Design of a Non-glycosylated Outer Domain-derived HIV-1 gp120 Immunogen That Binds to CD4 and Induces Neutralizing Antibodies*†‡

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The envelope glycoprotein of human immunodeficiency virus 1 (HIV-1) is composed of two polypeptide chains, gp120 and gp41, that are present on the surface of the virus as a trimer of heterodimers (1). As these are the primary viral components exposed to the external environment, they present the single best target for neutralizing antibodies. gp41 mainly contains the fusion machinery and a large part of the membrane proximal region and is transiently exposed during the fusion process (2, 3). gp120 remains largely exposed on the virus (4). However, the virus has evolved various mechanisms to evade the humoral immune response toward gp120. A very high rate of mutation, large conformational flexibility (5), and the extensive glycosylation of the surface (6, 7) are some of the important ways by which the virus has evaded a neutralizing antibody response (8). As a result, when recombinant gp120 is used as an immunogen, the antibodies generated are often directed to immunodominant epitopes present in variable loops and have limited breadth of neutralization (9–13). Core gp120 lacking the variable loops is poorly immunogenic, in part because of surface coverage by glycans (2, 14). It was recently shown that demangosylated full-length gp120 is much more immunogenic than wild-type gp120 in mouse immunization studies (15).

The structure of gp120, as seen from the crystal structure of gp120 complexed to CD4 and antibody 17b (PDB ID 1G9M) (16) reveals that the molecule can be subdivided into three distinct parts: the inner domain, the outer domain, and the bridging sheet. On monomeric gp120, the inner domain is well exposed and has little coverage by glycans (2). However, this domain is likely to be involved in interactions with gp41 (17) and is probably inaccessible on the intact envelope trimer on the virion surface and, hence, is not a good immunogen target. The bridging sheet is part of a cryptic epitope involved in coreceptor binding (16, 18, 19), which is transiently exposed upon receptor binding and is, therefore, difficult to target by antibodies. There are known antibodies to this target that have weak but relatively broad neutralizing activity.

Several conserved regions in gp120, including regions of the CD4 binding site and epitopes for the broadly neutralizing antibodies b12 (20–22) and 2G12 (20, 23, 24), are located on the outer domain. Thus, although sequence variability in some regions of this domain is quite high, it merits consideration as a possible immunogen. Previous attempts to develop an outer domain-based immunogen, however, met with little success. The outer domain construct (OD1)3 made by Yang et al. (25), composed of residues 252–482, included the V1V2 and V3 variable loops, was expressed in S2 Drosophila cells, and was glycosylated. The construct had the YU2 envelope sequence, which is deficient in T, helper epitopes, and thus, was poorly immunogenic. Antibody titers became high after attaching a

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†‡The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

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3The abbreviations used are: OD1, eukaryotic cell expressed outer domain of gp120; OD_{EC}, E. coli expressed outer domain of gp120; GdnCl, guanidine hydrochloride; ANS, 8-anilinonaphthalene-1-sulfonic acid; rcam-RNase A, reduced carboxymethylated RNase A; Mw, Mycobacterium w; env, envelope; Ni-NTA, nickel-nitrilotriacetic acid; TCID, tissue culture infectious dose.
Pan-reactive epitope for HLA-DR (PADRE) at the C terminus. However, when the V3 loop was deleted, the OD1-ΔV3-PADRE was again poorly immunogenic, indicating that the V3 loop was responsible for the observed high antibody titers. OD1 could bind 2G12 but not CD4. B12 binding also had a very high off-rate, and complete dissociation was observed within 50–60 s for all concentrations of OD1 used (26). The sera obtained after immunizing rabbits did not elicit significant neutralizing antibodies (25). Chen et al. (27, 28) expressed residues 251–481 of HIV-1 Clade C virus gp120 (clade C virus) as a C-terminal fusion to the Fc domain of human IgG1 using recombinant baculoviruses and used that to immunize mice. The monoclonal antibodies derived from the serum were mostly V3-directed. However, no neutralization studies were done with the sera.

In an alternate approach, we describe bacterial expression, biophysical and immunological characterization of an outer domain construct based on the HIV-1 HxB2 sequence. Such a bacterially expressed molecule is not glycosylated, and this may lead to better exposure of conserved epitopes, including the highly conserved CD4 binding site. This construct is hereby referred to as OD1EC. We show that in addition to binding CD4, it also binds the broadly neutralizing antibody b12 but not the non-neutralizing antibodies b6 and F105. When used as an immunogen in rabbits, the resulting sera showed neutralization against all viruses, a subtype C virus ZM109F (29) and a subtype B virus Tro.11 (30).

**EXPERIMENTAL PROCEDURES**

**Purification of Protein**—An Escherichia coli codon-optimized version of the OD1EC gene was synthesized and cloned into the pET28a(+) vector (Novagen) between the NdeI and BamHI sites and contained an N-terminal His tag. *E. coli* strain BL21(DE3) cells transformed with the plasmid were grown in 1 liter of Luria-Broth (LB) at 37 °C till an absorbance of 0.6. Cells were then induced with 1 mM isopropyl-β-thiogalactopyranoside and grown for another 6 h at 37 °C. Cells were harvested at 3500 × g and resuspended in 30 ml of phosphate-buffered saline (PBS), pH 7.4. The cell suspension was lysed by sonication on ice and centrifuged at 15,000 × g. The supernatant was discarded, and the pellet was washed in 30 ml of 0.1% Triton X-100, PBS, pH 7.4, and subjected to centrifugation at 15,000 × g. The pellet was solubilized in 25 ml of 8 M guanidine hydrochloride (GdnCl) in PBS, pH 7.4, overnight at room temperature. The solution was centrifuged at 15,000 × g for 30 min. The supernatant was bound to 3 ml of Ni-NTA beads (GE Healthcare) and washed with 30 ml 50 mM imidazole containing 8 M guanidine hydrochloride in PBS, and finally, denatured protein was eluted with 8 M GdnCl in PBS containing 500 mM imidazole at room temperature.

The first four eluted fractions (each 3 ml) were pooled together and then rapidly diluted 10-fold with PBS containing 1 mM EDTA to reduce the denaturant concentration from 8 to 0.8 M. The resulting solution was again concentrated back to the original volume in an Amicon concentrator. This was followed by desalting into PBS, pH 7.4, containing 1 mM EDTA using a HiTrap Desalting column (Amersham Biosciences) to remove the remaining denaturant. Protein was ~90% pure as assessed by SDS-PAGE. The desalted protein was concentrated to a final concentration of 0.5 mg/ml and flash-frozen in liquid nitrogen and stored in aliquots at −80 °C. The average yield was 5–6 mg/liter of culture. The yield was determined by densitometry analysis from SDS-PAGE using standard proteins of known concentrations.

**Far-UV Circular Dichroism (CD) and Fluorescence Spectroscopy**—CD spectra were recorded on a Jasco J-715C spectropolarimeter flushed with nitrogen gas. The concentration of protein sample was 10 μM and the buffer used was PBS, pH 7.4. Measurements were recorded in a 1-mm path length quartz cuvette with a scan rate of 50 nm/min, a response time of 4 s, and a bandwidth of 2 nm. Each spectrum was an average of three scans. Mean residue ellipticities were calculated as described previously (31). Buffer spectra were also acquired under similar conditions and subtracted from protein spectra before analysis.

All fluorescence spectra were recorded at 25 °C on a SPEX Fluoromax3 spectrophotometer. For intrinsic fluorescence measurements, protein concentration used was 1 μM. The excitation was at 280 nm, and emission was recorded from 300 to 400 nm. The excitation and emission slit widths were 3 and 5 nm, respectively. For the 8-anilinonaphthalene-1-sulfonic acid (ANS) binding study, the protein and ANS concentrations used were 1 and 100 μM, respectively. Samples were excited at 365 nm, and emission spectra were collected over the wavelength range 400–600 nm. Each spectrum was an average of three consecutive scans. Buffer spectra were also acquired under similar conditions and subtracted from protein spectra before analysis. All fluorescence experiments were carried out in PBS at pH 7.4.

**Proteolysis**—Proteolytic digestion of OD1EC and reduced carboxymethylated RNase A (rcam-RNase A) was carried out using a protease/substrate ratio of 1:1000 (w/w) for trypsin and 1:50 (w/w) for proteinase K. A total of 100 μg of protein was digested in 200 μl of digestion buffer (final concentration 50 mM HEPES, pH 8.0, 2 mM CaCl2) at 37 °C. At various times, 40 μl of sample was removed, and trypsin was deactivated with 0.1% formic acid, whereas proteinase K was deactivated by 5 mM PMSF. SDS-PAGE gel-loading dye (final concentration 50 mM Tris-HCl at pH 6.8 containing 2% SDS, 0.1% bromphenol blue, 10% glycerol, and 5% β-mercaptoethanol) was added, and samples were boiled for 10 min and stored at −20 °C till use. Samples collected at different time points were subjected together to SDS-PAGE on a 12% gel followed by staining with Coomassie Brilliant Blue R250.

**Trichloroacetic Acid Precipitation**—100 μl of denatured protein in 8 M GdnCl was diluted 12 times with PBS, pH 7.4. It was chilled on ice, and then 300 μl of trichloroacetic acid was added to bring it to a final concentration of 20% (v/v). It was incubated on ice for 10 min and then spun down at 14,000 rpm for 5 min. The protein pellet obtained was washed 4–6 times with 200 μl of ice-cold acetone and dried at 95 °C for 5–10 min to remove the acetone. To the dried pellet, 20 μl of 1× Tris-glycine electrophoresis buffer, pH 8.3, was added followed by 5 μl of 5× loading dye, boiled for 10 min, and then analyzed by SDS-PAGE.
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Gel Filtration Analysis—Approximately 20 μg of protein was analyzed under non-denaturing conditions by gel filtration chromatography in PBS buffer at room temperature on a Superdex-75 analytical gel filtration column. Chymotrypsinogen marker (25 kDa) having almost the same mass as OD_EC (24 kDa) was used to determine the expected position of the monomeric peak.

HPLC—25 μg of protein in PBS, pH 7.4, buffer was injected into a C18 analytical column (150 × 4.6 mm, 5-μm particle size) (Vydac) and eluted with a gradient of 5 to 95% acetonitrile containing 0.1% trifluoroacetic acid. For the reduced sample, 12 μg of protein was incubated with 6 mM GdnCl, 5 mM DTT at 37 °C before injection.

Surface Plasmon Resonance Experiments—All surface plasmon resonance experiments were performed with a Biacore 2000 (Biacore, Uppsala, Sweden) optical biosensor at 25 °C. 900 resonance units of four-domain CD4, monoclonal antibody b12, b6, or F105 was attached by standard amine coupling to the surface of a research-grade CM5 chip. A sensor surface (without CD4 or any antibody) that had been activated and deactivated served as a negative control for each binding interaction. Four different concentrations of WT gp120 or OD_EC were run across each sensor surface in a running buffer of PBS, pH 7.4, containing 0.01% P20 surfactant. Protein concentrations ranged from 12.5 to 75 nM for gp120 and from 2 to 10 μM for OD_EC. Both binding and dissociation were measured at a flow rate of 30 μl/min. In all cases the sensor surface was regenerated between binding reactions by 1–2 washes with 10 mM HCl for 30 s at 30 μl/min for b12 and b6 and with 10 mM NaOH for 30 s at 30 μl/min for CD4 and F105. Each binding curve was corrected for nonspecific binding by subtraction of the signal obtained from the negative-control flow cell. The kinetic parameters were obtained by fitting the data to the simple 1:1 Langmuir interaction model by using BIA EVALUATION 3.1 software.

Immunization Studies—Four New Zealand White female rabbits were injected subcutaneously with 100 μg of protein in phosphate buffer and boosted with 50 μg of protein 6 and 12 weeks after the initial immunization. Sera were collected 2 weeks after each injection. The animals were rested for 43 weeks after that. At week 55, they were boosted once more with 50 μg of protein and terminal bleed was collected at week 57. Two different adjuvants were used, Freund’s and an alternate mycobacterium-based adjuvant Mycobacterium w (Mw) (32, 33). Freund’s complete adjuvant was used for priming, whereas for subsequent boosts, Freund’s incomplete adjuvant was used. For the group immunized using Mw adjuvant, the same adjuvant was used throughout for priming and boosters. Mw is a heat-killed preparation of a non-pathogenic mycobacterium commercially available for immunization (Cadila Pharmaceuticals, Ahmedabad, India). For each adjuvant, two rabbits were used due to shortage of available animals. A similar study with four rabbits, two in each group for each adjuvant, was done using wild-type full-length JRCSF gp120 as the immunogen. However, one rabbit in each group for the gp120 immunogen died after the first boost.

Determination of titers of antibody against gp120 and OD_EC were carried out as follows. Enzyme-linked immunosorbent assays (ELISA) against native gp120 was performed in 96-well plates to which D7324 (2 μg/ml) (Cliniqa Corp., Fallbrook, CA) had been adsorbed after overnight incubation at 4 °C in PBS. D7324 is a sheep antibody against the C-terminal 15 amino acids of gp120 from the BH-10 strain of HIV-1 (34). Plates were washed 3 times with PBS containing 0.05% Tween 20 (PBST) and blocked with 200 μl of 3% BSA in PBST. After washing, 100 μl of JRCSF gp120 (2 μg/ml) was captured over the plate by incubating for 2 h. Alternatively, for determining the OD_EC titer, plates were directly coated with 500 ng of the native protein and then blocked with 3% BSA in PBST. Serial dilutions of serum in a total volume of 100 μl of blocking buffer were added in separate wells and incubated for 2 h at room temperature followed by 6 washes with PBS containing 0.05% Tween 20 at room temperature. For the negative control, the same serum dilutions were added to wells that did not have any gp120 or OD_EC immobilized but were blocked with 3% BSA in PBST. Bound sera were detected by using an alkaline phosphatase-conjugated goat anti-rabbit antibody (Sigma) at a dilution of 1:10,000 and the chromogenic substrate p-nitrophenyl phosphate. The reciprocal of the serum dilution showing an optical density reading more than twice that of the negative control and greater than 0.1 was taken as the antibody titer.

Depletion and Purification of IgG from Serum—The serum was incubated with Protein A-agarose beads (Amersham Biosciences) for 2 h at room temperature. The flow-through was collected. It was tested for gp120 and OD_EC binding in ELISA to confirm depletion of all antibodies.

The protein A beads obtained above were washed with two column volumes of PBS, and bound IgG were eluted with 10 mM glycine-HCl, pH 3, and immediately neutralized with 1 M phosphate buffer, pH 7. The eluates were then dialyzed against PBS, pH 7.4, concentrated 10-fold in a Centriprep concentrator (Amicon), and stored at −70 °C in aliquots. IgG concentration was estimated using SDS-PAGE by comparison of the band intensity to that of an IgG standard of known concentration.

Determination of Concentration of OD_EC-specific IgG in the Serum—300 μg of purified OD_EC in the presence of 1 mM EDTA was bound to 100 μl of Ni-NTA beads that were previously equilibrated with PBS, pH 7.4. These beads were incubated with 50 μg of purified IgG at 4 °C overnight. The beads were then washed, boiled with SDS-PAGE gel-loading buffer (final concentration 50 mM Tris-HCl at pH 6.8, containing 2.0% SDS, 0.1% bromophenol blue, 10% glycerol), and loaded on a 10% gel. The amount of IgG eluted from the beads was quantitated by comparison of the band intensity to that of an IgG standard of known concentration. A negative control experiment was performed in which the same amount of antibodies was incubated with 100 μl of Ni-NTA beads that was bound an irrelevant His-tagged protein in the presence of 1 mM EDTA. These beads were also subjected to SDS-PAGE after washing with PBS. From the difference in the amount of eluted antibodies, the specific IgG concentration in serum was calculated.

Neutralization Assays—The assay used measures neutralization of HIV as a function of reductions in Tat-regulated luciferase reporter gene expression after a single round of infection in TZM-bl cells (35, 36) essentially as described previously. Briefly, NL4-3 and 93IN101 viral stocks were made by trans-
fecting HEK 293T cells with plasmid DNA. JRFL was produced in peripheral blood mononuclear cells by infecting them with pre-existing viral supernatant. Pseudotyped viruses SC422661.8, TRO.11, REJO4541.67, and ZM109F.PB4 (from the standard reference panel of subtype B and C env clones) were made in HEK293 cells using a two-plasmid system, a backbone plasmid pSG3Δenv that expresses the entire HIV-1 genome except env along with an envelope expression vector. The env expression vectors were obtained from the NIH AIDS Research and Reference Reagent Program. The env expression vectors were co-transfected with the backbone plasmid at a ratio of 1:2 in HEK293 cells by calcium phosphate method. Spent medium was collected 48 h post-transfection, TCID-titered, and stored in aliquots at −80 °C. The sera were heat-inactivated at 56 °C for 1 h, spun briefly, and stored as aliquots at −80 °C. The virus was added to the corresponding antibody/sera dilutions to get a final virus concentration of 200 TCID₅₀ units and incubated at 37 °C for 1 h. All dilutions were performed in complete media. The lowest dilution tested was 1:10, and this was followed by sequential 4-fold dilutions. After this, the antibody/sera-virus mixture was added to 1 × 10⁵ cells/well of the 96-well plate and incubated at 37 °C in a CO₂ incubator for 48 h. The cells were washed in 1× PBS and lysed, and relative light units were measured in the luminometer after the addition of luciferin substrate (Bright-Glo™ luciferase assay system, Promega).

RESULTS

Construct Design—The design goal was an outer domain construct of gp120, which would include most of the CD4 binding site residues. The structure of gp120 (16) as it occurs when complexed to CD4 is shown in Fig. 1A. gp120 residues involved in CD4 binding are primarily located in the stretches 124–126, 279–283, 365–371, 425–430, 456–459, and 469–474. Two anti-parallel β-strands from 251–260 and 470–474 connect the inner domain with the outer domain (37). Thus, the fragment 255–474 would include the whole of the outer domain and most of the CD4 binding residues. This fragment is 191 residues long and contains three disulfide bonds between residues 296–331, 378–445, and 385–418. To determine which residues in this fragment were previously interacting with the rest of the molecule, the in-house software PREDBURASA was used in a manner similar to that described previously (38). Residues from 255–474 of gp120 were considered to be one chain (chain A) and the rest of the molecule as another chain (chain B). Accessible surface areas (ASA) of residues for chain A are calculated in the presence and absence of chain B. Residues that have a difference of 5 Å² or more in absolute ASA are considered as interacting residues. ODₑᶜ has a total accessible surface area of 9900 Å², of which 2400 Å² (24%) of this area was previously involved in interaction with other regions of gp120. This included 11 hydrophobic residues and 18 hydrophilic residues (excluding the CD4 binding site residues). Each hydrophobic residue was allowed to vary in identity to all polar residues, and the best substitution was selected on the basis of energy values. These mutations and subsequent energy calculations were done using the program ROSETTA DESIGN (Version 2.0) (39) (supplemental Table S1). The final mutations incorporated were V255T, L260N, L261D, V271S, V275N, I285T, F382R, M434K, Y435K, I439D, and L453N, as highlighted in Fig. 2, A and B. Residues 255–474, inclusive of all the mutations, is called ODEC. The WT sequence used in the design is based on the subtype B, CXCR4 tropic HXBc2 strain and, except for the indicated mutations, is identical to that of region 255–474 in PDB ID 1G9M (Fig. 1). It has the tripeptide Gly-Ala-Gly substi-
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Figure 2. A, a spacefill structure of ODEC (PDB ID 1G9M) shows the positions of the 11 surface mutations (in orange) that are present in ODEC. The residues in green are CD4 binding site residues, whereas those in blue are the asparagine residues involved in glycosylation in whole gp120. B, the same structure is shown, rotated by 180° along the vertical axis. C, shown is sequence alignment of YU2 outer domain sequence present in OD1 (25) with HxBc2 outer domain sequence in ODEC. The 11 engineered mutations are indicated in red, whereas the N-linked glycosylation sites are in blue. Besides these ROSETTA-predicted changes, there are a number of mismatches between the sequences that are the inherent differences between the YU2 and HxBc2 sequences. In addition, OD1 has the complete V3 loop, which is absent in ODEC. The region of the V4 loop is also very different in both sequences.

Protein Purification, Biophysical Characterization, and Binding Studies—The OD_EC gene was cloned in pET-28a(+) plasmid with an N-terminal His tag. The protein was expressed in E. coli BL21(DE3) cells and purified on a Ni-NTA affinity matrix after resolubilization from inclusion bodies. The yield was about 5–6 mg/liter of the culture. SDS-PAGE studies confirmed that the protein was at least 90% pure. Mass spectrometric analysis (electrospray ionization-mass spectroscopy) confirmed the identity of the protein.

The CD spectrum of the protein (Fig. 3A) showed that it has appreciable secondary structure. The protein shows a clear thermal unfolding transition with an apparent T_m of 62.3 °C (Fig. 3D). The fluorescence spectrum (Fig. 3B) of the protein shows an expected red shift and a change in emission intensity upon denaturation, showing that the protein is likely to be folded with burial of some tryptophan residues in the native state. ANS binding studies were also done with OD_EC. ANS is a fluorescent probe that binds to hydrophobic pockets present on partially denatured, molten-globule like states of proteins (40). When it binds to hydrophobic patches, there is a characteristic blue shift in its λ_max together with an increase in its fluorescence emission intensity. Relative to a previously characterized molten globule control (41), both OD_EC and full-length gp120 showed much weaker binding to ANS (Fig. 3C). This shows that OD_EC does not have large exposed hydrophobic patches. Tryptic digestion (Fig. 4, A and B) and proteinase K digestion (Fig. 4, C and D) studies of OD_EC at 37 °C showed that OD_EC is quite resistant to digestion relative to an unfolded protein (rcam-RNase A), confirming that OD_EC is a well ordered molecule.

The 11 hydrophobic to hydrophilic mutations in OD_EC were introduced to prevent the protein from aggregating. As a negative control, an E. coli codon-optimized version of the OD_EC gene without the 11 mutations (hereby referred to as WT OD_EC) was synthesized, and the protein was purified under denaturing conditions using Ni-NTA affinity chromatography as was done for OD_EC. However, on attempting to refold it by rapid dilution, there was considerable precipitation. For comparison, equal amounts of denatured OD_EC and WT OD_EC in 8 M GdnCl were diluted 10-fold, and then in both cases the pellet and the supernatant were loaded on the gel (Fig. 4E). Although OD_EC remained almost entirely in the soluble fraction, WT OD_EC was mostly in the precipitate. Thus, the 11 mutations contributed significantly to increasing the stability and reducing the aggregation propensity of the protein.

The OD_EC construct contains six cysteine residues. All six are involved in disulfide bond formation in the context of the native full-length gp120 molecule. A 5,5′-dithiobis(nitrobenzoic acid) assay showed that the percentage of free thiols in OD_EC is negligible, thereby showing the disulfides are formed. Analytical gel filtration (Fig. 5A) showed that OD_EC is a monomer. Native protein eluted from a C18 analytical reverse-phase column as a single peak (Fig. 5C), thereby showing that it is a homogeneous species in solution and not a mixture of different intensity...
disulfide bonded isomers. The denatured, reduced protein eluted at a different acetonitrile concentration than the native protein, re-confirming that native ODEC is well folded and oxidized.

**Binding Studies on Biacore**—ODEC bound soluble 4-domain CD4 with a $K_D$ of 3.3 nM (Fig. 6A), whereas the positive control full-length gp120 has a $K_D$ of 11 nM (Fig. 6B), and loop deleted core gp120 has a $K_D$ of 50 nM (26). Although ODEC binds CD4 with ~66-fold lower affinity relative to core gp120, the fact that there is measurable binding with micromolar $K_D$ is encouraging. There may be various reasons as to why ODEC shows poorer binding than full-length gp120. First, although our biophysical studies show that it is folded, it might not have adopted exactly the same structure as in the context of the whole molecule. In addition, ODEC lacks variable loops (V1V2 and V3), and as previously shown (42), the loops also contribute to binding affinity of gp120 for CD4.

Binding of ODEC to three CD4 binding site antibodies, b12, F105, and b6, was also examined (Figs. 7 and 8). It bound the broadly neutralizing antibody b12 with a $K_D$ of 12 nM at 25 °C (Fig. 7). The high $K_D$ was primarily due to the high $k_{off}$ for the binding. On lowering the temperature to 15 °C, there was a 10-fold decrease in $k_{off}$, and the $K_D$ was close to that obtained for CD4 binding at 25 °C. A comparison of all the kinetic parameters for binding is listed in Table 1. Most importantly, ODEC did not bind the CD4 binding site-directed non-neutralizing antibodies b6 and F105 to any appreciable degree. In contrast, monomeric gp120 bound both neutralizing and non-neutralizing antibodies with nanomolar values of $K_D$ (Fig. 8). To examine why ODEC does not bind F105 and b6 but binds CD4 and b12, the structures of gp120 in complex with each of CD4, b12, and F105 ligands were analyzed (Fig. 1, A–C). 28 of the 31 residues important for CD4 binding were included in ODEC; hence, CD4 binding is not surprising. All residues required for b12 binding are present in ODEC; thus, b12 binding is also expected. However, in the recently solved gp120-F105 structure (43), it is seen that F105 binds in a hydrophobic groove in between the inner and the outer domain, which is lined by res-
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idues Trp-112, Ile-109, Val-255, Met-475, Ile-371, Phe-382, Gly-473, Gly-366, Gly-367, and Pro-369. Of these residues, Trp-112, Ile-109, and Met-475 are absent in ODEC, and Val-255 and Phe-382 are mutated to threonine and arginine based on ROSETTA DESIGN calculations. Thus, with 5 of the 10 residues important for binding being absent, it is clear why ODEC does not bind F105. It is worth mentioning at this point that OD1, designed previously, also did not bind F105 and b6 (43).

**Immunization and Neutralization Studies**—Both ODEC and gp120 were used as immunogens in rabbit immunization studies. After the second booster immunization, rabbits immunized with full-length JRCSF gp120 showed titers about $8 \times 10^5$ with Freund’s adjuvant (rabbit 454) and $6.4 \times 10^6$ with the Mw adjuvant (rabbit 457). In comparison, with the ODEC immunogen, after the second boost ELISA titers were close to $2.5 \times 10^6$ with Freund’s adjuvant (rabbits 465, 466) and $2.5 \times 10^5$ with the Mw

**Figure 4**. Shown is SDS-PAGE analysis of proteolytic digests of ODEC at pH 8.0 by trypsin at 37 °C (A), rcam-RNase A at pH 8.0 by trypsin at 37 °C (B), ODEC at pH 8.0 by proteinase K at 37 °C (C), and rcam-RNase A at pH 8.0 by proteinase K at 37 °C (D). Lanes 1–6 (A and C) indicate aliquots of the ODEC digestion mixture at times 0 (undigested), 5, 15, 25, and 40 min and 1 h for trypsin and 0 (undigested), 5, 10, 25, and 40 min and 1 h for proteinase K, whereas lanes 1 and 2 (B and D) indicate those of rcam-RNase A digestion mixture are at time 0 (undigested) and at 5 min, respectively. Samples were mixed with formic acid at a final concentration of 0.1% to deactivate trypsin and 5 mM PMSF to deactivate proteinase K at the indicated times to stop the proteolysis and SDS-PAGE gel loading buffer was added. After electrophoresis, proteins were visualized by staining with Coomassie Blue. E, shown is SDS-PAGE analysis of ODEC and WT ODEC before and after refolding into PBS with 1 mM EDTA. The arrow indicates the expected position for ODEC, Lanes 1 and 4 indicate the denatured WT ODEC and ODEC, respectively, in 8 M GdnCl before refolding, whereas lanes 2 and 5 are the precipitate, and lanes 3 and 6 are the soluble fraction for WT ODEC and ODEC, respectively, obtained after refolding. The denatured protein in 8 M GdnCl was precipitated with trichloroacetic acid before loading on the SDS-PAGE.

**Figure 5**. A, analytical gel filtration analysis of ODEC on a Superdex 75 column in PBS buffer at room temperature is shown. For comparison, an equal amount of chymotrypsinogen protein, having almost the same mass (25 kDa), was separately loaded onto the column. The absorbance at 220 nm is shown as a function of the elution volume. The plot shows that ODEC elutes at the expected position for the monomer. B, shown is a calibration curve of the analytical Superdex-75 column with standard marker proteins albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (24 kDa), and RNase A (13 kDa). C, shown is reverse-phase HPLC for ODEC in an analytical C18 column (Vydac) at room temperature. 25 μg of oxidized protein (dashed line) was injected in PBS, pH 7.4, whereas for reduction, 12 μg of protein (solid line) was incubated with 6 M GdnCl and 1 mM DTT at 37 °C before loading. Proteins were eluted in a gradient of acetonitrile from 5 to 95% at a rate of 2% per minute. The intensity at 220 nm is shown as a function of the elution time. The reverse phase-HPLC shows that oxidized ODEC elutes as a single peak and is, therefore, unlikely to be a mixture of disulfide-bonded isomers. a.u., arbitrary units.
adjuvant (rabbits 467, 468). However, with both the adjuvants, for rabbits immunized with ODEC the anti-gp120 titers were about 5000 only. This may be due to either glycosylation of the gp120 surface or differences in conformation of ODIC and ODIC. Both adjuvants yielded sera with very similar neutralization titers (see below).

For the in vitro neutralization experiments, we used five subtype B viruses (NL4-3, JRFL, SC422661.8, TRO.11, REJO4541.67) and two subtype C viruses (93IN101 and ZM109F.PB4). Of the subtype B viruses, NL4-3 is a T-cell line adapted CXCR4 tropic virus and is classified as Tier-I. JRFL, SC422661.8, TRO.11, and REJO4541.67 are classified as Tier-II viruses. ZM109F.PB4 is a Tier-I subtype C virus, whereas 93IN101 is a primary Indian subtype C virus. Neutralization data are summarized in Table 2. The ODIC anti-sera neutralized four of the five subtype B and one of the two subtype C viruses tested, although with modest neutralization titers. In contrast, the gp120 anti-sera did not significantly neutralize any of the viruses tested. The lack of neutralization seen with gp120 is consistent with previous studies (31). It has been shown that immunization with gp120 protein alone does not result in neutralization of the viruses tested. The ODIC binds CD4 300-fold weaker as compared with full-length gp120 and ODIC shows binding to the neutralizing antibody b12, but shows a high koff at 25 °C. At a lower temperature (15 °C), the koff decreases 10-fold, thereby decreasing the KD.

TABLE 1

| Ligand | k_{on} | k_{off} | KD |
|--------|--------|---------|-----|
| gp120  | sCD4   | b12 (25 °C) | b12 (15 °C) | b6 | F105 |
| ODIC   | 1.9 × 10^{3} | 1.4 × 10^{3} | 1.1 × 10^{3} | 1.3 × 10^{3} | 1.5 × 10^{3} |
| gp120  | 6.8 × 10^{3} | 1.6 × 10^{3} | 2.2 × 10^{3} | 1.0 × 10^{3} | 1.5 × 10^{3} |
| ODIC   | 2.1 × 10^{-3} | 5.2 × 10^{-3} | 7.5 × 10^{-3} | 1.5 × 10^{-3} | 1.5 × 10^{-3} |
| gp120  | 2.2 × 10^{-2} | 1.9 × 10^{-2} | 7.0 × 10^{-3} | 1.5 × 10^{-3} | 1.5 × 10^{-3} |
| ODIC   | 11 | 36 | 3340 |
| ODIC   | 3300 |

# FIGURE 6

Biacore sensorgram overlays. A, shown is binding of different concentrations of full-length gp120 to surface-immobilized 4-domain CD4. Curves 1, 2, 3, and 4, respectively, indicate 75, 50, 25, and 12.5 nM concentrations of gp120. B, shown is binding of different concentrations of ODIC to surface-immobilized 4-domain CD4. Curves 1, 2, 3, and 4, respectively, indicate 9.6, 7.2, 4.8, and 2.4 μM concentrations of ODIC. Surface density, 900 resonance units; buffer, PBS, pH 7.4, 0.01% P20; flow rate, 30 μl/min. ODIC binds CD4 ~300-fold weaker as compared with full-length gp120 and 66-fold weaker relative to core gp120.

# FIGURE 7

Biacore sensorgram overlays for the binding of different concentrations of ODIC to surface-immobilized IgGb12 at 25 °C (A) and 15 °C (B). Surface density, 900 resonance units; buffer, PBS, pH 7.4, 0.01% P20; flow rate, 30 μl/min. In both the plots curves 1, 2, and 3, respectively, indicate 9.6, 7.2, and 4.8 μM concentrations of ODIC. ODIC shows binding to the neutralizing antibody b12, but shows a high koff at 25 °C. At a lower temperature (15 °C), the koff decreases 10-fold, thereby decreasing the KD.

# FIGURE 8

Biacore sensorgram overlays for the binding of 100 nM full-length gp120 (curve 1) and 8 μM ODIC (curve 2) to surface-immobilized monoclonal antibody IgGb6 (A) and monoclonal antibody F105 (B). Surface density for both the antibodies is about 900 resonance units; buffer PBS, pH 7.4, 0.01% P20; flow rate, 30 μl/min. ODIC does not bind the non-neutralizing antibodies b6 and F105, whereas for full-length gp120 there is substantial binding. RU, resonance units.
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TABLE 2
Neutralization titers (IC50) for OD EC and gp120 immune sera
Data are present by animal number.

| Virus/subtype | OD EC immunized | gp120 immunized |
|---------------|-----------------|-----------------|
|               | #465b | #466 | #467 | #468 | #454 | #457 |
| TRO11/B      | 22    | 28   | 19   | 43   | 10   | 10 |
| SC422661.8/B | 25    | 22   | 22   | 17   | 10   | 10 |
| REJO4541.67/B | 30    | 25   | 19   | 32   | 10   | 10 |
| ZM109F.PB4/C | 64    | 63   | 52   | 16   | 10   | 10 |
| NL4-3/B      | 75    | 54   | 28   | 75   | 10   | 10 |
| JRFL/B       | 10    | <10  | <10  | <10  | <10  | <10 |
| 93IN101/C    | <10   | <10  | <10  | <10  | ND*  | ND* |
| VSV-G pseudotyped virus | <10  | <10  | <10  | <10  | ND*  | ND* |

a Rabbits 465–468 were immunized with OD EC, and 454 and 457 were immunized with gp120.

b Neutralization assays were carried out using terminal bled sera and env-pseudotyped virus.

c Neutralization assays were carried out with post-dose 2 sera against infectious virus generated from full-length molecular clones of each isolate. Pre-immune sera as well as sera from rabbits immunized with an irrelevant bacterial antigen (CcdB) also showed no neutralization.

d ND, not done.

neutralization of homologous neutralization-resistant strains such as JRFL, although DNA prime-protein boost immunization results in low but measurable homologous neutralization even for JRFL (44).

To demonstrate that OD EC-directed antibodies are responsible for the observed neutralization, negative control experiments were done with pre-immune sera, serum-depleted antibodies and with nonspecific sera (obtained after immunizing rabbits with an unrelated bacterial protein CcdB). None of the negative control sera showed neutralization at dilutions of 1:10 or higher. As an additional negative control, the ability of the OD EC anti-sera to neutralize NL4-3 virus pseudo-typed with vesicular stomatitis virus envelope glycoprotein (VSV-G) was examined. No neutralization was observed. As it was not possible to test neutralization below 1:10 dilutions of the serum, we purified the whole IgG from the serum of OD EC immunized rabbit #466 and used the purified total IgG for neutralization of NL4-3 (positive control) as well as JRFL and 93IN101 viruses.

The IC50 values obtained with the purified IgGs were 266, 694, and 922 μg/ml for NL4-3, JRFL, and 93IN101, respectively. To interpret this result, it is useful to determine the approximate percentage of OD EC-specific IgG in the total purified IgG fraction. To accomplish this, OD EC was bound to Ni-NTA beads and incubated with a known amount of IgG. From the amount of antibodies that bound to the OD EC, we calculated that the amount of OD EC–specific IgG in the serum was ~9%. Thus, a 1 mg/ml concentration of the whole IgG would correspond to 90 μg/ml of the OD EC–specific IgG. All these data suggest that the OD EC immunogen is able to generate moderate levels of neutralization against primary HIV-1 isolates from both B and C clades.

DISCUSSION

The development of an effective AIDS vaccine requires a novel immunogen that would present the conserved epitopes of the envelope glycoprotein in the correct conformation. One of the most conserved regions is the receptor binding site, more commonly known as the CD4 binding site. Various monoclonal antibodies toward this site are known. The majority of these antibodies such as b6 and F105 are non-neutralizing (22, 45). IgGb12 is currently the best characterized broadly neutralizing monoclonal antibody against this site. In recent years much effort has been directed to determining commonly targeted epitopes in individuals where there is a broadly neutralizing immune response for HIV infection (46, 47). Sera have been collected from long term non-progressor patients, and adsorption of these sera to wild-type gp120 and mutants, which lack CD4 binding, showed that in several cases a large fraction of the neutralizing antibodies are directed against the CD4 binding site. These novel fractions of CD4 binding site antibodies were able to neutralize viruses that were partially or fully resistant to b12 (46). These data emphasize the importance of CD4 binding site-directed antibodies in virus neutralization.

Unfortunately, various studies have shown that full-length gp120 is not able to elicit such antibodies, although high titers of antibodies directed against the outer domain are obtained. Despite several attempts over the past decade with a variety of gp120 and gp140 derivatives, it has not been possible to design an immunogen that elicits significantly improved neutralization relative to full-length gp120 (48, 49). There have been previous attempts with gp120 immunogens toward using only the OD as the immunogen. However, these have not been successful. One such outer domain construct OD1 (25), composed of residues 252–482 of the YU2 gp120 sequence. The protein was expressed in S2 Drosophila cells and could bind 2G12 but could not bind CD4. b12 binding also had a very high off-rate of binding. In rabbit immunization studies, it was poorly immunogenic without the V3 loop, and the resulting sera did not show any significant neutralization against HIV-1KBR or HIV-1YU2.

There have been more recent attempts (50) to produce soluble OD1 constructs by expression on 293 cells. However, these aggregated and failed to bind b12. This aggregation is probably because of the large hydrophobic surface that is exposed upon removal of the inner domain. This problem could be overcome by making a membrane-anchored outer domain (50). This construct had residues 252–482 from the HIV-1 clade B virus, TAI R3A, that is highly sensitive to neutralization by IgGb12. The V1V2 loop and β20-β21 hairpin was deleted in this, and the V3 loop was truncated leaving nine amino acids on each side of the V3 stem. It was fused at its N terminus to the IL-2 signal peptide sequence and at its C terminus to the human CD4 transmembrane domain. It was able to bind b12 when expressed on the surface of 293F cells and could also selectively absorb the neutralizing antibody fraction from broadly reactive sera. However, it was not used as an immunogen.

In contrast to the above mammalian expressed OD constructs, the bacterial construct described in this work could be refolded easily to a monomer from inclusion bodies. This is likely to be a result of the 11 designed mutations in the interface of the inner domain and the outer domain that were intended to prevent aggregation. A control construct lacking these mutations was highly aggregation prone (Fig. 4E). Not only did OD EC bind CD4, it also bound the neutralizing antibody b12 and did not bind the non-neutralizing antibodies b6 and F105. Because the protein was expressed in E. coli, it was unglycosylated. Numerous previous studies have suggested that proper glycosylation is essential for gp120 fold-
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