Enhanced Expression, Native Purification, and Characterization of CCR5, a Principal HIV-1 Coreceptor*

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Tajib Mirzabekov‡§, Norbert Bannert‡§, Michael Farzan‡§, Wolfgang Hofmann‡§, Peter Kolchinsky‡§, Lijun Wu†, Richard Wyatt‡§, and Joseph Sodroski‡§**

From the ‡Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, the §Department of Pathology, Harvard Medical School, the ¶Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, Massachusetts 02115, and ¶Leukosite, Inc., Cambridge, Massachusetts 02142

Seven-transmembrane segment, G protein-coupled receptors (GPCRs) play important roles in many biological processes in which pharmaceutical intervention may be useful. High level expression and native purification of GPCRs are important steps in the biochemical and structural characterization of these molecules. Here, we describe enhanced mammalian cell expression and purification of a codon-optimized variant of the chemokine receptor CCR5, a GPCR that plays a central role in the entry of the human immunodeficiency virus-1 (HIV-1) into immune cells. CCR5 could be solubilized in its native state as determined by its ability to be precipitated by 2D7, a conformation-dependent anti-CCR5 antibody, and by the HIV-1 gp120 envelope glycoprotein. The 2D7 antibody recognized immature and mature forms of CCR5 equally, whereas gp120 preferentially recognized the mature form, a result that underscores a role for posttranslational modification of CCR5 in its HIV-1 coreceptor function. The methods described herein contribute to the analysis of CCR5 and are likely to be applicable to many other GPCRs.

The entry of human immunodeficiency virus-1 (HIV-1) into host cells usually requires the sequential interaction of the gp120 envelope glycoprotein with the CD4 coreceptor and a chemokine receptor on the cell membrane (1, 2). CD4 binding facilitates virus attachment to the cell surface and mediates conformational changes in gp120 that allow a high affinity interaction with the chemokine receptor (3, 4). Chemokine receptor binding is believed to trigger further conformational changes in the viral envelope glycoproteins that allow the gp41 transmembrane envelope glycoprotein to fuse the viral and host cell membranes (5, 6).

Chemokine receptors are members of the large family of seven-transmembrane segment, G protein-coupled receptors (GPCRs). The β-chemokine receptor CCR5 is the principal HIV-1 coreceptor used during natural infection (7–11). Individuals with genetic defects in CCR5 expression are relatively resistant to HIV-1 infection (12, 13). Some HIV-1 isolates can be adapted in tissue culture to replicate on cells lacking CD4 (14, 16), but binding to either CCR5 or to the other common HIV-1 coreceptor, CXCR4 (17), is essential for the entry of these viruses. The necessity of the HIV-1 gp120-chemokine receptor interaction to virus replication makes an understanding of the structural basis of this binding a high priority. Structures of unbound CD4 (18–20), an HIV-1 gp120 derivative complexed with CD4 (21, 22), and segments of the HIV-1 gp41 ectodomain (23–25) have been resolved by x-ray crystallography. Resolution of the structure of CCR5, either alone or in a complex with HIV-1 gp120, would provide information important for guiding attempts at intervention.

The GPCRs play central roles in a wide variety of physiological, neurological, and immunological processes and represent major targets for current pharmaceutical therapies (26–28). Nonetheless, structural information on this large family of proteins is very sparse. Generally low levels of expression and the dependence of the native conformation of these proteins on the hydrophobic, intramembrane environment have complicated attempts to study GPCR structure. With the exception of the light-sensitive opsins found in certain bacteria and in the retinae of higher organisms, most GPCRs are naturally expressed at low levels (29, 30). No system has reproducibly resulted in levels of protein expression suitable for the purification of most GPCRs (31). Bacterial, yeast, or insect cell expression of GPCRs can result in protein misfolding, aggregation, and heterogeneity (32). Furthermore, some GPCRs, such as CCR5, require posttranslational modification, which may exhibit significant differences in nonmammalian cell types, for efficient function (33). Irreversible protein denaturation occurring during the solubilization of the cell membrane, a process required for purification of the GPCR, represents another obstacle to biochemical, biophysical, and structural studies.

Here, we report the development of a system for efficient expression of CCR5, and the identification of an approach that allows solubilization and purification of CCR5 in its native state. We demonstrate a cell-free association between CCR5 and the HIV-1 gp120 glycoprotein. These methods should facilitate the identification of small molecules that block the HIV-1-CCR5 association, the determination of CCR5 structure, and the study of other GPCRs.

EXPERIMENTAL PROCEDURES

Construction and Expression of Codon-optimized CCR5 (synCCR5)—The analysis of codon usage for 45 GPCRs representing different pro-
tein subfamilies was performed with GenBank™ data and software developed by the University of Wisconsin Genome Sequence Group. The sequence encoding human CCR5 was optimized for mammalian cell codon usage (28), utilizing the following codons: alanine (GCC), arginine (CGG), asparagine (AAC), aspartic acid (GAC), cysteine (TGC), glutamine (CAA/CAG), glutamic acid (GAG), glycine (GCG), histidine (CAC), isoleucine (ATT), leucine (AAG), methionine (ATG), phenylalanine (TTT), proline (GCC), serine (TCT), threonine (ACC), tryptophan (TGG), tyrosine (TAC), and valine (GTG). The 5' and 3' sequences flanking the CCR5 coding sequence were modified. Following restriction sites for EcoRv, EcoRI and HindIII, the Kozak consensus (GCGGCCCATG) was replaced in each reading frame. A sequence encoding a single glycine residue followed by the bovine rhodopsin C9 peptide tag (TETTSQVAPA) was inserted immediately 5' to the natural stop codon of CCR5. At the 3' end of the epitope-tagged CCR5 gene, XbaI, SalI, and NotI restriction sites were introduced. Analogous constructs were made for the wild-type human CCR5 gene and the bovine rhodopsin gene, except that the codons were not altered and, in the latter case, the C-terminal C9 sequence was naturally present.

A total of 34 oligonucleotides, each approximately 70 nucleotides in length, corresponding to the complete sense and antisense strands of the synCCR5 gene and flanking sequences, were constructed so that approximately 50% of their sequences were complementary to those of each of the two complementary oligonucleotides from the opposite strand. Oligonucleotides were deposited in purified form at −70 °C. The DNA was melted at 65 °C for 4 h, after which the ammonium hydroxide was evaporated, and the oligonucleotides were dissolved in water at a final concentration of 2 mM. For gene synthesis, the 34 oligonucleotides were separated into five groups (6 or 8 oligonucleotides per group) and 25 cycles of polymerase chain reaction were performed using Pfu polymerase (Stratagene, La Jolla, CA) and a 3-fold molar excess of the 5' and 3' terminal oligonucleotides in each group. This step generated five small segments of the synCCR5 gene with complementary and overlapping ends. Equal amounts of each polymerase chain reaction product were combined with a 3-fold molar excess of the 5' and 3' terminal oligonucleotides of the complete synCCR5 sequence. A second round of 25 cycles of polymerase chain reaction yielded the complete synCCR5 sequence. The product was sequenced to ensure that the sequence was correct.

The synCCR5, wild-type CCR5, and bovine rhodopsin sequences were cloned into the following vectors: PMT4 (a gift from Dr. Reeves, Massachusetts Institute of Technology), PACH (a gift from Dr. Velan, Israel Institute for Biological Research), pcDNA 3.1 (+) and pcDNA/HisMax (Invitrogen), and PND (a gift from Dr. Rhodes, University of California, Davis). After cloning of the synCCR5 gene into the pcDNA3.1 (+) vector, the sequence encoding the N-terminal HisMax tags was removed using the polymerase chain reaction, and transiently transfected 293T cells expressing CCR5, are associated with codons that are more representative of a spectrum of different GPCR subfamilies. Opsin codon usage for opsins, the only GPCRs that are naturally expressed, with the codon usage for 45 other GPCRs representing a spectrum of different GPCR subfamilies. Opioid receptors are biased toward those shown to be optimal for efficient translation in mammalian cells (34), whereas other GPCRs, including CCR5, are associated with codons that are more random and, in many cases, inefficiently translated (data not shown). A codon-optimized CCR5 gene was designed, synthesized using the polymerase chain reaction, and transiently expressed in several different cell lines, using five different expression vectors (pcDNA 3.1, PACH, PND, PMT4, and pcDNA/HisMax). The level of CCR5 expression directed by the codon-optimized gene was 2–5 times that directed by the wild-type CCR5 gene (Fig. 1 A and B, and data not shown). Among the cell lines tested, CCR5 expression of the highest in Cf2Th canine thymocytes (data not shown), so these cells were used to generate stable cell lines. The PACH vector was used to express the codon-optimized gene encoding human CCR5 containing a 9-residue C-terminal epitope tag (the C9 tag) derived from bovine rhodopsin. The presence of the C9 tag allows recognition of the CCR5 protein by the 1D4 antibody (35). CCR5 expression in the stable cell line, designated C2Th/PACH/synCCR5, could be visualized by autoradiography or analyzed on a Molecular Dynamics PhosphorImager SI (Sunnyvale, CA).

A total of 18 oligomers were tested in the solubilization buffers. The oligomers, with abbreviations and critical micelle concentrations in parentheses, were n-octyl-β-D-glucopyranoside (25.4 mM), n-decyl-β-D-glucopyranoside (28.7 mM), n-dodecyl-β-D-maltoside (34.1 mM), n-cyclohexyl-buty1-β-D-maltoside (Cymal™7-4, 7.6 mM), cyclohexyl-pentyl-β-D-maltoside (Cymal™7-4, 2.4 mM), cyclohexyl-hexyl-β-D-maltoside (Cymal™7-4, 0.56 mM), cyclohexyl-heptyl-β-D-maltoside (Cymal™7-4, 0.19 mM), cyclohexyl-propanoyl-N-hydroxyethylglucamide (108 mM), cyclohexylbutanoyl-N-hydroxyethylglucamide (35 mM), cyclohexylpentyl-N-hydroxyethylglucamide (11.5 mM), N-decylphosphocholine (Fos-Choline™ 8, 114 mM), N-decylephosphocholine (Fos-Choline™ 9, 114 mM), N-decylephosphocholine (Fos-Choline™ 10, 114 mM), N-decylephosphocholine (Fos-Choline™ 12, 1.5 mM), N-tetra-decylphosphocholine (Fos-Choline™ 14, 0.12 mM), Triton X-100 (0.02 mM), CHAPS (8 mM), Nonidet P-40 (0.02 mM), and diheptanoyl-phosphohexethanolamine (DHPH) (1.4 mM). All detergents were purchased from Avanti Polar Lipids (Alabaster, AL).

**Binding of HIV-1 gp120 Envelope Glycoproteins to Solubilized CCR5—**Approximately 4 × 10^6 Cf2Th/PACH/synCCR5 cells were labeled for 12 h with [35S]Met/Cys and lysed in solubilization buffer containing 1% Cymal™-5. One ml of cleared cell lysate was incubated with 50 μl of 1D4-Sepharose beads on a rocking platform at 4 °C for 10–12 h. The Sepharose beads were washed five times with the washing buffer (100 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 7.5), 10% glycerol, and 1% Cymal™-5) and once with washing buffer plus 500 mM MgCl₂. CCR5 was eluted from the beads by three successive washes with 50 μl of medium containing 200 μM C9 peptide (TETTSQVAPA), 500 mM MgCl₂, 100 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 7.5), 10% glycerol, and 0.5% Cymal™-5. The total quantity of harvested CCR5 was estimated by Coomassie Blue staining of an SDS-polyacrylamide gel run with standard quantities of bovine serum albumin.

**Results**

**Expression of CCR5 in Mammalian Cells—**We compared the codon usage for opsins, the only GPCRs that are naturally highly expressed, with the codon usage for 45 other GPCRs representing a spectrum of different GPCR subfamilies. Opioid receptors are biased toward those shown to be optimal for efficient translation in mammalian cells (34), whereas other GPCRs, including CCR5, are associated with codons that are more random and, in many cases, inefficiently translated (data not shown). A codon-optimized CCR5 gene was designed, synthesized using the polymerase chain reaction, and transiently expressed in several different cell lines, using five different expression vectors (pcDNA 3.1, PACH, PND, PMT4, and pcDNA/HisMax). The level of CCR5 expression directed by the codon-optimized gene was 2–5 times that directed by the wild-type CCR5 gene (Fig. 1 A and B, and data not shown). Among the cell lines tested, CCR5 expression of the highest in Cf2Th canine thymocytes (data not shown), so these cells were used to generate stable cell lines. The PACH vector was used to express the codon-optimized gene encoding human CCR5 containing a 9-residue C-terminal epitope tag (the C9 tag) derived from bovine rhodopsin. The presence of the C9 tag allows recognition of the CCR5 protein by the 1D4 antibody (35). CCR5 expression in the stable cell line, designated C2Th/PACH/synCCR5, could be visualized by autoradiography or analyzed on a Molecular Dynamics PhosphorImager SI (Sunnyvale, CA).
be enhanced 2–3-fold by treatment of the cells with sodium butyrate (Fig. 1C). Following this treatment, approximately 3–5 μg of CCR5 of high purity could be isolated from 10^7 Cf2Th/PACH/synCCR5 cells, using techniques described below (Fig. 1D).

**Precursor and Mature Forms of CCR5—**CCR5 synthesis and turnover in Cf2Th cells were studied by pulse-chase analysis (Fig. 2). A precursor of approximately 40 kDa chased into the mature form of CCR5, which migrated as a wide band of approximately 43 kDa. The CCR5 precursor exhibited a half-life of approximately 25 min (Fig. 2A). The half-life of the mature form of CCR5 was 11–14 h, regardless of whether CCR5 expression was directed by the wild-type or codon-optimized CCR5 gene (Fig. 2, B and C). The half-lives of the precursor and mature forms of CCR5 in HEK-293 cells were similar to those in Cf2Th cells (data not shown). In several different cell lines, a lower molecular mass (approximately 36 kDa) form of CCR5 appeared in parallel with the mature protein (Fig. 2, B and C). This lower molecular mass form of CCR5 was expressed at lower levels than the mature form of CCR5 and has not been completely characterized. Its identity as a CCR5 isoform was confirmed by its precipitation by the 1D4 antibody and the anti-CCR5 antibody 2D7 and by mass spectrometry (Figs. 2 and 3A and data not shown).

**Solubilization of Native CCR5—**Membrane protein purification requires solubilization of the membranes, typically through the use of detergents. A broad spectrum of conditions was studied to arrive at the composition of the buffer that allowed solubilization and isolation of native CCR5. This optimization was guided by a comparison of the amount of solubilized CCR5 capable of being precipitated by the 2D7 antibody, which recognizes a conformation-dependent CCR5 epitope (36), with that able to be precipitated by the 1D4 antibody directed against the linear C9 epitope tag. In this manner, the percentage of solubilized CCR5 remaining in a native conformation could be estimated (Fig. 3A). Eighteen detergents, most of which were designed specifically for the extraction and purification of membrane proteins, were studied. In terms of the quantity of isolated CCR5 protein, as well as the percentage of protein in a conformation able to be recognized by the 2D7 antibody, the most effective detergents were DDM, CymalTM-5, and CymalTM-6 (Fig. 3B). Of these detergents, CymalTM-5 exhibits the highest critical micelle concentration (2.4 mM), facilitating dialysis of the detergent from the protein solution for the purposes of membrane reconstitution and/or crystallization. We also found that a CCR5 conformation competent for binding HIV-1 gp120 was best preserved in buffers containing CymalTM-5 (see below). Therefore, CymalTM-5 was used for further refinement of the CCR5 solubilization/isolation protocol, examining a number of variables (salt composition and concentration, pH, temperature, and minor additives) known to influence the stability of solubilized proteins (37).
sulfate and glycerol were found to prolong the existence of a CCR5 conformation capable of being recognized by the 2D7 antibody (data not shown). The optimized CCR5 solubilization buffer was composed of 100 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 7.5), 10% glycerol, and 1% Cymal TM-5.

Binding of Solubilized CCR5 to HIV-1 gp120—To examine whether the solubilized CCR5 was capable of binding the HIV-1 gp120 glycoprotein, coprecipitation experiments were conducted using three different gp120 glycoproteins. The JR-FL and ADA gp120 glycoproteins, like all characterized wild-type R5 HIV-1 envelope glycoproteins, bind CCR5 efficiently only in the presence of CD4 (3, 4). By contrast, the 190/197 R/S variant of the ADA gp120 glycoprotein was derived from a virus adapted in culture to replicate on CD4-negative, CCR5-positive cells, and it therefore binds CCR5 in the absence of CD4. The mature CCR5 protein was precipitated from cell lysates by the C11 anti-gp120 antibody when unlabeled JR-FL gp120 and soluble CD4 (sCD4) were added to the lysates (Fig. 4A), but not when either gp120 or sCD4 were left out of the mixture (data not shown). The C11 antibody precipitated the mature CCR5 protein when radiolabeled 190/197 R/S gp120 was added to the lysates without sCD4 (Fig. 4B). Conversely, the 1D4 antibody precipitated the labeled 190/197 R/S gp120 glycoprotein from these lysates. In the coprecipitation experiments using the C11 anti-gp120 antibody, only the mature form of CCR5, and not the 36-kDa CCR5 isoform, was precipitated (Fig. 4).

Sulfation of CCR5—CCR5 has been shown to be posttranslationally modified by O-linked carbohydrates and by tyrosine sulfation (33). The latter modification has been suggested to facilitate the efficiency with which CCR5 is utilized as an HIV-1 coreceptor (33). To examine the relationship of sulfation to the observed conversion of the CCR5 precursor to the higher molecular mass, mature form of the protein, a pulse-chase analysis of C2Tb/PACH/synCCR5 cells labeled with either [³⁵S]cysteine/methionine or [³⁵S]sulfate was performed. Sulfate label was incorporated only into the mature form of CCR5 (Fig. 5).

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of CCR5 by the 1D4 and 2D7 antibodies and by gp120-sCD4 complexes was examined (Fig. 5). The conformation-dependent 2D7 antibody precipitated the CCR5 precursor at an efficiency approximately 20% lower than that of the mature CCR5, whereas the half-lives of the two proteins were comparable. This difference was apparently compensated for by the establishment and selection of the stable CIITA/CYPH/synCCR5 cell lines. Scale-up of these cells could allow the purification of milligram quantities of the CCR5 protein.

We evaluated the conformational integrity of CCR5 solubilized in several different detergents, including DDM, which has been employed in the study of some other GPCRs (38, 39). We show that CymalTm-5 was at least as effective as DDM in solubilizing CCR5 in a native conformation. Human CCR5 retained a native conformation in CymalTm-5-containing buffers for a few days at 4 °C, and bound HIV-1 gp120 more efficiently than CCR5 solubilized in DDM (data not shown). A further advantage of CymalTm-5 is its critical micelle concentration, which is 14 times higher than that of DDM, the mild detergent commonly used for membrane protein biochemistry. The high critical micelle concentration of CymalTm-5 should facilitate its removal during reconstitution of the GPCR into membranes or for crystallization.

The C-terminal C9 peptide provides an easy and rapid means for purification of CCR5 using the anti-peptide antibody 1D4. The presence of the C9 epitope tag does not affect the ability of CCR5 to function as an HIV-1 entry cofactor or as a receptor for chemokine ligands (data not shown). The purified CCR5 can be gently eluted from the 1D4 antibody by using the free C9 peptide as a competitor (35, 38).

The synthesis and turnover of CCR5 were examined in the course of this study. Approximately 80% of the 40-kDa CCR5 precursor is competent for binding the conformation-dependent 2D7 antibody, indicating that a native structure is achieved rapidly after synthesis of the protein. The 40-kDa CCR5 precursor is rapidly converted into a more slowly migrating form. This 43-kDa form of CCR5 is evident within 10 min of labeling the CCR5 precursor. Most of this shift in molecular mass derives from the addition of O-linked carbohydrates to the CCR5 protein (27). The single potential N-linked glycosylation site on CCR5 is not utilized (27). Once synthesized, the mature CCR5 protein exhibits a half-life of 11–14 h. The tyrosines in the N terminus of the 43-kDa CCR5 form are modified by sulfation, suggesting that sulfation occurs after O-glycosylation. The incorporation of the sulfate label into the 43-kDa CCR5 protein continuously increases over a 4-h labeling period. Because tyrosine sulfation typically occurs in the trans-Golgi network, late in the secretory pathway (15), some sulfate addition may be occurring on CCR5 molecules that have been recycled from the cell surface back to the Golgi. Our results indicate that the HIV-1 gp120 glycoprotein preferentially recognizes the mature, sulfated form of CCR5, consistent with the proposed role of sulfated tyrosines in gp120 binding and HIV-1 entry (33).

The availability of methods to purify adequate amounts of CCR5 should expedite progress on understanding the structure and function of this key HIV-1 coreceptor and facilitate the search for effective inhibitors of virus-coreceptor interactions.

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