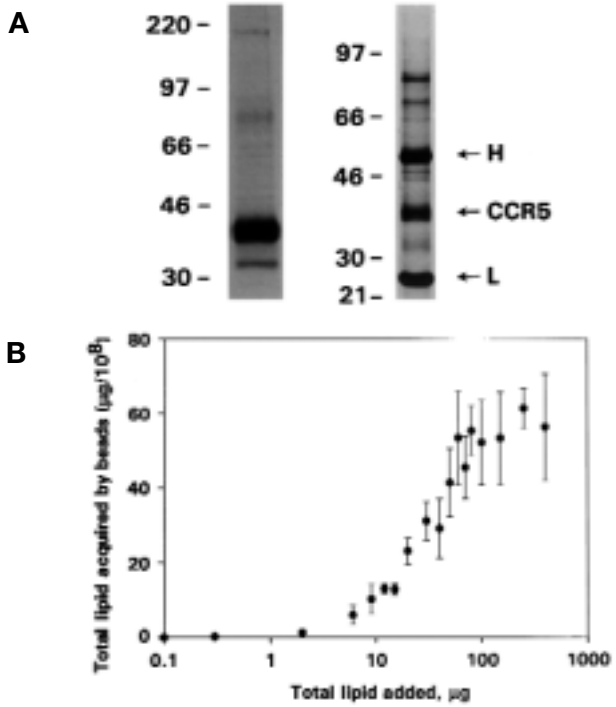

Figure 2. Protein and lipid composition of CCR5-proteoliposomes. (A) SDS–PAGE mini-gel analysis of protein composition of the CCR5 proteoliposomes. Left panel, [^{35}S]cysteine/methionine-labeled lysate from Cf2Th/SynCCR5 cells was used for CCR5 proteoliposome formation. The proteoliposomes were lysed in SDS-sample buffer and the labeled proteins analyzed by SDS-PAGE. Molecular weight markers (kDa) are shown. Right panel, approximately 10^7 CCR5 proteoliposomes were incubated in SDS–sample buffer and loaded onto an SDS–PAGE mini-gel. The gel was stained using a silver staining kit (Plus One, Pharmacia Upjohn). (B) Graphic representation of quantitation of lipid acquired by the paramagnetic CCR5 proteoliposomes. The data represent the averages derived from two independent measurements. The approximate mass of total lipid (m) necessary for complete encapsulation of a given number of beads (n) by a single lipid bilayer membrane was calculated by the formula $m = 2\,SnM/\rho N_A$. S is the estimated effective surface area of the 2.8 μm diameter Dynal bead, assuming that it is a smooth sphere. The approximate area occupied by one lipid molecule in the bilayer membrane (ρ) was considered to be 60 Å^2 (ref. 36). N_A is Avogadro's number. M is the average molecular weight of the lipids used for membrane reconstitution and was considered equal to 740.

SDS–sample buffer at 55°C for 1 h and the labeled proteins analyzed on polyacrylamide gels (Fig. 2A, left). Prominent bands associated with mature CCR5 (43 kDa) and a previously reported[24] CCR5 derivative (36 kDa) were observed, as well as faint bands associated with higher molecular weight aggregates of CCR5. Other cellular proteins were apparently present at only trace levels.

The proteins in the paramagnetic proteoliposomes were also examined by silver staining of polyacrylamide gels of the SDS lysates (Fig. 2A, right). The other major bands visible in addition to the CCR5 bands described above were those associated with the 1D4 antibody heavy and light chains (55 and 25 kDa, respectively). As expected, the 60 kDa band associated with streptavidin was less evident. Apparently, no cellular proteins other than CCR5 are incorporated stoichiometrically into the paramagnetic proteoliposomes.

Analysis of the lipid bilayer in CCR5 proteoliposomes. To obtain some insight into the nature of the lipid membrane in the CCR5 proteoliposomes, the total quantity of lipid incorporated into the proteoliposomes was determined. Analysis of CCR5 proteoliposomes formed with increasing amounts of lipid containing 1% rhodamine–DOPE revealed that 80–90 μg of lipid were acquired per 10^8 beads (Fig. 2B). This is higher than the amount of lipid (~40 μg) that is theoretically needed to form bilayers surrounding beads of 2.8 μm diameter (see formula in Fig. 2B, legend). This difference can be explained by the irregularity of the bead surface, which was documented by scanning electron microscopy (data not shown). Fluorescence-activated cell-sorting (FACS) analysis revealed a very narrow peak of fluorescence (data not shown), indicating the high degree of homogeneity of the paramagnetic proteoliposome population.

The CCR5-proteoliposomes were also studied by confocal microscopy (Fig. 3A, B). The control paramagnetic beads did not exhibit fluorescence indicative of rhodamine–DOPE incorporation. By contrast, the CCR5 proteoliposomes that had been formed with 1% rhodamine–DOPE fluoresced intensely and uniformly. No lipid vesicles or other structures >0.1 μm were observed on the surface of the fluorescently labeled CCR5 proteoliposomes. These data are consistent with the CCR5 proteoliposomes being surrounded by a single lipid bilayer membrane with at most small irregularities. Some discontinuities in the lipid bilayer probably exist, as the CCR5 proteoliposomes exhibit weak staining by anti-mouse IgG antibodies, which apparently recognize the 1D4 antibody on the bead surface (data not shown).

Ligand-binding properties of CCR5 proteoliposomes. The binding of anti-CCR5 monoclonal antibodies to the CCR5 proteoliposomes was examined. CCR5 proteoliposomes efficiently bound the 2D7 antibody (Fig. 3C, D), which recognizes a conformation-dependent epitope that includes the second extracellular loop of CCR5 (ref. 25). The recognition of CCR5 proteoliposomes by three anti-CCR5 monoclonal antibodies was equivalent to that of CCR5-expressing cells (Fig. 4). In addition to 2D7, the 3A9 antibody, which recognizes the CCR5 N terminus[25,26], and the 45523 antibody, which recognizes a discontinuous epitope dependent on multiple CCR5 extracellular loops[26], were used in this study.

To examine the ability of the CCR5 proteoliposomes to bind the HIV-1 exterior envelope glycoprotein, the gp120 glycoprotein from

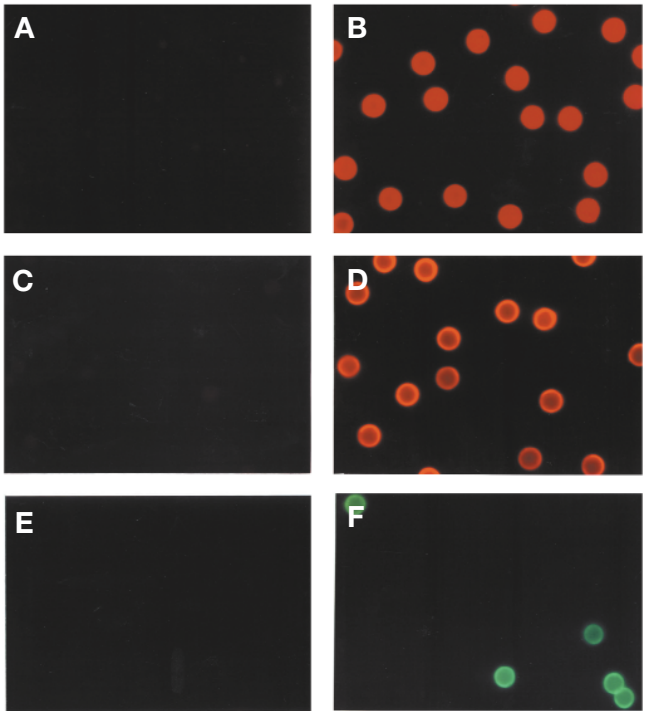

Figure 3. Confocal microscopy of fluorescently labeled CCR5 proteoliposomes. Excluding the control beads (A), all beads were reconstituted with a POPC/POPE/DMPA lipid mixture (in a 6:3:1 molar ratio) containing 1% Biotinyl-DOPE. (B) The lipid membrane surrounding the CCR5 proteoliposomes was visualized by using the fluorescent lipid Rhodamine-DOPE, which had been added at 1% concentration during proteoliposome formation. In a control experiment (C), CCR5 proteoliposomes were treated with an irrelevant antibody against CXCR4, 12G5-PE. (D) CCR5 proteoliposomes were labeled with the anti-CCR5 antibody 2D7 conjugated with phycoerythrin (2D7-PE). Control beads with membrane only (E) and CCR5 proteoliposomes (F) were incubated with the JR-FL gp120-soluble CD4 complex, the C11 antibody against gp120, and goat anti-human IgG-FITC. Samples were analyzed using the Nikon Diaphot 300 Inverted Confocal Microscope and Oncor Image Software.

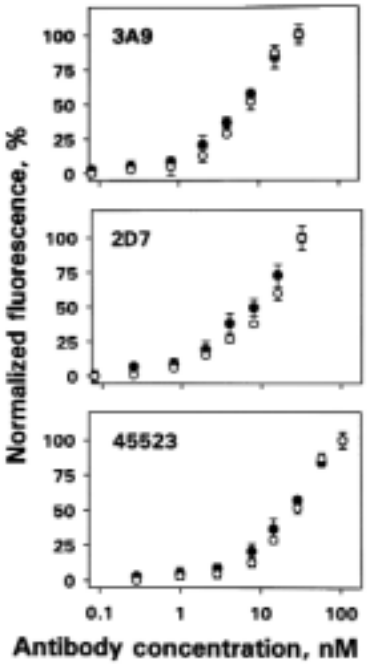

Figure 4. Binding of anti-CCR5 antibodies to CCR5 on cells and proteoliposomes. The indicated concentrations of anti-CCR5 antibodies 3A9-PE, 2D7-PE, and 45523-PE were incubated with either Cf2Th/synCCR5 cells (●) or CCR5-proteoliposomes (○) for 1 h at 22°C. The cells and proteoliposomes were then washed and analyzed by FACS. The mean fluorescent intensities for the cells and proteoliposomes were normalized to the respective values observed at the highest antibody concentrations.

the CCR5-using strain JR-FL was preincubated with a soluble form of CD4 (sCD4) to induce the high-affinity interaction with CCR5. The gp120–sCD4 complex was incubated with CCR5 proteoliposomes, after which the bound complexes were detected by the C11 anti-gp120 antibody. Binding of the gp120 glycoprotein–sCD4 complexes to the CCR5 proteoliposomes, but not to control liposomes lacking CCR5, was readily detected (Fig. 3E, F).

The binding of the HIV-1 gp120 glycoprotein to the CCR5 proteoliposomes was also examined in a different assay. Equivalent amounts of metabolically labeled gp120 glycoproteins from an HIV-1 strain, HXBc2, which does not use the CCR5 protein as a coreceptor, and from the ADA strain, which uses CCR5 as a coreceptor, were added to the CCR5 proteoliposomes. Only the ADA gp120 glycoprotein detectably bound the CCR5 proteoliposomes (Fig. 5A). This binding was enhanced by the addition of sCD4. The binding of the ADA gp120–sCD4 complex to the CCR5 proteoliposomes was inhibited by preincubation of the proteoliposomes with the 2D7 anti-CCR5 antibody. These results indicate that the gp120 glycoprotein from a CCR5-using HIV-1 specifically binds CCR5 in the proteoliposome, and that CD4 binding enhances the gp120–CCR5 interaction, as has been observed with cell surface CCR5 (refs 15,16).

To compare the binding of a chemokine to the CCR5 proteoliposomes and to CCR5-expressing cells, radiolabeled MIP-1α was incubated with target liposomes or cells in the presence of increasing concentrations of unlabeled MIP-1α (Fig. 5B). MIP-1α specifically bound to CCR5-expressing cells and not to control cells expressing another chemokine receptor, CXCR4. The binding of radiolabeled MIP-1α to the CCR5-expressing cells was subject to competition from the unlabeled MIP-1α. The specific binding of radiolabeled MIP-1α to CCR5 proteoliposomes was dependent upon the presence of up to 30 nM additional unlabeled MIP-1α. At higher concentrations, the unlabeled MIP-1α inhibited the binding of the radiolabeled MIP-1α. Control liposomes lacking CCR5 did not specifically bind radiolabeled MIP-1α. These results suggest that MIP-1α binding to CCR5 proteoliposomes is highly dependent upon ligand concentration and cooperativity. Because of the complex nature of the binding characteristics, it is difficult to assess the relative affinities of MIP-1α for CCR5 on the surfaces of cells and proteoliposomes.

Stability of CCR5 proteoliposomes. The effects of alterations in pH, ionic strength, and temperature on the stability of the CCR5 proteoliposomes were examined. Rhodamine–DOPE-labeled CCR5 proteoliposomes were exposed to acidic (pH 3) or basic (pH 10) conditions for 30 min, after which they were returned to a neutral pH environment. The fluorescence intensity measured by FACS was comparable to that observed for untreated control CCR5 proteoliposomes (data not shown). Fluorescence intensity was also unaffected by incubation in solutions of different ionic strengths, ranging from <1 mM to 4 M NaCl (data not shown). The binding of the 2D7 antibody to CCR5 proteoliposomes was completely disrupted by incubation of the antibody–proteoliposome complex at pH 3.0 for 30 min (Fig. 6A). However, the ability of the 2D7 antibody to rebind the CCR5 proteoliposomes was completely restored by returning the pH to 7.5. The CCR5 proteoliposomes were stable at room temperature for days and could be stored for at least six months in PBS +0.1% BSA at 4°C without loss of binding properties.

Screening a phage library with CCR5 proteoliposomes. To illustrate the ability of CCR5 proteoliposomes to identify CCR5-specific ligands in a complex mixture, we used the CCR5 proteoliposomes to select recombinant antibodies from a human single-chain antibody

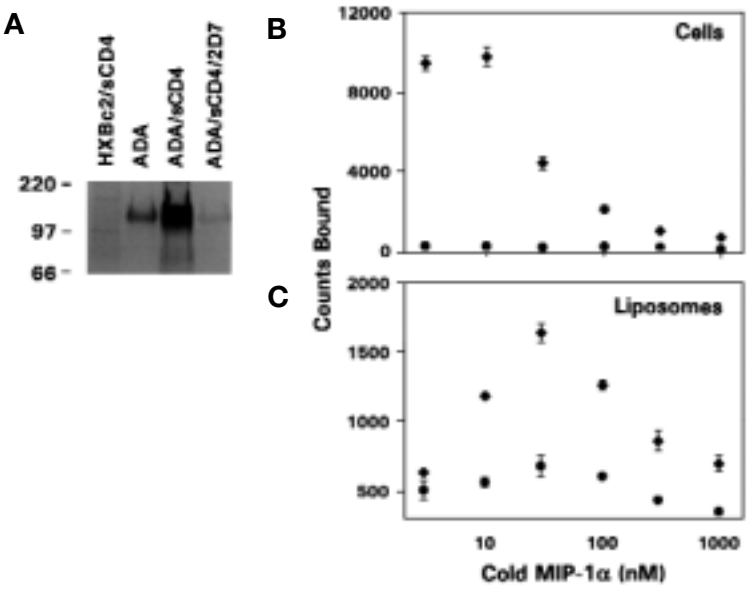

Figure 5. Ligand-binding properties of CCR5 proteoliposomes. (A) Binding of [^{35}S]cysteine/methionine-labeled gp120 to the CCR5 proteoliposomes. Equivalent amounts of [^{35}S]cysteine/methionine-labeled gp120 glycoproteins from the CXCR4-using HXBc2 isolate or the CCR5-using ADA isolate were incubated with CCR5 proteoliposomes in the absence or presence of soluble CD4 (sCD4). In one experiment, the CCR5 proteoliposomes were incubated with the 2D7 anti-CCR5 antibody before incubation with the ADA gp120–sCD4 complexes. Proteins bound to the CCR5 proteoliposomes are shown, with molecular weight markers (in kDa) indicated on the left. (B) Binding of MIP-1α to either cells (B, upper panel) or liposomes (C, lower panel). Approximately 0.1 nM ^{125}I-labeled MIP-1α was incubated with either Cf2Th/synCCR5 cells (◆) or control Cf2Th/ synCXCR4 cells (●), in A, or with CCR5 proteoliposomes (◆) or liposomes lacking CCR5 (●), in B, in the presence of the indicated concentrations of unlabeled MIP-1α. Bound counts are indicated.

phage display library of ~1.5×10^{10} members (S. Mehta et al., unpublished data). After three rounds of panning, the phage were counterselected with control liposomes lacking CCR5. After four rounds of panning, 96 of the selected phage were examined for the ability to bind CCR5- and CXCR4-expressing cells. Of the 96 phage, 19 specifically bound CCR5-expressing cells. After five rounds of panning, 49 out of 96 phage tested specifically bound CCR5-expressing cells. Several single-chain antibodies from the selected phage were synthesized and exhibited the ability to bind native CCR5 expressed on the surface of the cells (H. Kontos et al., unpublished data). Figure 6B shows the binding of one selected single-chain antibody to Cf2Th/synCCR5 cells but not to the parental Cf2Th cells. Thus, the CCR5 proteoliposomes can select ligands that react specifically with native CCR5 from a complex mixture of potential ligands.

Discussion

We have previously reported that the GPCR CCR5 can be expressed at reasonably high levels in mammalian cells and purified in its native state in detergent-containing solutions[27]. Here we show that the purified CCR5 can be reconstituted into a native lipid membrane environment formed on the surface of paramagnetic beads. With minor adjustments, our approach should be applicable to many integral membrane proteins, such as other GPCRs, transporters, and ion channels. The method for the production of CCR5-proteoliposomes allows purification of the protein before reconstitution into lipid membranes. This feature permitted us to examine HIV-1 gp120 binding to essentially pure CCR5 in the proteoliposomal membrane. No other cellular protein appeared to be required in stoichiometric amounts for the efficient binding of gp120 to CCR5, other than the sCD4 glycoprotein.

The purity of the reconstituted protein is advantageous for the use of the paramagnetic proteoliposomes to identify ligands from libraries of recombinant proteins, peptides, or chemical compounds. The presence of pure protein antigen in the paramagnetic proteoliposomes might also promote the generation of more specific immune responses in animals immunized with these preparations.

The purity of the reconstituted membrane protein in the proteoliposomes may be a disadvantage for some applications. For example, if the binding of a ligand of interest is dependent upon other cellular moieties, studies with proteoliposomes may imperfectly mimic the binding events that occur at the surface of a cell. Although HIV-1 gp120 and conformation-dependent anti-CCR5 antibodies bound CCR5 proteoliposomes efficiently, some differences in the binding of the β-chemokine, MIP-1α, to CCR5 on the surface of cells and proteoliposomes were observed. It has been suggested that the efficient binding of some ligands to their GPCRs can be influenced by proteoglycans on the cell surface[27–29] or by dimerization[10,30] or G protein coupling of the receptor[6,10].

The concentration and orientation of the reconstituted protein in the paramagnetic proteoliposomes can be manipulated. The concentration of the reconstituted protein is determined by the density of the conjugated capture antibody on the bead surface and the concentration of the protein of interest in the cell lysates. The conjugation to

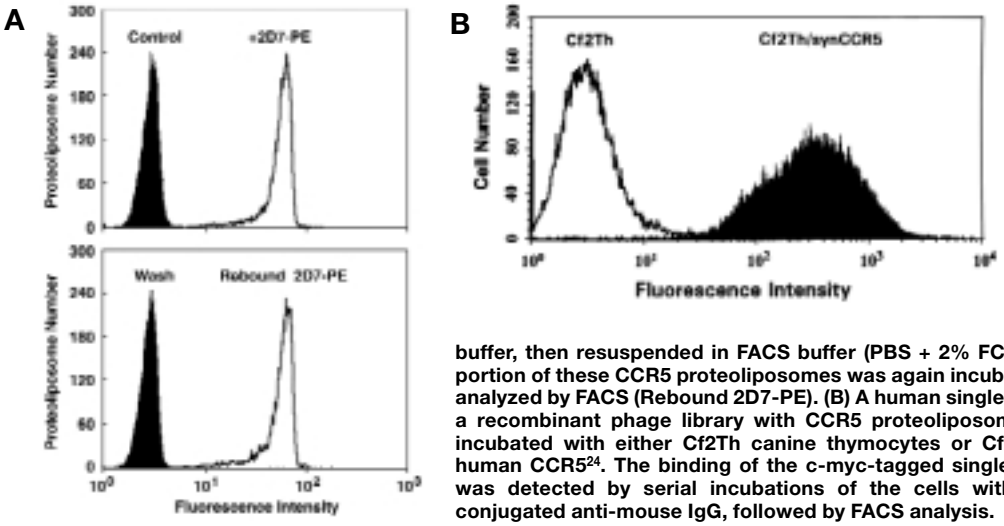

Figure 6. FACS analysis of antibody binding to CCR5. (A) Reversible binding of the conformation-dependent antibody 2D7 to CCR5 proteoliposomes. CCR5 proteoliposomes were incubated for 1 h at 22°C with an irrelevant control antibody, IgG-PE (Control), or with the phycoerythrin-conjugated 2D7 antibody against CCR5 (+2D7-PE). A fraction of the proteoliposomes with bound 2D7-PE was incubated for 30 min in 100 mM glycine-HCl (pH 3.0), washed twice in the same buffer, then resuspended in FACS buffer (PBS + 2% FCS) and analyzed by FACS (Wash). A portion of these CCR5 proteoliposomes was again incubated with 2D7-PE for 1 h at 22°C and analyzed by FACS (Rebound 2D7-PE). (B) A human single-chain antibody selected by panning a recombinant phage library with CCR5 proteoliposomes was synthesized, purified, and incubated with either Cf2Th canine thymocytes or Cf2Th/synCCR5 cells, which express human CCR5[24]. The binding of the c-myc-tagged single-chain antibody to the cell surface was detected by serial incubations of the cells with mouse anti-c-myc IgG and PE-conjugated anti-mouse IgG, followed by FACS analysis.

the beads of particular antibodies that recognize the extracellular or intracytoplasmic portions of the reconstituted protein of interest allow its orientation in the proteoliposome membrane. Most of the studies described herein used the 1D4 antibody against the CCR5 C-terminal epitope tag to orient CCR5; however, we have also conjugated the 2D7 antibody to the bead surface, allowing an inside-out orientation of CCR5 in the proteoliposome (data not shown). Thus, the binding of intracellular as well as extracellular ligands can be studied.

The integrity of the conformation-dependent CCR5 epitope recognized by the 2D7 antibody was preserved after exposure of the CCR5 proteoliposomes to harsh conditions (high or low pH, extremes of ionic strength, ranges of temperature, 0–50°C). Because most of these conditions have been shown to denature detergent-solubilized CCR5[24], the observed conformational stability indicates that CCR5 in the proteoliposomes is in an environment, presumably within the lipid membrane, that is strongly conducive to the preservation of native structure. This property allows the rapid exchange of external buffers that is useful for functional studies of several types of integral membrane proteins, as well as long-term storage of the proteoliposomes.

Other approaches to immobilizing GPCRs on solid surfaces have been devised. Small amounts of rhodopsin have been reconstituted in a membrane supported on a surface plasmon resonance (SPR) chip[31–33]. For this purpose, rhodopsin exhibits several advantages over most GPCRs, including a high natural level of expression, stability during purification, covalent binding of a small ligand (*cis*-retinal), and an identified and available G protein, transducin. Although SPR-based approaches will be useful in certain instances, it should be possible to apply the paramagnetic proteoliposome technology to any protein predicted to reside in a membrane. Information on the protein's ligands, orientation, and configuration in the membrane is not required for the production of paramagnetic proteoliposomes. In fact, by using epitope tags to capture and monitor expression of the protein of interest, it is possible to generate paramagnetic proteoliposomes with no information other than the primary amino acid sequence of the protein. Thus, paramagnetic proteoliposome technology could provide a useful path from cDNA identification to understanding protein function by furnishing adequate amounts of native membrane proteins for characterization of ligands, generation of antibodies, and other applications.

Experimental protocol

CCR5-expressing cells. A cell line (Cf2Th/synCCR5) stably expressing human CCR5 was used in these studies[24]. The C terminus of the expressed CCR5 consists of a glycine residue followed by the C9 nonapeptide TETSQVAPA, which contains the epitope for the 1D4 antibody[34]. Wild-type and C-terminally tagged CCR5 molecules have been shown to be functionally comparable[24]. Cf2Th/synCCR5 cells grown to full confluency in 150 mm dishes were harvested using 5 mM EDTA in PBS, washed in PBS, pelleted, and frozen until needed.

Radiolabeling of cells expressing CCR5 or gp120. Cf2Th/synCCR5 cells were radiolabeled in 150 mm dishes for 12 h with 10 ml/dish of methionine/cysteine-free DMEM supplemented with 400 μCi each of [^{35}S]methionine and [^{35}S]cysteine (NEN Life Science Products, Boston, MA). Labeled cells were harvested using 5 mM EDTA in PBS, pelleted, and frozen until needed.

To label the HIV-1 gp120 envelope glycoprotein, HEK-293T cells (American Type Culture Collection) grown to 70–80% confluence were transfected (GenePorter transfection reagent; Gene Therapy Systems, San Diego, CA) with plasmids expressing secreted gp120 from HIV-1 strains ADA and HXBc2. The medium was replaced 24 h after the transfection with labeling medium, as described above. The cell supernatants containing [^{35}S]cysteine/methionine-labeled gp120 were harvested every 48 h, a total of three times. The labeled gp120 was purified from the pooled supernatants using a Protein A Sepharose-F105 antibody column, as described[15].

Coating of Dynabeads M-280 by antibodies and streptavidin. Tosyl-activated Dynabeads M-280 (Dynal, Inc., Lake Success, NY) were conjugated with 1D4 antibodies (National Cell Culture Center, Minneapolis, MN), and streptavidin (Vector Laboratories, Inc., Burlingame, CA) at a molar ratio of 10:1 unless specifically mentioned. Approximately 6×10^8 beads in 1 ml volume were vortexed, pelleted on a magnetic separator (Dynal), and resuspended in 1 ml of 0.1 M sodium phosphate, pH 7.4 containing 1 mg of 1D4 antibody and 40 μg of streptavidin. After incubation on a rocking platform for 20 h at 37°C, the unbound surface reactive groups on the beads were inactivated by treatment with 0.2 M Tris-HCl (pH 8.5) for 4 h at 37°C. The non-covalently absorbed proteins were removed by a 1 h incubation in medium composed of 1% cyclohexyl-pentyl-β-D-maltoside (Cymal-5) detergent (Anatrace, Maumee, OH), 20 mM Tris-HCl (pH 7.5), 100 mM $(NH_4)_2SO_4$, and 1M NaCl. Then the 1D4–streptavidin-beads were washed twice and stored at 4°C in PBS. The efficiency of antibody conjugation to the beads, which was estimated by FACS using anti-mouse R-phycoerythrin-conjugated IgG (IgG-PE) (Boehringer Mannheim, Indianapolis, IN), was approximately 5×10^4-10^5 antibody molecules/bead. The 2D7–streptavidin conjugation was accomplished using the same protocol.

Preparation of lipid solutions for liposomal membrane reconstitution. All lipids were obtained as chloroform solutions from Avanti Polar Lipids (Alabaster, AL). A total of 10 mg of chloroform-dissolved lipids (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and dimyristoylphosphatidic acid (DMPA)), mixed in a molar ratio of 6:3:1, were dried in a 2 ml polyethylene tube under a vacuum until all of the solvent was removed. One milliliter of PBS was added to the tube, and a liposomal solution was obtained by 1–2 min ultrasonication in an ice bath using the Ultrasonic Processor (Heat Systems, Inc., Farmingdale, NY). Liposomal solutions of total lipids from membranes of Cf2Th cells, which was extracted with chloroform–methanol[35], were prepared similarly, using a final lipid concentration of 10 mg ml^{-1}. Liposomal solutions of the head group-modified synthetic lipids 1,2-dioleyl-*sn*-glycero-3-phosphoethanolamine-n-(biotinyl) (Biotinyl-DOPE) and dioleoylphosphoethanolamine-lissamine rhodamine B (Rhodamine-DOPE), at a final concentration of 1 mg ml^{-1}, were prepared separately using the same protocol. All liposomal solutions were kept in liquid N_2 until use.

Formation of proteoliposomes with purified CCR5. Approximately 10^8 Cf2Th/CCR5 cells were lysed in 10 ml of solubilization buffer (S-buffer) composed of 100 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 7.5), 10% glycerol, 1% (wt/vol) Cymal-5, and Protease Inhibitor Mixture (one tablet of Complete (Boehringer Mannheim) per 50 ml) for 30 min at 4°C. Cell debris was removed by 30 min centrifugation at 150,000 *g*. Approximately 5×10^8 1D4–streptavidin-coated beads washed in S-buffer were added to the cleared cell lysate and incubated in it for 3 h at 4°C on a rocking platform. The CCR5-bound beads were then removed from the cell lysate and extensively washed in S-buffer. For formation of the lipid membrane around the CCR5-containing beads, 1 mg of liposomes composed of either synthetic lipid mixtures or Cf2Th cellular lipids was combined with 10 μg of liposomes made from Biotinyl-DOPE and solubilized in 1 ml S-buffer. When fluorescent labeling of the lipid membrane was desired, 10 μg of Rhodamine-DOPE was added to the mixture. This detergent-containing mixture was added to CCR5-containing beads and, after 1 h incubation at 4°C, the detergent was slowly removed by dialysis for 24 h at 4°C against 100 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 7.5), and 10% glycerol, using a 10 kDa molecular weight cutoff (Slide-A-Lyzer 10 K; Pierce, Rockford, IL). The excess of unbound lipid and residual detergent was removed on a magnetic separator and proteoliposomes were stored in PBS with 0.1% BSA at 4°C for up to six months.

The protein composition of CCR5 proteoliposomes was analyzed by silver staining or, when [^{35}S]cysteine/methionine-labeled CCR5 was used, by autoradiography. For these purposes, $1–3 \times 10^7$ proteoliposomes were resuspended in 2% SDS–sample buffer and, after 1 h incubation at 55°C, the eluted sample was run on an 11% SDS–PAGE mini-gel under reducing conditions. The gel was treated for 1 h with Enhance (NEN), dried, and autoradiographed.

Analysis of the lipid bilayer in CCR-5 proteoliposomes. Approximately 10^8 1D4–streptavidin-conjugated beads were reconstituted with CCR5 and different quantities of lipids. The lipid mixtures contained POPC/POPE/DMPA in a 6:3:1 molar ratio, as well as 1% each (by weight) of Biotinyl-DOPE and Rhodamine-DOPE. After separation from unbound lipid and washing with PBS, the proteoliposomal membranes were dissolved in 0.5 ml of S-buffer and separated from the beads. According to FACS analysis, removal of the membrane from the beads was complete (excluding, probably, the small amount of biotinyl-DOPE bound to streptavidin). The light absorption by resolubilized lipid samples at 550 nm, the wavelength of rhodamine lissamine B excitation, was measured. The total amounts of lipids in the samples were estimated using the calibrated light absorptions observed for solubilized standard concentrations of the same lipid mixtures (Fig. 2B).

Ligand binding to CCR5-proteoliposomes. The binding of anti-CCR5 antibodies was analyzed by FACS and confocal microscopy, using R-phycoerythrin (PE)-conjugated antibodies 3A9, 2D7 (both from Pharmingen, San Diego, CA) and 45523 (R&D Systems, Minneapolis, MN). CCR5 proteoliposomes were suspended in 2% fetal calf serum (FCS) in PBS or, in some experiments, in binding buffer (50 mM HEPES-NaOH (pH 7.5), 2% dialyzed FCS (Gibco BRL, Grand Island, NY), 1 mM $CaCl_2$, 5 mM $MgCl_2$, and a 1:800 dilution of MegaBlock (Cell Associates, Houston, TX)), and incubated with fluorescently labeled antibodies for 1 h at 22°C. The proteoliposomes were then washed in the same buffer, fixed in 0.2% formaldehyde in PBS, and analyzed by FACS or confocal microscopy.

The binding of the HIV-1 gp120 glycoprotein to CCR5 proteoliposomes was analyzed by FACS using unlabeled gp120 (JR-FL strain) or by SDS–PAGE analysis of bound, radiolabeled gp120 proteins. For the FACS analysis, CCR5 proteoliposomes were suspended in 0.1 ml binding buffer and incubated for 1 h at 22°C with 3–5 μg JR-FL gp120 or with JR-FL gp120 that had been preincubated for 1 h at 37°C with an equimolar concentration of sCD4. Afterwards, the C11 anti-gp120 antibody (kindly provided by Dr. James Robinson, Tulane University) and a fluorescein-conjugated goat anti-human IgG (Pharmingen) were added, each at a final concentration of 3–5 μg ml^{-1}. Following incubation at 22°C for 1 h, the CCR5 proteoliposomes were washed in the binding buffer, fixed in 2% formaldehyde in PBS, and used for FACS and confocal microscopy.

For the studies of radiolabeled HIV-1 gp120 binding to CCR5 proteoliposomes, the metabolically labeled gp120 glycoproteins from a CCR5-using HIV-1 strain, ADA, and from a CXCR4-using HIV-1 strain, HXBc2, were employed. The gp120 glycoproteins were incubated in either the presence or absence of sCD4 (10 nM final concentration) for 1 h at 37°C. Approximately 10^7 CCR5 proteoliposomes were resuspended in 1 ml of binding buffer and incubated with the gp120 glycoproteins for 1 h at 22°C. The proteoliposomes were extensively washed in the binding buffer and then resuspended in SDS–sample buffer containing 5% β-mercaptoethanol. After boiling for 2 min, the samples were loaded on 10% polyacrylamide mini-gels and analyzed by autoradiography.

Approximately 10^6 Cf2Th/synCCR5 or control Cf2Th/synCXCR4 cells, or 10^7 CCR5 proteoliposomes or control liposomes, were incubated in binding buffer (0.1 ml total volume) supplemented with 0.1 nM ^{125}I-MIP-1α (NEN), and different concentrations of unlabelled MIP-1α (R&D Systems) for 1 h at 37°C. After three washes with 0.5 ml binding buffer, the counts bound to the cells or liposomes were measured using a γ-counter.

Identification of a CCR5-specific single-chain antibody. The paramagnetic CCR5 proteoliposomes were used to screen a human single-chain antibody phage display library of approximately 1.5×10^{10} members(S. Mehta et al., unpublished data; H. Kontos, unpublished data). After the fourth and fifth rounds of panning, 96 selected individual phage were tested for ability to bind Cf2Th/synCCR5 cells relative to binding of Cf2Th cells. Selected single-chain antibodies containing His_6 and c-myc tags were produced in *Escherichia coli*, passed over nickel–nitrilotriacetic acid agarose columns, and eluted with EDTA. The single-chain antibodies (at a concentration of ~30 nM) were incubated with either Cf2Th/synCCR5 cells or the parental Cf2Th cells for 1 h on ice. After washing once with 2% BSA in PBS, the cells were incubated with mouse anti-c-myc IgG followed by anti-mouse PE-conjugated IgG and analyzed by FACS.

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