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Purified complexes of HIV-1 envelope glycoproteins with CD4 and CCR5(CXCR4): production, characterization and immunogenicity

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Abstract

The ability to readily elicit broadly neutralizing antibodies to HIV-1 remains elusive. We and others have hypothesized that interaction of the viral envelope glycoprotein (Env, gp120-gp41) with its receptor molecules could enhance the exposure of conserved epitopes that may facilitate the elicitation of broadly neutralizing antibodies. The Env-CD4-coreceptor complexes mediate HIV-1 entry into cells and serve as a major target for inhibitors of this process. To begin to evaluate their potential also as vaccine immunogens we produced relatively large amounts of complexes of purified recombinant soluble truncated Env, $gp140_{89.6}$ or $gp120_{89.6}$, with CD4 and CCR5 or CXCR4. We found that gp140(gp120)-CD4-CCR5 complexes are stable and immunogenic in mice transgenic for human CD4 and CCR5. They elicited anti-gp120 and anti-gp140 antibodies that inhibited an heterologous primary HIV-1 isolate (JR-FL) with two- to threefold higher neutralizing activity than those elicited by gp120 and gp140. The antibodies elicited by the complexes competed better with the antibodies X5 and CG10 but not with b12 for binding to gp120 and gp120-CD4 complexes compared to those elicited with gp140(120) alone. These findings suggest that stable purified Env-CD4-CCR5(CXCR4) complexes can be produced in relatively large amount sufficient for their further characterization that may help in the development of novel vaccines candidates.
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1. Introduction

Binding of the human immunodeficiency virus type-1 (HIV-1) envelope glycoprotein (Env, gp120-gp41) to receptor molecules (CD4 and coreceptors) initiates a series of conformational changes that are at the heart of the fusion machinery driving viral entry [1–3]. It has been proposed that CD4 interaction with gp120 may enhance exposure of structures conserved among various HIV-1 isolates and stabilize them; thus complexes of gp120 with recombinant soluble CD4 (sCD4) have been tested as immunogens in mice [4–7] and in goats [8]. Recently, immunization of monkeys with gp120(gp140)-sCD4 complexes suggested the possibility that such complexes can elicit broadly HIV-1 neutralizing antibodies in non-human primates [9]. After the discovery of the HIV-1 coreceptors it was hypothesized that complexes of Env with CD4 and coreceptors could exhibit conserved epitopes and may have potential for development of vaccines [10]. Experimental evidence appears to support the notion for Env conformational changes induced by coreceptors [2,11] although the epitopes induced by coreceptors have not been characterized. Given the role of the interactions of the Env with receptor molecules for the mechanisms of HIV-1 entry into cells and the potential for development of vaccines, based on conserved fusion intermediate structures, it is of paramount importance to produce relatively large amounts of purified Env-CD4-coreceptor complexes, and characterize their properties for use as a virus entry model system and immunogens. Recently, gp120-CD4-CCR5 complexes have been used as an antigen for selection of a potent broadly HIV neutralizing human monoclonal antibody Fab, X5 [12]. The selection of a broadly neutralizing antibody by Env-CD4-coreceptor complexes suggested the possibility that these complexes may have the potential to elicit broadly neutralizing antibodies in vivo. Such possibility is further supported by the experimental demonstration of conformational changes induced by coreceptors [2]. A major

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obstacle for testing this hypothesis has been the generation of sufficient amounts of stable Env-CD4-CCR5 complexes in conformation close to the native one.

Unlike purified complexes between Env and CD4, which have been extensively characterized, purified complexes of Env and CD4 with the major HIV-1 coreceptors, CCR5 and CXCR4, have not been well characterized primarily because of difficulties in their production, isolation and purification. This is due to the extremely hydrophobic nature of the coreceptors and the sensitivity of their three-dimensional structure to perturbations by detergents used for their solubilization [13,14]. Immunopurified complexes between gp120, CD4 and CXCR4 [12,15–17] or CCR5 [12,17] have been previously produced in small quantities and partially characterized. Here we describe the production of relatively large amounts of purified complexes of gp120 or gp140 (gp120-truncated gp41) with CD4 and CCR5 or CXCR4. These complexes were stable, showed conformational integrity, and elicited HIV-1 neutralizing antibodies.

2. Materials and methods

2.1. Cells, viruses, plasmids, recombinant proteins and mAbs

3T3 cells expressing CD4 and CCR5 were gift from D. Littman (New York University, New York, NY). Cf2ThCCR5 cells expressing high amounts of CCR5 were gift from J. Sodroski (Dana Farber Institute, Boston, MA); the parental Cf2Th cells were purchased from ATCC and used as negative control. The stable cell line TF228 expressing LAI Env was a gift from Z. Jonak (SmithKline Beechman Pharmaceuticals, Philadelphia, PA) through R. Blumenthal (NCI-Frederick, Frederick, MD). HeLa cell lines stably transfected with CD4 and/or CCR5 were gift from David Kabat (Oregon Health and Science University, OR). Recombinant vaccinia viruses, vCB21R and vTF7.3 were used for the reporter gene fusion assay [18,19]. Plasmids expressing various Envs were obtained through the NIH AIDS Research and Reference Reagent Program or were gift from M.A. Martin (NIAID, Bethesda, MD). Plasmid S-tag CXCR4 was obtained from J. Nunberg (University of Montana, Missoula, MT). The anti-CD4 polyclonal antibody T4-4 was obtained through the AIDS Research and Reference Reagent Program from R. Sweet (SmithKline Beechman Pharmaceuticals). Two-domain soluble CD4 (2DsCD4) was a gift from E. Berger (NIAID, Bethesda, MD); four-domain soluble CD4 (sCD4) was obtained through the NIH AIDS Research and Reference Reagent Program from N. Schuelke. Recombinant $gp120_{89.6}$ and $gp140_{89.6}$ were produced by vaccinia virus (gift of R. Doms (University of Pennsylvania, Philadelphia, PA)) and purified with a combination of lentil lectin affinity chromatography and size exclusion chromatography, as previously described [20,21]. The anti-CCR5 mAbs 5C7 and 2D7 were gifts from L. Wu (Millenium Pharmaceuticals, Cambridge, MA) and b12 from D. Burton. The 1D4 MAb against the bovine rhodopsin C9 peptide (TETSQVAPA) tag was purchased from Biovest International Inc./National Cell Culture Center (Minneapolis, MN). The goat polyclonal anti-CCR5 antibody CKR5(C20) was purchased from Santa Cruz Biothechnology Inc. (Santa Cruz, CA). Recombinant $gp120_{JR\text{-}FL}$ was provided by A. Schultz and N. Miller (NIAID).

2.2. Production, purification and quantification of gp120-CD4-CCR5 complexes

NIH 3T3 transfectants (10^9 in 100 ml) expressing high levels of CD4 and CCR5 were washed twice with cold (4 °C) phosphate-buffered saline (PBS) then pelleted by centrifugation and resuspended in 100 ml lysis buffer (1% Brij97, 5 mM iodoacetamide, added immediately before use, 150 mM NaCl, 20 mM Tris (pH 8.2), 20 mM EDTA, and protease inhibitors) at 4 °C for 40 min with gentle mixing. The nuclei were pelleted by centrifugation at 14,000 rpm for 25 min in a refrigerated centrifuge. Anti-CCR5 antibody 5C7 at 2 μg/ml and protein G-Sepharose beads (Sigma, St. Louis, MO) (1 ml) prewashed with PBS were added to the cell lysate and incubated at 4 °C for 14 h. The beads were then washed five times with 100 ml of ice-cold lysis buffer and incubated with gp120 at 5 μg/ml in 20 ml lysis buffer for 1 h at 4 °C. They were again washed five times with 100 ml of cold lysis buffer, once with cold PBS, and incubated with 1% formaldehyde overnight, followed by washing twice with cold lysis buffer and used. They contained approximately 0.01 mg CD4 and 0.02 mg gp120 as quantified by calibrating amounts of soluble CD4 and gp120. For quantification of CD4 and gp120 two duplicated samples each containing 0.1% of the total amount of bead-associated gp120-CD4-CCR5 complexes were used. They were eluted by adding sample buffer for SDS-PAGE gel and kept overnight at 37 °C. They were run on a 10% SDS-PAGE gel simultaneously with calibrating amounts (1, 3, 10, 30, 100 ng) of soluble four-domain CD4 (sCD4) or gp120 and were electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with TBST buffer containing 20 mM Tris–HCl (pH 7.6) 140 mM NaCl, 0.1% Tween-20 and 5% nonfat powdered milk. For Western blotting these membranes were incubated with anti-CD4 or anti-gp120 antibodies, then washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies. The blots were developed by using the supersignal chemiluminescent substrate from Pierce (Rockford, IL). The images were acquired using a Bio-Rad phosphoimager (BioRad, Hercules, CA). The signal from the calibrating molecules was integrated for each band and plotted in a calibration curve for the signal versus amount dependence. The amounts of CD4 and gp120 were then calculated by interpolation using the calibration curve.

To test the association between Env gp120 and gp140 with CXCR4, we expressed CXCR4 by transfecting HEK293T cells with pCXCR4-Spep followed by infection with vaccinia virus encoding T7 RNA polymerase (vTF7.3). Cells were then lysed using 1% cymal-5 lysis buffer, and then 2 μg of gp140 and 10 μl of S-protein beads (Novagen, Madison, WI) were added and incubated at 4 °C for overnight. To test the association between gp140 and CXCR4 in the presence of sCD4, 2 μg of sCD4 and 2 μg of gp140 was pre-incubated in 50 μl of PBS at room temperature for 1 h before incubation with the cell lysate and S-protein beads. The samples were analyzed as described above.

2.3. Mice immunizations

Balb/c mice, transgenic for human CD4 and CCR5 (lck-hu-CD4/CCR5) [22], were immunized four times in 1-month interval by $gp120_{89.6}$-CD4-CCR5 or $gp140_{89.6}$-CD4-CCR5 beads mixed with 20 μg QS-21 as adjuvant. Blood was collected 7–10 days after each immunization. The antibody response was evaluated by gp120 or gp140 captured ELISA assay. For this assay 96 well plates were coated with purified $gp120_{89.6}$ and $gp140_{89.6}$ protein at 100 ng per well concentration in bicarbonate buffer for 4 h at room temperature. Then they were blocked by PBS with 0.05% Tween-20 and 10% FBS. Sera was added and incubated at 4 °C overnight. The plates were washed with wash buffer (PBS with 0.05% Tween-20) five times. One hundred microliters of appropriate secondary antibody (0.3 μg/ml) was added and incubated for 1 h at room temperature. Plates were washed five times with washing buffer, ABTS substrate solution (Boehringer Mannheim) was added and optical density (OD) at 405 nm was measured.

2.4. ELISA determination of the anti-gp120/gp140 antibody titer of mouse sera

Microtiter plates were coated with purified $gp120_{89.6}$ or $gp140_{89.6}$ at 100 ng per well in bicarbonate buffer for 4 h at room temperature. The plates were washed twice with PBS/Tween-20 0.05% (v/v) and blocked with 10% bovine calf serum (PBS with 0.05% Tween-20 and 10% FBS) for 2 h at room temperature. Following blocking, the plates were washed twice and serially diluted serum samples and standards, in duplicate, where added and incubate overnight at 4 °C. One hundred microliters of secondary goat anti-mouse antibody conjugate with HRP (0.3 μg/ml) was added to each well and incubated 1 h at room temperature. Following three washings, ABTS substrate was added (Boehringer Mannheim) and absorbance at 405 nm was measured using an ELISA plate reader. For competition ELISA, biotinylated X5, CG10 and b12 antibodies were used along with the sera at different dilutions. The amount of bound antibody was detected using Streptavidin-HRP.

2.5. Inhibition of virus infection by mouse sera

Evaluation of HIV-1 neutralization by mouse sera was performed using infection with a luciferase reporter HIV-1 Env-pseudotyped system. Viral stocks were prepared by transfecting 293T cells with plasmids encoding the luciferase virus backbone (pNL-Luc-ER) [23] and full-length gp160 encoding expression plasmids from several HIV-1 isolates obtained from the NIH AIDS Research and Reagent Program (Rockville, MD): 89.6 [24], JR-FL [25], NL4-3 [26]. The resulting supernatant was clarified by centrifugation for 10 min at 2000 rpm in a Sorvall RT-7 centrifuge (RTH-750 rotor) and stored at 4 °C. The virus was mixed with various concentrations of diluted mice sera for 30 min at room temperature. Cells were then infected with 100 μl of virus preparation containing DEAE-dextran (8 μg/ml) for 4 h at 37 °C. After five washes with PBS fresh medium (0.2 ml) was added to each well in a 96-well plate. Cells were lysed 44 h later in 100 μl of cell lysis buffer (Promega, Madison, WI), and 50 μl of the lysate was assayed for luciferase activity, using an equal volume of luciferase substrate (Promega).

3. Results

3.1. Production of purified $gp120_{89.6}$($gp140_{89.6}$)-CD4-CCR5(CXCR4) complexes

For production of purified Env-CD4-CCR5 complexes, CCR5 was coimmunoprecipitated with cellular CD4 by an anti-N-terminus antibody, 5C7, selected for its immunoprecipitating efficiency from a battery of several other anti-CCR5 antibodies [17] followed by incubation with protein G-Sepharose beads, washing and subsequent incubation with purified gp120 (Section 2). The major advantage of this approach is the ability to study native CCR5 expressed in any cell of choice. In addition, the use of anti-CCR5 antibody guarantees that only those CD4 molecules associated with CCR5 will be coimmunoprecipitated. Cells with CD4 concentrations significantly higher than the CCR5 concentrations were used so the vast majority of CCR5 molecules are associated with CD4. As a result most of the immunoprecipitated CCR5 molecules are complexed with CD4. In addition, because gp120 binds with high affinity only to CCR5 complexed with CD4 this procedure also ensures that most of the gp120 molecules will be in the trimolecular complex. A disadvantage of this approach is that the immunoprecipitating mAb may interfere although weakly with binding to portions of the CCR5 N-terminus. This possible problem, however, can be solved by using antibodies against the C-terminus although the efficiency of immunoprecipitation was lower for the antibody (CKR5(C20)) we tested. Fig. 1 shows gp120 and CD4 coimmunoprecipitated with CCR5 by the anti-CCR5 mAb 5C7 from two different cell lines expressing CD4 and CCR5 (3T3

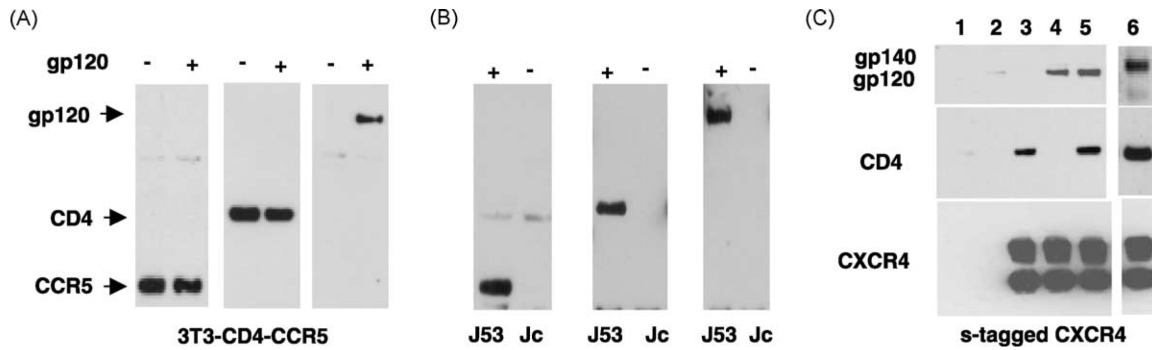

Fig. 1. Formation of purified complexes between gp120(140), CD4 and CCR5(CXCR4) demonstrated by Western blotting. (A) Complexes obtained from NIH 3T3 CD4.CCR5 cells, these cells expressing high levels of CD4 and CCR5 were lysed using 1% Brij97 lysis buffer at 4 °C for 40 min with gentle mixing. Cell lysate was incubated at 4 °C for 14 h after addition of anti-CCR5 antibody 5C7 at 2 μg/ml and protein G-Sepharose beads. The beads were then washed five times and incubated with gp120 at 5 μg/ml in lysis buffer for 1 h at 4 °C. (B) Complexes obtained from a HeLa transfectant, JC.53 following the same procedure as described above. Lack of complexes for control cells (HI-JC), which do not express CCR5 but do express CD4 at the same levels as JC.53. For both (A) and (B), CD4 and CCR5 were membrane associated. (C) Purified complexes between gp140, sCD4 and s-peptide tagged CXCR4. CXCR4 was immobilized on S-protein beads first. SCD4 and gp120(140) pre-incubated in PBS was then added to CXCR4 on beads and further incubated in Brij97 buffer for 1 h at 4 °C followed by washing with the same buffer three times. The beads were then analyzed for the presence of both CXCR4 and CD4. Lanes 1 and 2 represent beads incubated with cell lysates from cells transfected with pCDNA3 control plasmid plus sCD4 and Envs, respectively. Lanes 3–6 represent beads incubated with cell lysate from cells transfected with S-tag CXCR4, and sCD4, IIIB gp120, sCD4 + IIIB gp120, or sCD4 + IIIB gp140 added, respectively.

CD4.CCR5 and a HeLa transfectant, JC.53). There was no gp120-CD4-CCR5 coimmunoprecipitation for a control HeLa transfectant (HI-J) that expresses CD4 at about the same levels as JC.53 but not CCR5. Silver staining of the gels revealed no other major bands but only two minor bands (data not shown) suggesting relatively high purity of the complexes. Similar results were obtained by using another approach to produce gp120-CD4-CCR5 complexes based on the use of streptavidin bead-associated CCR5 through biotinylated anti-CCR5 antibodies (see Section 2, data not shown). The major advantage of this approach compared to the use of protein G beads is the ability to test complexes by antibodies, which would bind to protein G. In yet another approach coreceptors tagged by a bovine rhodopsin C9 peptide as previously described [13,14] or s-peptide were used. A major advantage of this approach is the easier release of soluble complexes by elution with the tags. In addition, the C9 tagged coreceptor genes were codon optimized resulting in significantly higher levels of expression than in available cell lines [13] which allows easier up-scaling of the complex production. Fig. 1C shows gp140 (containing gp120 and gp41 without transmembrane domain and cytoplasmic tail) and CD4 coimmunoprecipitated with CXCR4 tagged with s-peptide. These results suggest that purified Env-CD4-coreceptor complexes can be produced by different approaches appropriate for solving a variety of research problems.

3.2. Quantification of gp120-CD4-CCR5 complexes

The quantities of gp120 and CD4 in purified gp120-CD4-CCR5 complexes were measured by using calibrating amounts of soluble molecules of known concentrations and Western blotting quantified by a phosphorimager (Fig. 2A). Ten micrograms complexed gp120 was obtained from this procedure starting with 3×10^8 3T3 CD4.CCR5 cells. The molar ratio of the two molecules in the gp120-CD4-CCR5 complexes was about 1:1 as expected. Experiments are in progress to determine the molecular weight of the complexes and the CCR5 stochiometry.

3.3. Conformation of CCR5 and its interaction with gp120 in purified gp120-CD4-CCR5 complexes

To test the conformation of bead-associated dsCCR5, in particular, its binding to gp120-sCD4 complexes, we used

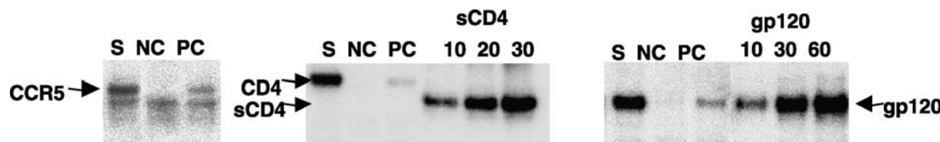

Fig. 2. Quantification of gp120, CD4 and CCR5 in purified gp120-CD4-CCR5 complexes by Western blotting. Quantification of gp120 and CD4 in complex was done using purified JR-FL gp120 and sCD4 as standard as described in Section 2. PC and NC represents the products from the purification procedure using 3T3-CD4-CCR5 cells and 3T3-CD4 cells respectively in a small scale and serve as positive and negative controls. S represents 0.1% of the total products from one large preparation.

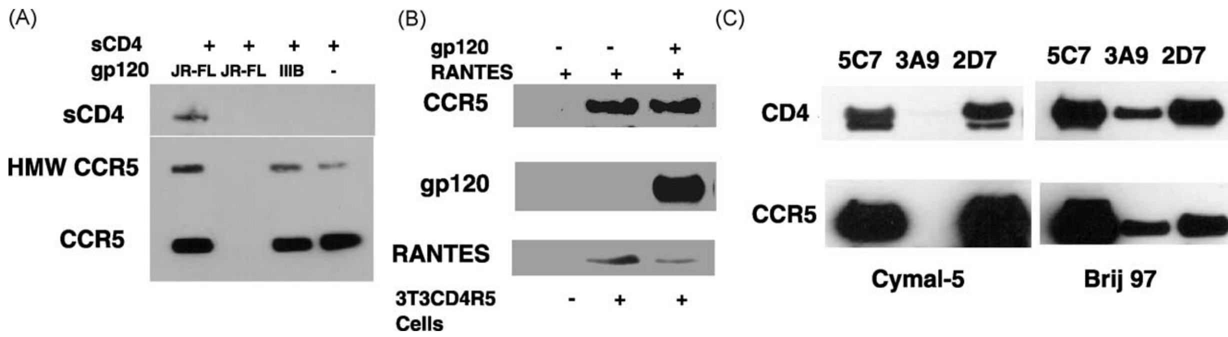

Fig. 3. Native conformation of dsCCR5. (A) sCD4-gp120$_{JR-FL}$ but not sCD4-gp120$_{IIIB}$ complex binds dsCCR5, as indicated by the binding of sCD4. This experiment was performed in similar fashion as in Fig. 1C. Protein G beads were incubated with the anti-CCR5 mAb 5C7, lysates from CCR5 cells or control cell lysate without CCR5 (second lane from left), and sCD4 complexed with JR-FL or IIIB gp120 as indicated. HMW CCR5 denotes higher molecular weight species, e.g. dimers of CCR5. (B) Direct interaction of gp120 with CCR5 demonstrated by displacement of RANTES bound to CCR5 by gp120. RANTES (1 μg/ml) was added to CCR5 immobilized on beads in the presence of 1 μg/ml of BSA and with or without 5 μg/ml of gp120 in Brij97 buffer, and incubated at 4 °C for 1 h, washed three times with the Brij97 buffer and analyzed by Western blotting. (C) Testing of the CCR5 native conformation in two different detergents, Cymal-5 and Brij97, by immunoprecipitation with three different anti-CCR5 monoclonal antibodies (5C7, 3A9 and 2D7) from NIH3T3-CD4-CCR5 cells.

mixtures of sCD4 and gp120 from two different HIV-1 Envs—JR-FL and IIIB. Binding of sCD4-gp120$_{JR-FL}$ but not of sCD4-gp120$_{IIIB}$ complex to dsCCR5 was detectable suggesting stronger binding of the R5 Env-sCD4 complex to CCR5 than the X4 Env-sCD4 complex (Fig. 3A). To further test the ability of CD4-associated CCR5 to bind gp120 in the gp120-CD4-CCR5 complexes we designed an experiment where gp120 mediated displacement of RANTES bound to CCR5 was tested. Fig. 3B shows displacement of RANTES by gp120 demonstrating direct interaction of gp120 with CCR5. It was previously shown that cell surface-associated CD4 binds to CCR5 even in the absence of gp120. Therefore these results suggest that in the gp120-CD4-CCR5 complex all three molecules interact with each other. The relative affinities of CD4-CCR5 versus (gp120-CD4)-CCR5 is currently under investigation but they are likely to be comparable. We also tested the effect of the detergent we routinely employ, Brij97, on CCR5 epitopes in comparison with another detergent, Cymal-5, which was found to preserve best the 2D7 epitope on CCR5 among various other detergents [14]. As shown in Fig. 3C immunoprecipitation by 2D7 is better in Cymal-5 solutions than in Brij97, but the immunoprecipitation by 5C7 is worse and by 3A9 is not detectable. Thus, it appears that overall Cymal-5 and Brij97 are comparable in preserving some epitopes on CCR5. The binding of RANTES to CCR5 and the association between CCR5 with CD4 and gp120 suggest that the molecules in the complex maintained their function and native conformation.

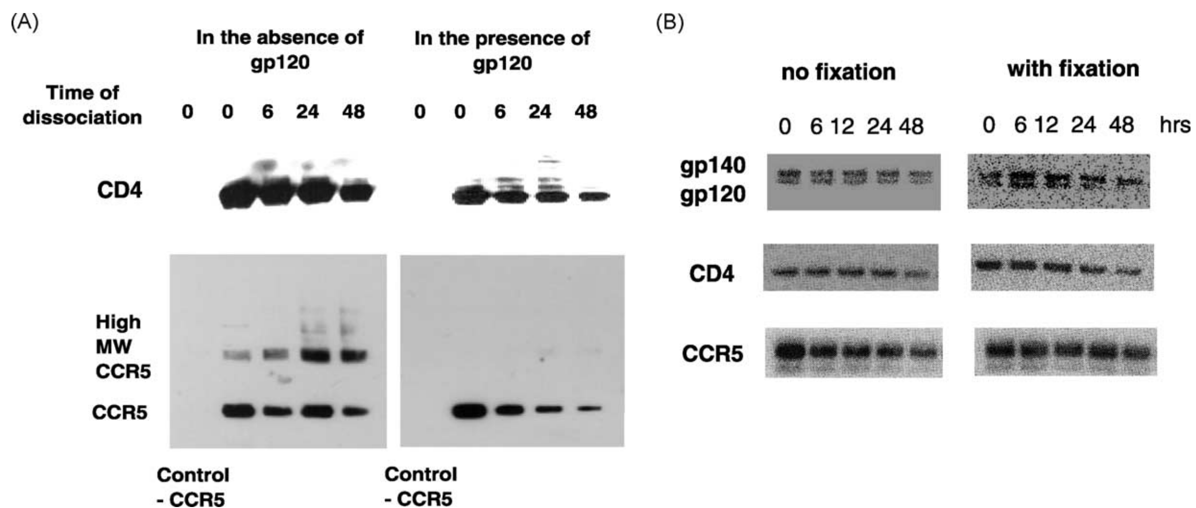

Fig. 4. Stability of gp120-CD4-CCR5 complexes with and without formaldehyde fixation. (A) The stability of CD4-CCR5 and CD4-CCR5-gp120 was tested using bead-bound molecules as indicators. CD4-CCR5 or gp120-CD4-CCR5 complexes on beads were prepared as described in Section 2 and kept at 4 °C for indicated period of time and then washed once with brij97 buffer and analyzed by Western blotting. (B) The effect of fixation on complex stability was tested using bead-bound molecules as indicators. Gp120-CD4-CCR5 complexes were prepared and fixed as described in Section 2 and kept at 4 °C for different length of time, and washed once with Brij97 buffer and analyzed by Western blotting.

3.4. Stability of the gp120-CD4-CCR5 complexes

We next tested the stability of the gp120 and CD4 interactions with CCR5 by measuring the amounts of bound molecules as function of time. To distinguish between the amounts of bound versus dissociated gp120 and CD4 we measured by Western blotting the amount left on the beads. CD4 and gp120 amounts did not decrease markedly for the time period we tested (Fig. 4A and B, respectively). Note that in the absence of gp120 the amount of the high molecular weight form of CCR5 left on the beads increases probably due to aggregation. In the presence of gp120 such high molecular weight CCR5 forms (aggregates) were absent. In another experiment we tested the effect of formaldehyde, which is routinely used to preserve conformations and in immunization experiments. Fixation with formaldehyde

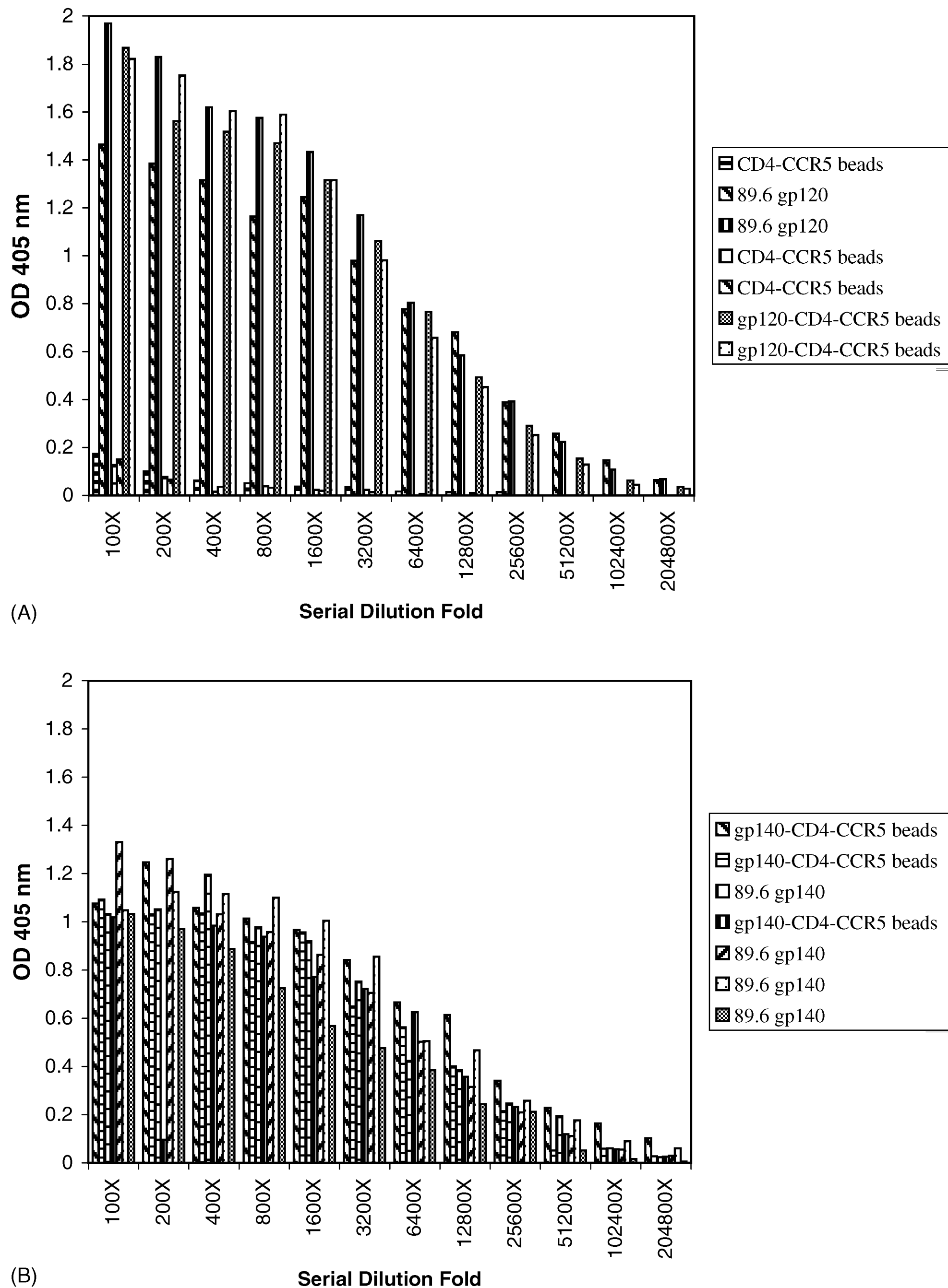

Fig. 5. Antibody titer in the sera determined by ELISA. (A) sera from gp120 and gp120 complexes and (B) sera from gp140 and gp140 complexes. Micro titer plates were coated with purified $gp120_{89.6}$ or $gp140_{89.6}$ at 100 ng per well. The plates were washed and blocked with 10% bovine calf serum (PBS with 0.05% Tween-20 and 10% FBS). Following blocking, the plates were washed and serially diluted serum samples and standards, in duplicate, where added and incubated. The amount of bound antibody is detected using secondary goat anti-mouse antibody conjugate with HRP.

maintained the stability reasonably well (Fig. 4B). Importantly, decreasing concentrations of detergent and its complete removal and substitution of the lysis buffer with PBS, a step necessary for fixation, did not significantly reduce the antibody binding to the complexes. These results demonstrate that formaldehyde-fixed gp120-CD4-CCR5 complexes are sufficiently stable to be used as model systems and for immunization.

3.5. Immunogenic activity of purified gp120(gp140)-CD4-CCR5 complexes

To examine the ability of Env-CD4-coreceptor complexes to induce an immune response we used mice transgenic for human CD4 and CCR5 (lck-hu-CD4/CCR5). Sera from different mice in the same treatment group were pooled before all subsequent analysis was performed. The titer of anti-gp120 antibodies induced by the complexes was very high and similar to the titer induced by uncomplexed gp120 or gp140 suggesting that bead-associated complexed gp120(gp140) is as good immunogen as free gp120(gp140) (Fig. 5). There were no anti-gp120 antibodies induced by bead-associated CCR5-CD4 complexes. These results suggest that purified Env-CD4-CCR5 complexes can be produced in sufficient amounts and are in appropriate conformation to induce immune responses. Neutralizing activities of the induced antibodies by gp120(gp140)-CD4-CCR5 complexes were compared to that induced by pure gp120(gp140). Infection of CEM-R5 cells by pseudo-viruses carrying NL4-3, 89.6, and JR-FL Env was inhibited to a greater extent by sera generated against gp120(gp140)CD4-CCR5 complexes compared to sera generated by gp120(gp140) alone (Fig. 6). The sera was not cytotoxic as observed by trypan blue exclusion and did not affect cell growth during the duration of the experiment. A novel Fab (X5) selected from a phage display library against an immobilized form of the gp120-CD4-CCR5 complex that exhibited broad neutralizing ability against infection by various HIV-1 primary isolates was used in gp120 binding competition assay. Sera generated against complexed gp120(gp140) competed efficiently with X5, while sera against uncomplexed gp120(gp140) showed reduced competition (Fig. 7). Similar pattern was observed with the gp120-CD4 complex specific antibody CG10 but not for b12 that binds to an epitope overlapping with the CD4 binding site. These data suggested that sera generated against the Env-CD4-CCR5 complexes recognizes epitope(s) that were not available on free gp120(gp140), and that their exposure is likely enhanced by the Env interaction with receptor molecules.

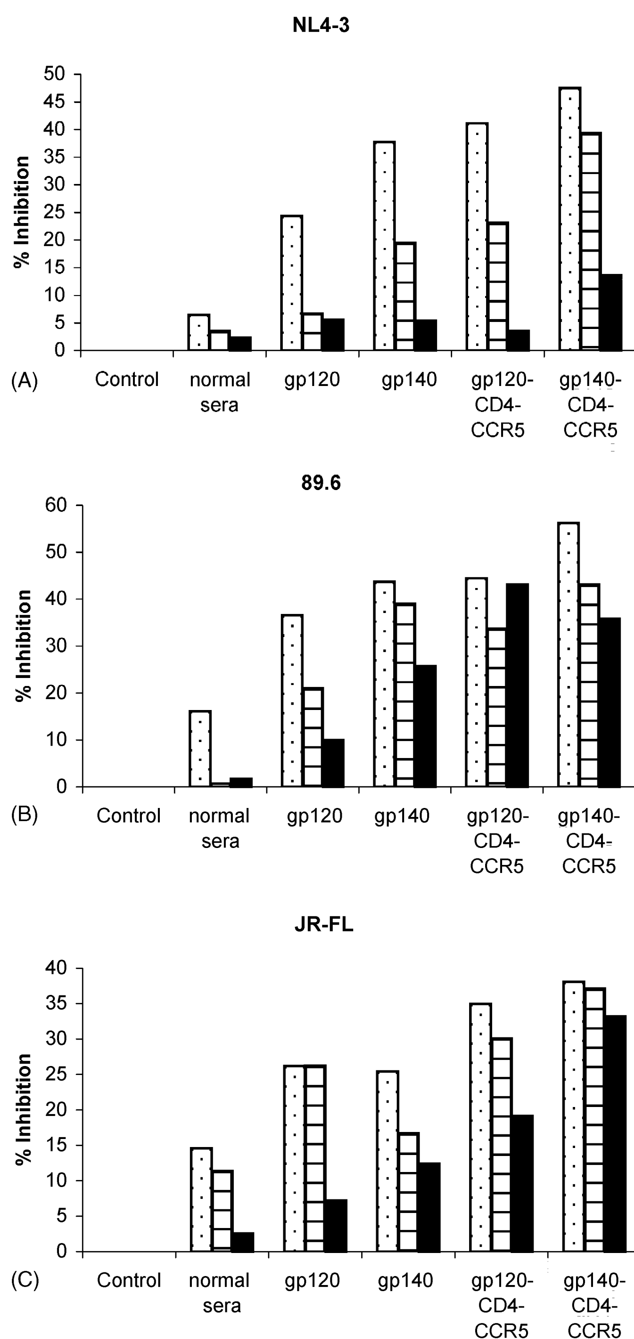

Fig. 6. Evaluation of HIV-1 neutralization by mouse sera using a reporter HIV-1 Env-pseudotyped system with a NL4-3 backbone where the env is replaced by a luciferase gene. Inhibition of pseudotyped: (A) NL4-3 Env; (B) 89.6 Env and (C) JR-FL Env was performed using sera from all samples at 1:20 (dotted), 1:100 (lined) and 1:500 (filled) dilutions. Viral stocks were prepared by transfecting 293T cells with plasmids encoding the luciferase virus backbone (pNL-Luc-ER) [23] and full-length gp160 encoding expression plasmids from several HIV-1 isolates obtained from the NIH AIDS Research and Reagent Program (Rockville, MD): 89.6 [24], JR-FL [25], NL4-3 [26]. The virus was mixed with various concentrations of diluted mouse sera for 30 min at room temperature. Cells were then infected with 100 μl of virus preparation containing DEAE-dextran (8 μg/ml) for 4 h at 37 °C. After five washes with PBS, fresh medium (0.2 ml) was added to each well in a 96-well plate. Cells were lysed and analyzed 44 h later for luciferase activity. All experiments were performed in duplicates using pooled sera from each treatment group.

4. Discussion

The ability to readily elicit broadly neutralizing antibodies to HIV-1 remains an elusive task in spite of significant efforts and ingenious approaches employing a myriad of antigen

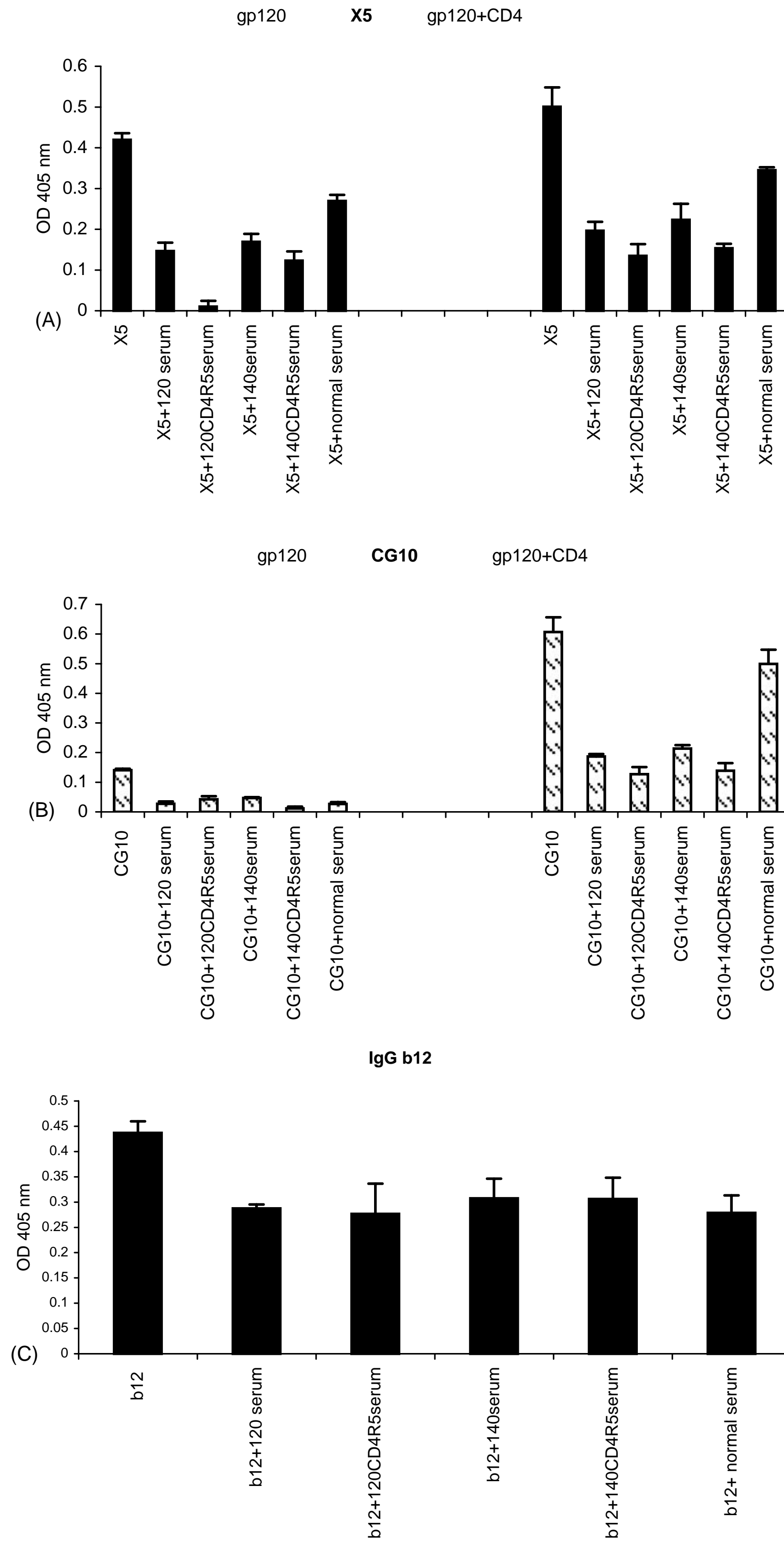

Fig. 7. Sera competition with: (A) FabX5; (B) IgG CG10 and (C) IgG1 b12 measured by ELISA The left panel shows binding of the respective mAb to gp120 in absence or presence of sera from different groups of mice including serum obtained before immunization (indicated as normal (preimmune serum)); the right panel shows the results for binding to gp120-sCD4 complexes. The micro titer plate was coated with either Env gp120 or Env gp120-CD4 complex at 100 ng per well. The plates were washed and blocked with 10% bovine calf serum (PBS with 0.05% Tween-20 and 10% FBS). Following blocking, the plates were washed, and diluted serum samples mixed with biotinylated X5, CG10 or b12 and standards, in duplicate, where added and incubated. The bound antibody was detected using Streptavidin-HRP. The experiments were performed three times.

preparations [27]. Others and we have hypothesized that interaction of Env with receptor molecules could enhance the exposure of conserved epitopes that may elicit broadly neutralizing antibodies. Although such epitopes could be only transiently exposed and difficult to access by antibodies, some of the receptor-induced epitopes may be highly conserved due to the conserved nature of the virus entry mechanism, and elicitation of high affinity antibodies to those structures could allow efficient neutralization of HIV-1 isolates in a broad fashion. In addition, recent work by Kwong et al. [28] suggested that the use of gp120-CD4 complexes as immunogens may avoid the conformational masking mechanism of evasion of humoral immune responses that is operating for gp120. Because of the complex nature of the entry process, and its neutralization, only experimentation can answer the question about the potential of receptor-induced epitopes for vaccine development. Recent experiments in non-human primates suggesting broad neutralizing activity of sera raised against gp120(gp140) complexes with sCD4 seems to support the hypothesis for immunogenicity of receptor-induced epitopes on the Env [9].

We have hypothesized that not only Env-CD4 complexes but also the Env-CD4-coreceptor complexes could expose conserved epitopes that may elicit broadly neutralizing antibodies. The Env-CD4-coreceptor complexes mediate virus entry into cells and serve as a major target for inhibitors. To begin to evaluate the potential of these complexes as vaccine immunogens, we proposed to produce relatively large amounts of purified Env-CD4-CCR5 complexes and immunize mice transgenic for human CD4 and CCR5. The use of such mice would avoid a possibility frequently discussed that anti-human CD4 antibodies are dominant in the broad neutralizing activity of antibodies elicited by Env-CD4 complexes. However, production of Env-CD4-coreceptor complexes in relatively large amounts and their characterization as immunogens remains a challenging task. We proposed several approaches for production of purified Env-CD4-coreceptor complexes in relatively large amounts sufficient to allow their characterization. We showed that the complexes are stable and retain the immunogenic properties of the HIV-1 envelope after it interacts with CD4 and CCR5. The interaction of CCR5 with gp120 in the trimolecular complex was shown by the ability of gp120 to displace RANTES (Fig. 3B). Appropriate use of mild detergent (Brij97) allowed the maintenance of the functional integrity of the coreceptor molecules. The complexes were relatively stable and did not show marked dissociation at 37 °C even for 48 h. Immunization with purified Env or Env-CD4-coreceptor complexes in Balb/c CD4/CCR5 (lck-hu-CD4/CCR5) transgenic mice generated sera with similar titers for both immunogens, suggesting that complexed gp120(gp140) retains its immunogenicity. Control CD4-CCR5 beads did not show any cross-reactive immune responses. The sera raised by Env-CD4-CCR5 complexes had on average higher neutralizing activity against several HIV-1 isolates compared to sera against Env alone.

Recently, we identified and characterized a broadly cross reactive HIV-1 neutralizing human monoclonal antibody Fab X5, selected from a phage display library using $gp120_{JR\text{-}FL}$-CD4-CCR5 complexes [16]. The exposure of the X5 epitope was enhanced by the interaction of CD4 with the Env and not affected by CCR5. We hypothesized that the role of the coreceptor in this case was to mask those CD4-induced (CD4i) epitopes that overlap the CCR5 binding site rather than induce new epitopes. The finding that sera from mice immunized with Env-CD4-CCR5 complexes elicited antibodies that competed with X5 better than sera raised against Env alone suggest that this sera may contain X5-like antibodies which may contribute to the neutralizing activity. It would be interesting to find out whether such antibodies could also be elicited by Env-CD4 complexes in these mice and whether they would have neutralizing activity similar to the sera from mice immunized with Env-CD4-CCR5 complexes (unfortunately sufficient numbers of transgenic mice for these experiments were not available). Further evaluation of Env-CD4-coreceptor complex as vaccine immunogen is required to answer this and other questions.

Recently, we described the development of tethered Envs where gp120 and gp41 are joint with flexible linkers of varying length hypothesizing that the linker would allow some degree of conformational changes induced by receptor molecules but will impose constraints thus “freezing” entry intermediates [29]. We further hypothesized that these intermediates could expose conserved Env structures that may elicit broadly neutralizing antibodies. Experiments are in progress to evaluate the potential of tethered Env alone and in complex with receptor molecules as vaccine immunogens.

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