THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 290, NO. 14, pp. 9195–9208, April 3, 2015


# Antigenicity and Immunogenicity of a Trimeric Envelope Protein from an Indian Clade C HIV-1 Isolate*

Received for publication, October 23, 2014, and in revised form, February 9, 2015 Published, JBC Papers in Press, February 17, 2015, DOI 10.1074/jbc.M114.621185

**Rangasamy Sneha Priya[‡], Menon Veena[§], Irene Kalisz[§], Stephen Whitney[§], Dhopeshwarkar Priyanka[§], Celia C. LaBranche[¶], Mullapudi Sri Teja[‡], David C. Montefiori[¶], Ranajit Pal[§], Sundarasamy Mahalingam[‡1], and Vaniambadi S. Kalyanaraman[§]**

*From the [‡]Laboratory of Molecular Virology and Cell Biology, Department of Biotechnology, Bhupat and Jyoti Mehta School of Biosciences, Indian Institute of Technology Madras, Chennai 600036, India, [§]Advanced Bioscience Laboratories Inc., Rockville, Maryland 20850, and [¶]Department of Surgery, Duke University Medical Center, Durham, North Carolina 27710*

**Background:** The RV144 trial has established the importance of studying diverse HIV-1 envelopes for immunogen design.
**Results:** HIV-1 93IN101 gp145 exposes conserved regions of envelope protein and induces broadly neutralizing antibodies.
**Conclusion:** Characterization of trimeric gp145 from an Indian isolate demonstrates its potential use as a multiclade candidate HIV-1 vaccine to combat AIDS.
**Significance:** Findings encourage studies on various Indian clade C envelope proteins.

**Human immunodeficiency virus type 1 (HIV-1) isolates from India mainly belong to clade C and are quite distinct from clade C isolates from Africa in terms of their phylogenetic makeup, serotype, and sensitivity to known human broadly neutralizing monoclonal antibodies. Because many of these properties are associated with the envelope proteins of HIV-1, it is of interest to study the envelope proteins of Indian clade C isolates as part of the ongoing efforts to develop a vaccine against HIV-1. To this end, we purified trimeric uncleaved gp145 of a CCR5 tropic Indian clade C HIV-1 (93IN101) from the conditioned medium of 293 cells. The purified protein was shown to be properly folded with stable structure by circular dichroism. Conformational integrity was further demonstrated by its high affinity binding to soluble CD4, CD4 binding site antibodies such as b12 and VRC01, quaternary epitope-specific antibody PG9, and CD4-induced epitope-specific antibody 17b. Sera from rabbits immunized with gp145 elicited high titer antibodies to various domains of gp120 and neutralized a broad spectrum of clade B and clade C HIV-1 isolates. Similar to other clade B and clade C envelope immunogens, most of the Tier 1 neutralizing activity could be absorbed with the V3-specific peptide. Subsequent boosting of these rabbits with a clade B HIV-1 Bal gp145 resulted in an expanded breadth of neutralization of HIV-1 isolates. The present study strongly supports the inclusion of envelopes from Indian isolates in a future mixture of HIV-1 vaccines.**

The development of an effective vaccine against HIV-1[2] has been a major challenge in the containment and eradication of the acquired immunodeficiency syndrome (AIDS) pandemic. Although a significant broadly effective cellular immune response has been achieved using HIV-1 *gag* gene-based viral vectors (1–3), it has been difficult to elicit a significant humoral immune response resulting in induction of broadly neutralizing antibodies (bNAbs) capable of conferring sterilizing immunity against HIV-1. Efforts toward the latter have been mainly directed against the HIV-1 envelope (Env) protein, which consists of glycoproteins gp120 and gp41 existing as non-covalently bound trimers on the surface of the virus. The vaccine strategies have been complicated by the high genetic variability of *env* among the global isolates of HIV-1 as well as the evolution of neutralization-resistant viruses within an individual during the course of infection. Most of the Env-based vaccines, which have been tested in preclinical studies with non-human primates and in human clinical trials, have failed to generate bNAbs (4–6). However, ~20% of individuals chronically infected with HIV-1 develop bNAbs over a period of 3 years. Several monoclonal antibodies that neutralize a broad spectrum of isolates from different clades of HIV-1 have been isolated from such individuals (7, 8). Interestingly, it has been shown in macaque animal models that a transfusion of a mixture of such bNAbs can protect against viral transmission if they are present at the time of challenge (9–15). Thus it should be possible to achieve protective immunity against HIV-1 with an appropriate vaccine regimen involving induction of both strong humoral and cellular immune responses against HIV-1. These bNAbs mostly bind to the conserved sites on the Env gp120 or gp41 essential for viral fitness, such as the CD4 binding site, co-receptor binding site, or fusion intermediate state (7). Although a lot of emphasis is directed toward the CD4 binding site antibodies based on gp120 immunogens (16–18), the conserved CD4-induced transition form of gp120-gp41 trimer has not received enough attention. The gp120-gp41 complex becomes a six-helical bundle at the time of virus attachment to the cells through interaction with the primary receptor CD4 and the co-receptor CCR5 or CXCR4. This transitional state, which occurs during the process of infection, lends itself to attack by neutralizing antibodies and thereby prevention of infection. However, many of

* This work was supported in part by Grant DST/TSG/ME/2010/50(G) from the Department of Science and Technology, Government of India (to S. M.) and a fellowship from the Ministry of Human Resource and Development, Government of India (to R. S. P.).
[1] To whom correspondence should be addressed. Tel.: 91-44-2257-5116; Fax: 91-44-2257-4102; E-mail: mahalingam@iitm.ac.in.
[2] The abbreviations used are: HIV-1, human immunodeficiency virus type 1; bNAb, broadly neutralizing antibody; Env, envelope; $IC_{90}$, concentration for 90% inhibition of infection; gp, glycoprotein; sCD4, soluble CD4; $K_D$, dissociation constant; $TCID_{50}$, median tissue culture infective dose.

these conserved sites are not easily accessible as they are protected by extensive glycosylation and are presented as conformation-specific quaternary epitopes on the native trimer. To generate recombinant stable trimeric immunogen, various strategies have been used so far. Most studies have relied on abolishing the gp120-gp41 cleavage of precursor gp160 to express their soluble form, gp140, with or without additional trimerization domains (16). These immunogens, which form stable gp140 trimers, have been in animal model systems (19). Recent reports on the antigenicity of disulfide-linked cleaved trimers called SOSIP trimers of an African clade A Env have been shown to bind well with a number of potent neutralizing human monoclonal antibodies (20).

The partial success of the RV144 HIV-1 vaccine trial has demonstrated a vital role of purified envelope proteins in future AIDS vaccine design (21). The vaccine regimen used in the RV144 trial consisted of a recombinant canary pox vector expressing the *gag* and *env* genes of HIV-1 and a mixture of clade B and E HIV-1 envelope proteins. The trial was conducted in 14,000 volunteers from a high risk population in Thailand and had an efficacy of 31.2% (22). Such a protective response was not seen when the recombinant canary pox vector or the HIV-1 gp120 proteins alone were used in human clinical trials (5). Although neutralizing antibodies to HIV-1 were not detected in the RV144 vaccinees, a strong positive correlation was seen between levels of binding antibodies to the V2 domain of gp120 with protection in the trial participants (23). This showed that, in addition to the neutralizing antibodies, non-neutralizing binding antibodies to gp120 play a poorly understood but important role in the efficacy of the AIDS vaccine. Thus the search is still on to identify a potential envelope immunogen that either alone or as a mixture will enhance the efficacy of such vaccines.

In view of the extensive variations in the sequences of HIV-1 isolates of different clades, it is important to understand the antigenic and immunogenic properties of the envelope proteins of HIV-1 from different geographical areas and serotypes. In this regard, the most widely studied envelope proteins are from HIV-1 clade B followed by the African clades A and C (17, 20, 24–30). Indian clade C envelope proteins have not been well characterized for their structural and immunological properties. Genetic analysis of the gp160 sequences shows that the Indian clade C HIV-1 isolates have not diverged much and form a closely associated branch in the phylogenetic tree (31–33). They are also unique in that they are difficult to neutralize by many bNAbs and are categorized mostly as Tier 1B or Tier 2 (34). Recent studies indicate that the Indian clade C viruses are mostly resistant to the CD4 binding site antibody b12 (32, 35–37), suggesting possible differences in the Env conformation. The membrane-proximal external region-targeting antibody 10E8 has a unique feature of no autoreactivity, and it neutralizes 98% of tested viruses (38). Mutation of a highly conserved Asp at position 671 renders the virus resistant to 10E8. Sequence analysis of Indian clade C HIV-1 showed the presence of Ser at position 671 instead of Asp, indicating that these variant viruses may belong to the 1.17% 10E8-resistant group (32, 36). Hence it is of interest to study the gp120 and gp145 trimers from Indian isolates of HIV-1 to understand their role in global AIDS vaccine strategies.

In the present study, we produced conformationally stable trimers of uncleaved gp145 (gp120 with the ectodomain of gp41) of HIV-1 93IN101 isolated from a chronically infected Indian patient (39). The stability of trimeric envelope and its binding to known bNAbs, which are essential criteria for mimicking the native viral surface trimers, were examined. Immunization of rabbits with 93IN101 gp145 trimer elicited a high titer antibody response capable of neutralizing a number of clade B and clade C isolates. Moreover, sequential boosting of 93IN101 gp145-immunized rabbits with clade B HIV-1 Bal gp145 protein broadened the neutralizing antibody response to a few Tier 2 isolates. These results suggest that trimeric Env protein of Indian HIV-1 isolates may represent a unique candidate to include in future vaccine designs to combat HIV-1 infection.

## EXPERIMENTAL PROCEDURES

*Antigens, Antibodies, Plasmids, and Cell Lines*—Broadly neutralizing human monoclonal antibodies (mAbs) PG9, PG16 (40), 4E10 (41), and 2F5 (42) were purchased from POLYMUN Scientific GmbH. mAb 17b (43) was a gift from Dr. James E. Robinson. HIV-1 gp120 mAb b6 was a gift from Dr. Dennis Burton. The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health: HIV-1 gp120 mAb (2G12) from Dr. Hermann Katinger (44), HIV-1 gp120 mAb (IgG1 b12) from Dr. Dennis Burton and Carlos Barbas (45), HIV-1 gp120 mAb (VRC01) from Dr. John Mascola (46), HIV-1 mAb NIH 45–46 G54W IgG (47), pCAGGS SF162 gp160 from Drs. L. Stamatatos and C. Cheng-Mayer (48), and TZM-bl cells from Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme Inc. (49). GS015 (50) envelope plasmid was provided by Dr. Vicky Polonis. Four-domain human soluble CD4 (sCD4) purified from stable CHO cells was obtained from Advanced BioScience Laboratories Inc. (51). HIV-1 Bal gp145 (GenBank™ accession number M63929) was also obtained from Advanced BioScience Laboratories Inc.

*Envelope Plasmid Construction*—HIV-1 gp120 and gp145 sequences used in the study were derived from the infectious molecular clone 93IN101, a CCR5 tropic clade C virus isolated from a patient chronically infected with HIV-1 in India (GenBank accession number AY669738.1). The schematic representations of DNAs used in the study are shown in Fig. 1. The codon-optimized gp120 and gp145 sequences were cloned into the expression vector under the control of a CMV promoter in-frame with an N-terminal tissue plasminogen activator signal peptide sequence. The vector also encodes a puromycin resistance gene as a selectable marker. The gp120-gp41 primary and secondary cleavage sites were mutated by replacing arginine with serine residues at positions 503 and 511 to abolish the cleavage of gp145 protein. The modifications were introduced by means of site-directed mutagenesis using *Pfu* Turbo polymerase (Stratagene).

*Development of HEK-293 Cells Expressing 93IN101 gp120 and gp145*—HEK-293 cells were transfected with the expression plasmid containing gp120 or gp145 using Lipofectamine

2000 (Life Technologies) following the manufacturer's protocol. After 24 h of transfection, the cells were trypsinized and seeded into 96-well flat bottom plates with 1000 cells/well. Twenty-four hours later puromycin was added to the medium to a final concentration of 5 μg/ml. After 4 days, cells were fed by replacing 100 μl of medium with 100 μl of fresh medium containing puromycin. The wells with stable clones were screened for gp120 or gp145 expression using an HIV-1 gp120 antigen capture assay (Advanced BioScience Laboratories Inc.). High envelope-producing single cell clones were selected by limiting dilution and adapted to grow in serum-free medium (293-SFMII, Life Technologies) with 10 μg/ml puromycin. For gp145 production, the cells were grown in hollow fiber units (Fibercell Systems) with 30,000-Da molecular mass-cutoff filters. The supernatants were harvested from the extracellular space and used for the purification of gp145 trimer.

*Purification of 93IN101 gp120 and gp145*—The gp120 and gp145 proteins were purified by affinity chromatography from the clarified culture using *Galanthus nivalis* lectin-agarose beads (Vector Laboratories) in buffer with 0.1% Triton X-100, 500 mM NaCl, and 10 mM Tris-HCl, pH 8. A Poly-Prep column (Bio-Rad) was packed with 5 ml of *G. nivalis* lectin-agarose beads, and the concentrated, buffered culture supernatant was passed through the column under gravity flow. After washing with PBS, gp120 and gp145 were eluted using 400 mM α-methyl mannopyranoside (Sigma). Nonspecific proteins were further removed by anion-exchange chromatography using Mono Q-Sepharose beads (GE Healthcare). At 150 mM NaCl, only the nonspecific proteins bound to the Mono Q beads, and gp145 was recovered in the flow-through fraction. A gel filtration chromatography step was introduced to selectively purify the trimeric gp145 fraction (52). Two columns were connected in tandem: Superdex 200 followed by Superose 6 (GE Healthcare). Columns were equilibrated using 1× PBS with 0.5 M NaCl followed by injection of sample containing 12–13 mg of gp145 in a 1-ml volume. Fractions were separated by blue native PAGE as described below. The fractions with only trimers were pooled together and concentrated using Amicon 50-kDa-cutoff centrifugal filters (Millipore). The concentrated protein fraction was passed through PD-10 gel filtration column (GE Healthcare) to buffer-exchange into PBS. The purified 93IN101 gp120 and gp145 were stored in aliquots at −80 °C. All protein concentrations were determined by a Bradford protein assay kit (Pierce). Endotoxin levels were checked in the purified trimeric 93IN101gp145 using a *Limulus* amebocyte lysate assay (Lonza).

*SDS-PAGE, Blue Native PAGE, and Ethylene Glycol Bis(succinimidyl succinate) Cross-linking Analysis*—Purified proteins were resolved in 4–16% Tris-glycine-SDS Criterion™ gels (Bio-Rad). For native PAGE, Novex 4–15% Bis-Tris gels (Life Technologies) were used according to the manufacturer's instructions. Ethylene glycol bis(succinimidyl succinate) cross-linking was performed as described by Chen *et al.* (53). The cross-linked proteins were analyzed on 3–8% Tris acetate-SDS gels under reducing conditions.

*Circular Dichroism*—Far-UV spectral analysis was performed using a J815 CD spectrometer (Jasco). Each protein was loaded at a concentration of 0.35 mg/ml in water, and the spectrum was recorded from 250 to 190 nm at 20 °C. An average value of three scans was plotted after subtracting the background value taken in water.

*ELISA*—For testing antigenicity, Nunc 96-well Maxisorb Immuno Module (Thermo Scientific) plates were coated overnight at 4 °C with 125 ng/well gp145 plus 125 ng/well bovine serum albumin (BSA) in a final volume of 100 μl/well using 50 mM bicarbonate buffer, pH 9.6. The coating solution was aspirated, and the wells were blocked for 1 h at room temperature with 200 μl of SuperBlock solution (GE Healthcare). Serial dilutions of human monoclonal antibodies in the diluent (Advanced BioScience Laboratories Inc.) were added to the wells and incubated for 1 h at 37 °C. The plates were then washed four times in PBST (1× PBS, 0.05% Tween 20) followed by 1-h incubation at 37 °C with HRP-conjugated antibody to human IgG (Kirkegaard and Perry Laboratories, Inc.). The wells were washed four times with PBST, and 100 μl/well 3,3′,5,5′-tetramethylbenzidine substrate (Kirkegaard and Perry Laboratories, Inc.) was added to the wells. The reaction was stopped after 30 min with 100 μl/well 2 N sulfuric acid. Absorbance at 450 nm was then measured using a Molecular Devices spectrophotometer. For 17b ELISA, 17b antibody was coated along with BSA as described above. After blocking, various dilutions of gp145 or gp120 with or without preincubation with CD4 were added to the wells. The bound gp120 or gp145 was detected with human anti-gp120-HRP conjugate (Advanced BioScience Laboratories Inc.). For peptide ELISA, 96-well Nunc plates were coated with 200 ng/well peptide, and the ELISA was performed as described above. The V1, V2, and V3 peptides of 93IN101 gp120 were used in ELISA and neutralization assays (V1 peptide, RNVSRNVSSYNTYNGSVEEIKNC; V2 peptide, SFNATPEVRDRKQRMYALFYGLDIVPLNKKNSSENSSEYRLINC; V3 peptide, TRPNNNTRKSIRIGPGQTFYATGDIIGDIRQAHC).

For full-length gp160 linear peptide ELISA, 15-amino acid-long linear peptides with 11-amino acid overlap were synthesized for the complete 93IN101 gp160 protein sequence (Infinity Biotech and Resource Inc.). Each peptide was coated in individual wells of 96-well ELISA plates overnight at a concentration of 500 ng/well. Plates were blocked for 1 h at room temperature. Rabbit serum diluted 1:50 in diluent was added to all wells of the peptide coated 96-well plates. After incubation at 37 °C for 1 h, the plates were washed four times with PBST buffer. Secondary incubation was performed in the presence of HRP-conjugated anti-rabbit antibody. The wells were then washed with PBST four times followed by addition of 100 μl/well aqueous 3,3′,5,5′-tetramethylbenzidine substrate. Color development was stopped using 2 N sulfuric acid after 30 min. The absorbance was measured at 450 nm.

*Kinetics of gp145 Binding to Antibodies and CD4*—The interaction of 93IN101 glycoproteins with CD4 and various monoclonal antibodies was measured using the Octet RED biolayer interferometry system (Pall ForteBio Corp.). The proteins were biotinylated (1:1 ratio) with EZ-Link NHS-PEG-4-biotin according to the manufacturer's protocol (Thermo Scientific). Streptavidin sensors were loaded at 1000 rpm shaking using 10 μg/ml biotinylated gp120 or gp145. Baseline was established using 1× kinetics buffer (PBS with 0.02% Tween and 0.1 mg/ml BSA). Association was carried out by dipping the loaded sensors in various concentrations of IgG in 1× kinetics buffer for

300, 600, or 900 s depending on the on-rate of each IgG. Dissociation was observed by dipping in buffer alone. Alternatively, biotinylated sCD4 was attached to the streptavidin sensor, and the kinetics of binding was analyzed with varying concentrations of gp120 or gp145 in solution.

*Rabbit Immunizations*—Rabbits were housed in the animal facility of Advanced BioScience Laboratories Inc. under protocols approved by the Institutional Animal Care and Use Committee. Four weight- and age-matched female New Zealand White rabbits were prescreened for background reactivity before being immunized thrice with 50 $\mu$g of 93IN101 gp145 in 1 ml of Adjuplex (Sigma) adjuvant once every 4 weeks. Inoculation was carried out as follows: 300 $\mu$l intradermally (50 $\mu$l into each of six sites), 600 $\mu$l intramuscularly (300 $\mu$l into each hind leg), and 100 $\mu$l subcutaneously (neck region). Serial bleeds were collected 2 weeks after each immunization. After the last 93IN101 gp145 immunization, gp145-specific IgG levels were monitored for 10 weeks followed by four HIV-1 Bal gp145 protein immunizations in a similar fashion. Another group of four rabbits was immunized with HIV-1 Bal gp145 four times with an interval of 4 weeks each as an additional control.

*gp145-specific IgG Quantification*—93IN101 gp145 was coupled to cyanogen bromide-activated Sepharose beads (GE Healthcare) according to the manufacturer's guidelines. Poly-Prep columns were packed with the gp145-coupled Sepharose beads. Pooled terminal bleeds from all four rabbits (1 ml each) were loaded onto the column and eluted in MAPSII elution buffer (Bio-Rad). The purified IgG was quantified by Bradford assay and used as a standard curve for gp145-specific serum IgG quantification in ELISA. gp145 (125 ng/well) and BSA were coated on 96-well plates overnight. Following 1 h of blocking at 37 °C, various dilutions of serial bleeds of rabbits postimmunization were incubated in 37 °C. After four PBST washes, anti-rabbit IgG-HRP conjugate (Kirkegaard and Perry Laboratories, Inc.) was added at 1:20,000 dilution, and absorbance was measured as described above.

*Pseudovirus Neutralization*—To make autologous pseudovirus, a *rev-vpu-env* cassette was amplified from the full-length molecular clone of 93IN101 and cloned using the pcDNA3.1 Directional TOPO cloning kit (Invitrogen) as described previously (54). HEK-293T cells were co-transfected with Rev/Env-containing vector and pSG3Del Env. Supernatant was harvested 48 h after transfection and stored in aliquots at −80 °C. Serial dilutions of purified rabbit IgG were mixed with 200 tissue culture $ID_{50}$/ml pseudovirus and incubated for 1 h followed by addition of 10,000 TZM-bl cells/well in 96-well plates along with DEAE-dextran at a final concentration of 10 $\mu$g/ml. Luciferase activity was measured 48 h after infection using the Bright-Glo luciferase assay system (Promega).

*Peptide Absorption of Neutralizing Antibodies*—IgG from various rabbit sera was purified using a Protein A-agarose column (Bio-Rad). In 96-well plates, 50 $\mu$g/ml IgG was serially diluted in DMEM (1:2) followed by incubation at 37 °C for 1 h with and without 15 $\mu$g/ml V1, V2, or V3 peptide. GS015 pseudovirus was diluted in DMEM to 200 tissue culture $ID_{50}$/ml and added to the wells followed by 1-h incubation at 37 °C in a $CO_2$ incubator. TZM-bl cells (10,000 cells/well) were then added with DEAE-dextran at a final concentration of 10 $\mu$g/ml. Luciferase activity was measured after 48 h of infection as described above.

## RESULTS

*Expression and Purification of 93IN101 gp120 and gp145*—Indian clade C HIV-1 gp120 and gp145 proteins (see schematic representation in Fig. 1*A*) were expressed in HEK-293 cells and purified as described under "Experimental Procedures." The initial step of purification by lectin affinity chromatography resulted in a mixture of dimers and trimers as shown in native PAGE analysis (Fig. 1*B*). The trimeric fraction was then purified by gel filtration on Superdex 200 and Superose 6. The SDS-PAGE analysis indicates that the purified gp120 and gp145 proteins are more than 90% pure. The structure of the pooled trimeric gp145 was further established by intramolecular cross-linking of gp145 with the bifunctional cross-linker ethylene glycol bis(succinimidyl succinate) followed by SDS-PAGE analysis under reducing conditions. Under saturating amounts of the cross-linker, only the trimer band was noted (Fig. 1*B*), thereby confirming that the purified gp145 was predominantly trimeric. The purified trimeric gp145 was used for all further studies described below and is referred to as gp145. The purified trimeric 93IN101 gp145 contained less than 20 units of endotoxin/mg (as measured using a *Limulus* amebocyte lysate assay), which did not cause any adverse effects during animal immunizations.

*Analysis of gp120 and gp145 by Circular Dichroism*—The secondary structure analysis and the proper folding of purified gp120 and gp145 were examined by circular dichroism at room temperature. Both gp120 and gp145 had similar folded structures typically consisting of $\alpha$ helices and $\beta$ sheets (Fig. 2), indicating that during purification the structures of these proteins were preserved. This was critical for the protein-protein interaction experiments.

*Characterization of CD4 and Co-receptor Binding Sites on Purified Env*—Interaction of CD4 with the purified gp120 and gp145 was examined by biolayer interferometry (Octet RED) with biotinylated CD4 bound to the sensors and 93IN101 gp120 and gp145 in solution. Serial dilutions of either 93IN101 gp120 or gp145 were incubated with the CD4-bound sensors and the dissociation constant ($K_D$) was determined. The results in Fig. 3 indicate that 93IN101 gp145 binds sCD4 with 200-fold higher affinity than gp120 (0.15 *versus* 30.73 nM). This higher affinity is due to 10-fold faster association (1.8E+05 *versus* 0.2E+05 1/Ms) and 4-fold slower dissociation rate (0.24E−04 *versus* 6.62E−04 1/s) of gp145 from the CD4-bound sensor. This may be due to the cooperative binding of CD4 to the trimeric gp145 compared with gp120 as reported previously (55).

The human monoclonal antibody 17b binds to the CD4-induced co-receptor overlapping site on gp120. The conformational change in gp120-gp41 trimers on the virion surface induced by CD4 binding leads to the exposure of the CCR5 or CXCR4 co-receptor binding site. To explore the co-receptor binding site in the purified Envs, the binding of 17b to 93IN101 gp120 and gp145 was examined. For this purpose, the 17b antibody was immobilized on Octet Protein *G* sensors, and the interactions were measured with both gp120 and gp145 in the

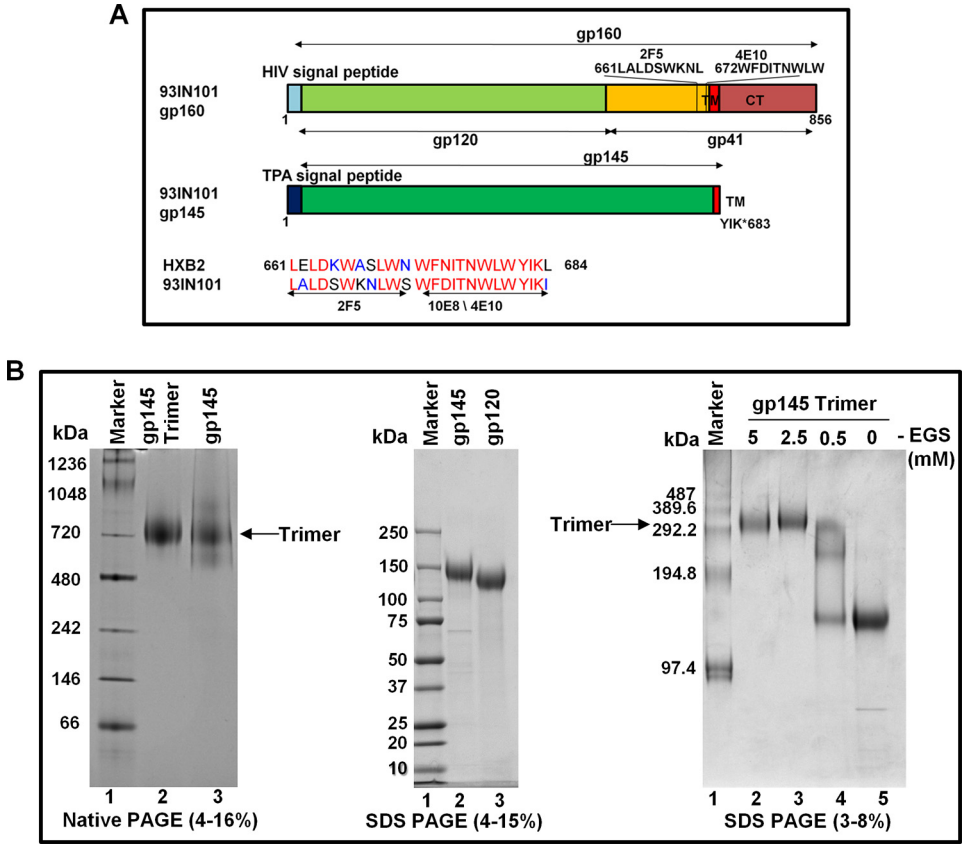



FIGURE 1. **Design and purification of trimeric 93IN101 envelope immunogen.** *A*, schematic representation of 93IN101 full-length gp160 showing the presence of the original signal peptide and intact 4E10 epitope. *TM*, transmembrane; *CT*, cytoplasmic tail. The amino acid residues are numbered according to HXB2 numbering. 93IN101 gp145 immunogen with a tissue plasminogen activator (*TPA*) signal peptide, abolished protease cleavage site, and cytoplasmic tail truncation is shown. A sequence alignment of 2F5-10E8 and 4E10 epitopes from HXB2 and 93IN101 is also shown. *B*, gel electrophoresis of purified gp120 and gp145 proteins. In native PAGE, pooled trimeric fractions of gp145 after gel filtration (*lane 2*) were compared with starting material (*lane 3*). 93IN101 gp145 (*lane 2*) and gp120 (*lane 3*) proteins were purified from stable HEK-293 cells and resolved by SDS-PAGE. Purified gp145 was analyzed under reducing conditions in SDS-PAGE after cross-linking with the indicated concentrations of ethylene glycol bis(succinimidyl succinate) (*EGS*). In all gels, 5 $\mu$g of protein was loaded per lane.

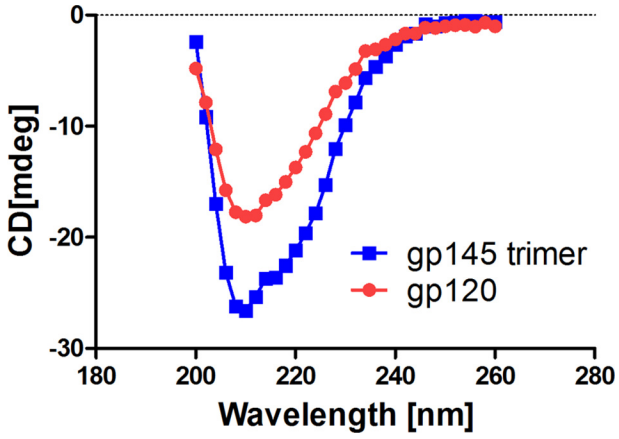



FIGURE 2. **Far-UV circular dichroism spectral analysis.** Purified 93IN101 gp120 and gp145 trimers were analyzed by CD at room temperature in PBS. *mdeg*, millidegrees.

presence of a 2-fold molar excess of sCD4. As seen from the sensorgrams in Fig. 3*B*, the rate of dissociation of bound gp120 and gp145 from the 17b-immobilized sensors is very slow both in the presence and the absence of CD4. Nevertheless it is evident that in the presence of sCD4 there is an increase in the rate of association of 17b with gp120 as well as gp145, demonstrating an intact co-receptor binding site and conformational flexibility of the purified proteins.

The CD4 binding site is the target for a number of broadly neutralizing human monoclonal antibodies (56). This site is highly conserved among HIV-1 isolates across different clades from various geographical regions. It is of interest to compare the affinity of known CD4 binding site antibodies to gp120 and gp145 of 93IN101 virus. The binding of neutralizing (b12, VRC01, and NIH 45–46 G54W) and non-neutralizing (b6) CD4 binding site antibodies to 93IN101 gp120 and gp145 was analyzed by ELISA using gp120- or gp145-coated plates (Fig. 4*A*). Comparable binding of b6 and NIH 45–46 G54W to both gp120 and gp145 was observed, but the neutralizing monoclonal antibodies b12 and VRC01 bound only to gp145, suggesting a conformational exposure of the site in gp145 but not in gp120. When the binding kinetics was assayed using Octet, all four CD4 binding site antibodies had a 2-fold faster $k_{on}$ to gp145 as compared with gp120 (Fig. 4*B*). Thus both gp120 and gp145 seem to have an intact CD4 binding site with better exposure in gp145 as it bound consistently to all antibodies tested.

*Recognition of 93IN101 gp145 by Glycosylation-dependent Human Monoclonal Antibodies*—It is well known that lentivirus envelope proteins are glycosylated, and in particular, this post-translational modification helps HIV-1 to escape from

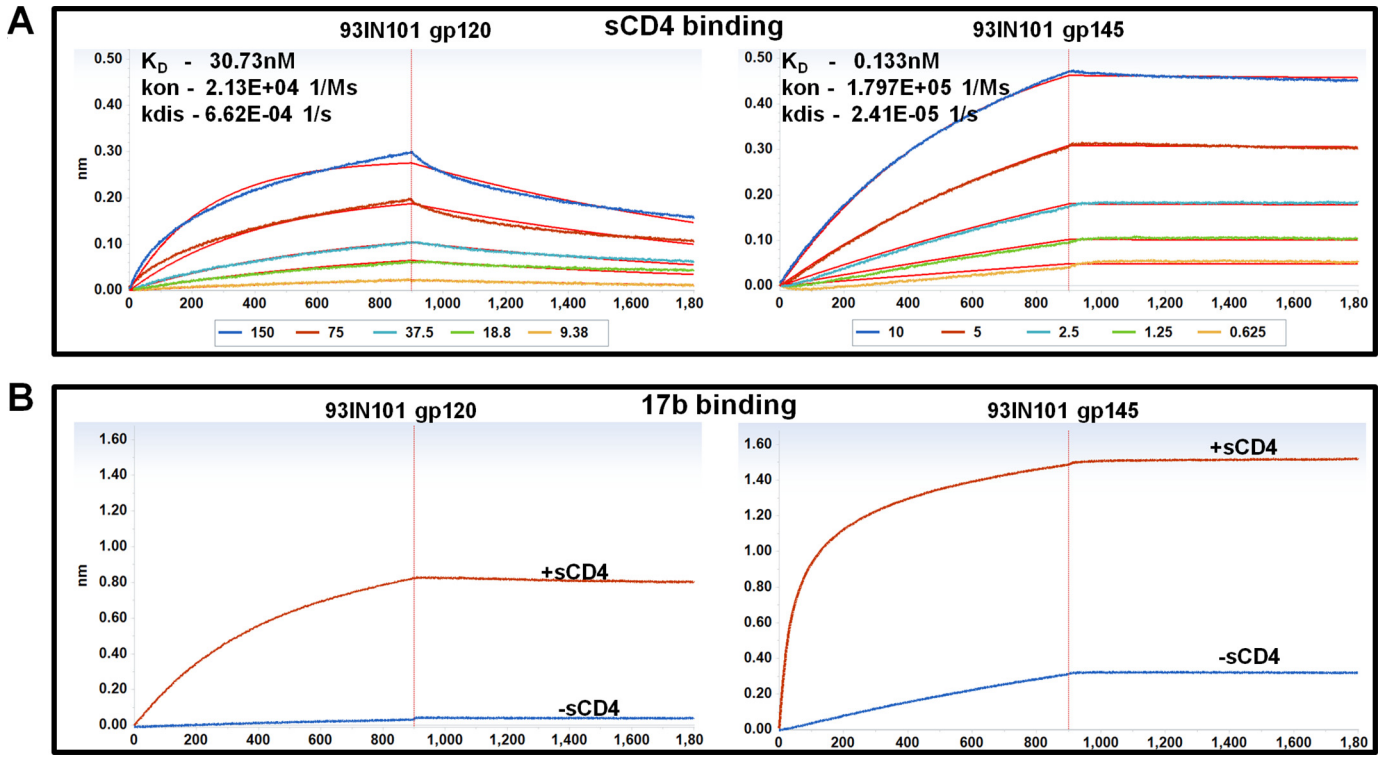
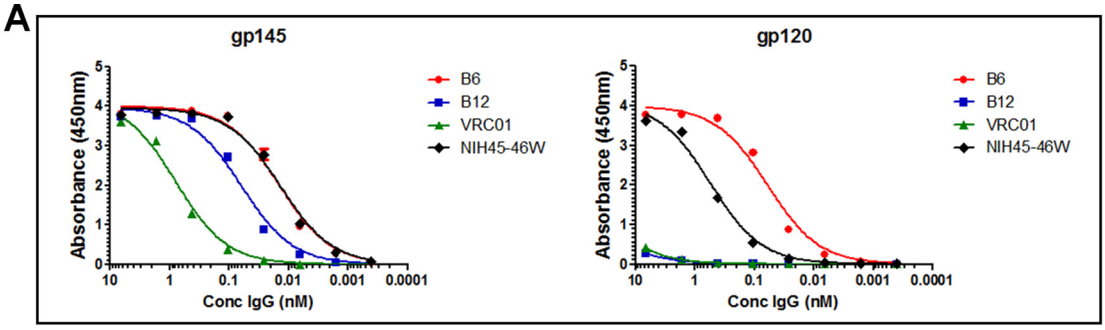



FIGURE 3. **Receptor and co-receptor binding site analysis using Octet RED biolayer interferometry.** *A*, Octet sensorgrams generated by binding of serial dilutions of purified envelope gp120 and gp145 in solution to biotinylated sCD4 immobilized on streptavidin sensors. *kon*, rate of association; *kdis*, rate of dissociation. The *red lines* denote 1:1 global curve fits. *B*, exposure of co-receptor binding site antibody 17b epitope upon CD4 binding to gp120 and gp145 proteins of 93IN101. Env concentrations were 100 nM, and the Env:sCD4 molar ratio was 1:2.



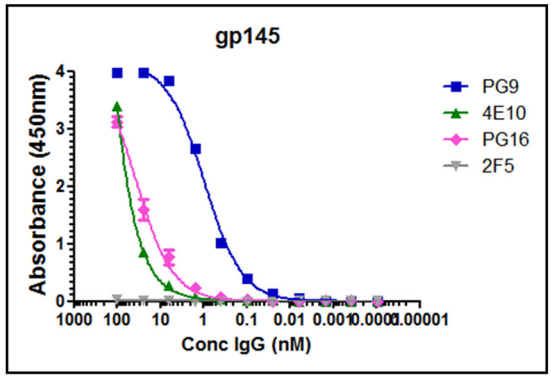

B

| Ligand | Sample ID | KD (nM) | kon(1/Ms) | kon Error | kdis(1/s) | kdis Error | Full X^2 | Full R^2 |
|---|---|---|---|---|---|---|---|---|
| 93IN101 gp145 trimer | B6 | 0.057 | 3.95E+05 | 1.94E+03 | 2.24E-05 | 1.39E-06 | 0.025 | 0.996 |
| | B12 | 0.233 | 1.81E+06 | 1.59E+04 | 4.22E-04 | 2.51E-06 | 0.031 | 0.984 |
| | VRC01 | 0.022 | 4.88E+04 | 4.95E+02 | 1.10E-06 | 3.10E-06 | 0.035 | 0.987 |
| | NIH45-46W | 0.549 | 2.51E+05 | 2.93E+03 | 1.38E-04 | 2.48E-06 | 0.022 | 0.99 |
| 93IN101 gp120 | B6 | 0.293 | 2.61E+05 | 3.52E+03 | 7.65E-05 | 2.82E-06 | 0.044 | 0.985 |
| | B12 | 1.225 | 1.07E+06 | 1.49E+04 | 1.31E-03 | 7.12E-06 | 0.066 | 0.928 |
| | VRC01 | NB | - | - | - | - | - | 0 |
| | NIH-45-46W | 0.510 | 1.43E+05 | 2.49E+03 | 7.29E-05 | 2.36E-06 | 0.066 | 0.992 |

FIGURE 4. **Binding of 93IN101 gp120 and gp145 to CD4 binding site antibodies.** *A*, binding curves generated by coating ELISA wells using gp120 or gp145 and incubating with serial dilutions of b6 (non-neutralizing antibody) or b12, VRC01, and NIH 45–46 G54W (neutralizing antibodies). *B*, table comparing kinetic parameters. *kon* is the rate of association and *kdis* is the rate of dissociation for immobilized gp145 and gp120 binding to various CD4 binding site antibodies in solution using Octet RED biolayer interferometry. *X^2* and *R^2* are measures of the goodness of fit.

immune pressure. It is worthwhile to test the binding affinity of purified Env with broadly neutralizing human monoclonal antibodies 2G12, PG9, and PG16, which target specific glycosylation sites on gp120. The purified 93IN101 gp145 did not bind with the 2G12 antibody in ELISA. This is not surprising in view of the lack of glycosylation at position 295 of 93IN101

A




B

| Epitope | Sample ID | KD (nM) | Kon (1/Ms) | kon Error | Kdis (1/s) | kdis Error | Full X^2 | Full R^2 |
|---|---|---|---|---|---|---|---|---|
| gp120-V2/V3 | PG9 | 8.086 | 9.98 E+04 | 2.18 E+03 | 8.07 E-04 | 1.33 E-05 | 0.007 | 0.944 |
| gp41-MPER | 4E10 | 57.8 | 1.94 E+04 | 2.86 E+02 | 1.12 E-03 | 7.44 E-06 | 0.086 | 0.937 |
| gp120-V2/V3 | PG16 | 34.820 | 1.99 E+05 | 6.87 E+03 | 6.94 E-03 | 6.69 E-05 | 0.021 | 0.933 |

FIGURE 5. **Analysis of 93IN101 gp145 binding to known broadly neutralizing human monoclonal antibodies.** *A*, ELISA binding curves generated by coating the plate with gp120 or gp145 and incubating with serial dilutions of various human monoclonal antibodies. *B*, summary of kinetic parameters. *kon* is the rate of association and *kdis* is the rate of dissociation calculated using Octet RED biolayer interferometry for immobilized gp145 binding to PG9, 4E10, and PG16 in solution. *X^2* and *R^2* are measures of the goodness of fit. *MPER*, membrane-proximal external region.

gp160, which is required for the binding of the neutralizing human monoclonal antibody 2G12 (57). The quaternary V2/V3 epitope- and glycosylation (Asn-160)-dependent antibodies PG9 and PG16 did not bind with 93IN101 gp120. Although PG9 bound with gp145 very well, PG16 binding was very poor in ELISA (Fig. 5*A*), similar to the observations of Barouch and co-workers (19) and Stamatatos and co-workers (58). The interaction was further examined by Octet with gp145 immobilized to the sensors (Fig. 5*B*), which showed that both PG9 and PG16 IgGs interacted with gp145, but the dissociation rate is 10 times faster with PG16 compared with PG9. This may account for the lack of binding in ELISA with PG16.

*Binding to Anti-gp41 Membrane-proximal External Region Antibodies*—Sequence comparison between the clade B HXB2 gp41 and 93IN101 (Fig. 1*A*) shows that the 2F5 epitope is not conserved in 93IN101 gp41. As expected, 2F5 did not bind 93IN101 gp145 either in ELISA or Octet. The other membrane-proximal external region-directed bNAb, 4E10, bound poorly in ELISA (Fig. 5*A*) with 93IN101 gp145 and had a high $K_D$ (57.8 nM) as determined by Octet (Fig. 5*B*). This shows that although the site is present in purified gp145 it is not optimal for high affinity binding to 4E10. The neutralizing human monoclonal antibody 10E8 against HIV-1 gp41 bound poorly in ELISA and did not show any binding to 93IN101 gp145 trimer in Octet binding studies (data not shown).

*Neutralization of 93IN101 Pseudovirus*—In view of the differences in the binding of purified trimeric 93IN101 gp145 to well characterized neutralizing human monoclonal antibodies, the ability of these antibodies to neutralize 93IN101 pseudovirus was tested in TZM-bl cells (Table 1). Those antibodies that showed strong binding to gp145, namely the CD4 binding site antibodies VRC101 and NIH 45–46 G54W, also neutralized the pseudovirus with lower $IC_{90}$ values (concentrations for 90% inhibition of infection) than others. In support of results obtained from ELISA and Octet binding studies, PG9 monoclonal antibody neutralized 93IN101 virus strongly (1.6 μg/ml), whereas only weak neutralization was observed with PG16 (20.9 μg/ml). The $IC_{90}$ value for sCD4 was very high (57.2 μg/ml), similar to that reported by Binley *et al.* (35), indicating that perhaps 93IN101 is partly CD4-resistant.

**TABLE 1**
**Neutralization sensitivity of 93IN101 pseudovirus to sCD4 and various known conformational broadly neutralizing antibodies to HIV-1**

| Sample | IC90 (μg/ml) |
|---|---|
| NIH45-46 G46W | 0.38 |
| PG9 | 1.6 |
| VRC01 | 3.2 |
| 4 E 10 | 10.3 |
| PG16 | 20.9 |
| B12 | 41.4 |
| 2G12 | 43.5 |
| 1.7b | 52.3 |
| VRC03 | 52.8 |
| sCD4 | 57.2 |

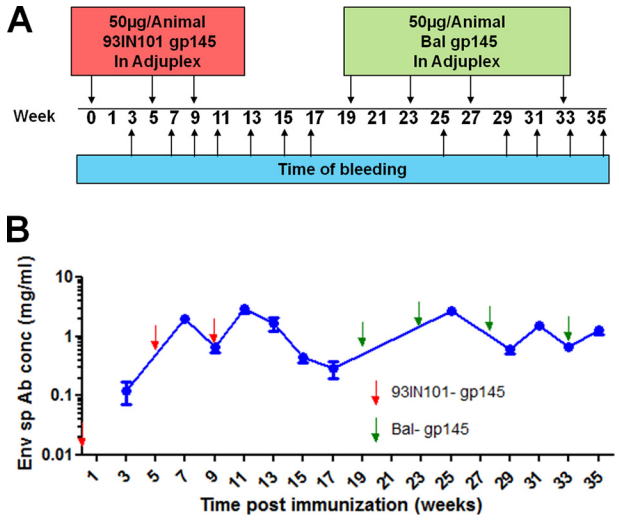



FIGURE 6. **Protein immunization in New Zealand White rabbits.** *A*, schematic representation of the immunization schedule showing the time of immunization and bleed. *B*, serial dilutions of rabbit sera were tested for reactivity by ELISA against 93IN101 gp145 protein bound on a plate. Specific IgG quantification was calculated by generating a standard curve. *Env sp Ab conc*, Env-specific antibody concentration.

*Immunogenicity in Rabbits*—Although a number of clade B and African clade C gp120 and gp140 proteins have been tested in animal models and human clinical trials, the envelope proteins of HIV-1 isolates from India, which form a unique serotype, have not been studied for immunogenicity in detail. A study using gp120 from two Indian isolates reported a limited immune response in guinea pigs (59), whereas there are no reports on the immunogenicity of Indian gp140 proteins. To this end, four New Zealand White rabbits were immunized with 50 $\mu$g of purified 93IN101 gp145 trimers in Adjuplex adjuvant at 4-week intervals as depicted in the schematic representation of the immunization scheme (Fig. 6*A*). Adjuplex has been shown to elicit high titer antibodies in rabbits and rhesus macaques (16, 60). Significant antibody titers were seen in all the rabbits even after the second immunization and remained high during the course of the study (Fig. 6*B*). To delineate the immune response to linear epitopes of gp145, the sera were analyzed in ELISA with overlapping peptides (15-mers with 11-amino acid overlap) of 93IN101 gp160 (Fig. 7*A*). Similar profiles are seen in all the rabbits. As can be seen from Fig. 7*A*, there was a robust immune response to gp120 domains C1, V1, V2, and V3 and the immunodominant region of gp41. A similar peptide scan with clade B HIV-1 Bal peptides was also carried out (data not shown) that showed high titer immune responses to the V1 and V2 domains of HIV-1 Bal gp120. We synthesized the complete linear 93IN101 V1, V2, and V3 peptides and tested serial dilutions of the rabbit sera by ELISA for reactivity with these peptides. A strong reactivity was observed with V3 peptide, moderate reactivity was observed with V2 peptide, and less reactivity was observed with the V1 peptide (Fig. 7*B*).

To determine the neutralization efficiency of these antibodies, we purified total IgG from the rabbits and tested it in the TZM-bl-based pseudovirus neutralization assay. As shown in Table 2, neutralization of the homologous 93IN101 pseudovirus was observed in one immunized rabbit. In addition, purified IgG from all four animals neutralized clade B viruses SF162, Bal, and pNL4-3 as well as African clade C virus GS015. The specificity of the neutralization of HIV-1 by the rabbit sera was also analyzed by absorption with V1, V2, and V3 peptides of 93IN101 gp120. The neutralization of the GS015 pseudovirus by IgG from all four of the immunized rabbits was completely absorbed by the V3 peptide (data not shown). It is well known than Tier1 neutralization is due to V3-specific antibodies (35, 61). Collectively these data demonstrate that the 93IN101 gp145 is a potent immunogen, and it elicits a high titer humoral immune response to all the regions of gp120 and gp41 of HIV-1.

To test whether the neutralizing activity can be broadened by a combination of heterogenous envelope proteins, the 93IN101 gp145-immunized rabbits were subsequently immunized four times with HIV-1 Bal gp145 in Adjuplex at 4-week intervals (Fig. 6*A*). A strong neutralization of HIV-1 Bal was seen in 93IN101-immunized rabbits, and this was enhanced after boosting with HIV-1 Bal gp145 (Table 2). This enhanced HIV-1 Bal neutralization was markedly higher than the neutralization observed using IgG from rabbit sera immunized with Bal gp145 alone (Table 2). Weak neutralization of the difficult to neutralize autologous 93IN101 virus was also observed by IgG purified from all rabbits. Immune sera collected 2 weeks after the last Bal gp145 immunization were also tested for their ability to neutralize a panel of Tier 1 and Tier 2 pseudoviruses (62, 63). As shown in Table 3, a high neutralizing antibody titer was observed in these sera to Tier 1 HIV-1 isolates. In addition, Tier 2 isolates were also neutralized by serum from two of the immunized rabbits after Bal gp145 with low to moderate titers. In this assay, significant neutralizing antibody titers were observed against clade B and African clade C HIV-1 isolates following just 93IN101 gp145 immunization (week 13), suggesting that the Indian clade C envelope proteins may be strong candidates for future vaccine design to fight the global AIDS epidemic.

## DISCUSSION

One of the major problems to be addressed in AIDS vaccine development is the variability of the envelope protein among the global isolates of HIV-1. The envelope proteins are the targets of well characterized human antibodies that broadly neutralize HIV-1 from different clades. Vaccine development is further made difficult by the evolution of the virus in response to the early development of neutralizing antibodies to HIV-1 in infected individuals. In the recently concluded RV144 human clinical trial, a mixture of clade B and clade E gp120 proteins was used in a prime boost regimen, presumably to generate a broadly reactive protective immune response in the vaccinees. The study was conducted in Thailand where HIV-1 clade E is widely prevalent. In other geographical regions of the AIDS pandemic like Africa and Asia where there is a great need for an AIDS vaccine, the most dominant forms of the virus belong to the clade C genotype. Although the immunogenicity of purified envelope proteins from African clade C have been well studied, little is known about the purified envelope proteins of clade C HIV-1 isolates from India. A limited immunogenicity study of Indian clade C gp120 along with other clade C gp120 isolates has been performed, but there has been no characterization of gp145, which is the target of gp120- and gp41-neutralizing antibodies. To achieve these stated goals, we expressed and purified

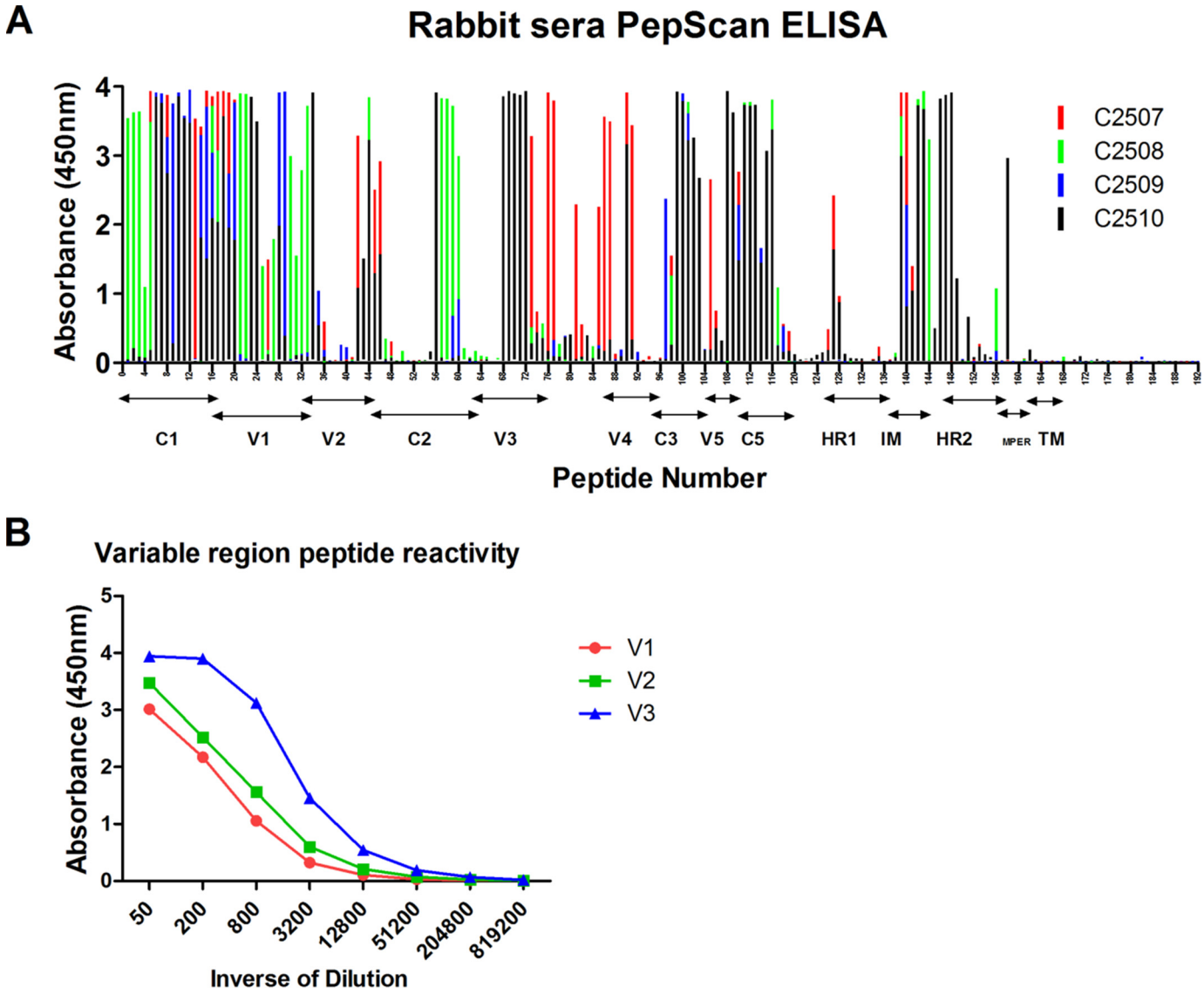



FIGURE 7. **Linear epitope mapping of 93IN101 gp145-immunized rabbit sera by ELISA.** *A*, a total of 192 peptides 15 amino acids long with 11-amino acid overlapping sequence covering the entire 93IN101 gp160 protein were used. Sera from 2 weeks after the final protein immunization were tested for reactivity at a dilution of 1:50. *C2507*, *C2508*, *C2509*, and *C2510* are rabbit identification numbers used in the study. *B*, titers to V1, V2, and V3 linear peptides of 93IN101 gp120. *MPER*, membrane-proximal external region; *TM*, transmembrane domain; *HR*, heptad repeat; *IM*, immuno dominant region.

stable trimeric gp145 envelope protein from a patient chronically infected with HIV-1 from India. The infectious molecular clone of the CCR5 tropic 93IN101 HIV-1 has been well characterized (35, 39). In this study, we showed for the first time that the 93IN101 gp145 protein purified from the conditioned medium of HEK-293 cells forms a highly stable trimer. For structural comparison, 93IN101 gp120 was also purified and studied simultaneously. The gp145 had better exposure of the CD4 binding site epitopes than gp120. Previous reports show a complex interaction between CD4 and the gp160 trimers present on the virion surface of HIV-1 isolates from India in pseudovirus neutralization assays by monoclonal antibodies. In addition, Berman and co-workers (59) observed that one of the purified Indian clade C gp120s did not bind CD4, similar to the 93IN101 gp120 reported in the present study. The purified 93IN101 gp120 had a 200-fold lower $K_D$ than gp145. Studies with other clade B and African clade C isolates indicate that CD4 binding with gp120 is of higher or equivalent affinity as compared with gp140 (19, 64), suggesting that the Indian clade C is distinct. Furthermore high affinity binding of 93IN101 gp145 to CD4 binding site monoclonal antibodies b12, VRC01, and NIH 45–46 G54W suggests that gp145 has the conformational integrity to expose the CD4 binding site to the immune system. As expected from this conformational integrity of the CD4 binding site of gp145, the antibodies VRC01 and NIH 45–46 G54W neutralized the 93IN101 pseudovirus at low concentrations (Table 1). As reported previously, most primary HIV-1 isolates from India are resistant to neutralization by b12 (32, 35–37). We also observed poor neutralization of HIV-1 93IN101 by b12. The gp41-directed monoclonal antibodies 2F5 and 10E8 did not bind to 93IN101 g145. This is not surprising because of the absence of critical residues (K665S in 2F5 epitope and N671S for 10E8 epitope) in the membrane-proximal external region of 93IN101. The 93IN101 virus was mod-

**TABLE 2**

**$IC_{50}$ values for neutralization of various HIV-1 pseudoviruses as well as autologous pseudovirus by purified IgG from immunized rabbit sera**

| IC50 (μg/ml) | | | Tier 1 | | | Autologous pseudovirus | |
|---|---|---|---|---|---|---|---|
| | | | Clade B | | Clade C | Clade B | Clade C |
| Animal Number | | Bleed Week | NL4-3 | SF162 | GS015 | BaL | 93IN101 |
| 93IN101 gp145 only | C2507 | 13 | >250 (12%) | >250 (43%) | 0.0007 | 215 | 30 |
| | C2508 | 13 | >250 (11%) | >250 (36%) | 0.31 | >250 (40%) | >250 (35%) |
| | C2509 | 13 | >250 (22%) | 30 | 0.0002 | 35 | >250 (42%) |
| | C2510 | 13 | 103 | 40 | 0.13 | 20 | >250 (18%) |
| 93IN101 gp145 + Bal gp145 | C2507 | 35 | 262 | 2 | 0.22 | 0.1 | >250 (38%) |
| | C2508 | 35 | >250 (41%) | 0.05 | 0.15 | 0.001 | >250 (40%) |
| | C2509 | 35 | 50 | 5.8 | 0.01 | 0.92 | 267 |
| | C2510 | 35 | 19 | 3 | 0.03 | 0.06 | 200 |
| Bal gp145 Only | L50680 | 13 | >250 (48%) | 31 | 12 | 34 | >250 (19%) |
| | L50681 | 13 | 239 | 4 | 5 | 0.00006 | >250 (17%) |
| | L50682 | 13 | 100 | 1 | 0.36 | 4.7 | >250 (26%) |
| | L50685 | 13 | >250 (44%) | 0.98 | 1.7 | 4.5 | >250 (18%) |

IC50- The IgG concentration at which relative luminescence units (RLUs) were reduced 50% compared to pre-bleed IgG.
If >250μg/ml then, percentage neutralization at 250μg/ml has been mentioned within bracket.

| >250 | 270-50 | 50-1 | <1 |
|---|---|---|---|

erately neutralized by the broadly neutralizing antibody 4E10 ($IC_{90}$ at 10 $\mu$g/ml). Additional evidence of the conformational integrity of 93IN101 gp145 was provided by the strong binding of the quaternary epitope-dependent broadly neutralizing antibody PG9 to gp145. This was observed both in ELISA and in Octet studies. The absence of ELISA reactivity of the closely related PG16 antibody may be due to rapid dissociation of PG16 compared with PG9 as supported by Octet binding studies. A corresponding difference in the neutralization of the homologous 93IN101 pseudovirus was also observed between PG9 and PG16 (Table 1; $IC_{90}$, 1.6 $\mu$g/ml PG9 and 20.9 $\mu$g/ml PG16). All the above studies with ELISA, $K_D$ measurements with Octet, and neutralization of the 93IN101 pseudovirus demonstrated that 93IN101 has a properly folded structure to expose a variety of epitopes in the gp120 and gp41 domains to the host immune system.

The RV144 HIV-1 vaccine trial has also demonstrated that a prime boost regimen with a recombinant viral vector results in a protective immune response because the proteins alone were not sufficient to prevent virus acquisition (22). The trial also showed that the protection correlated with the presence of non-neutralizing antibodies to the V1 and V2 domains of gp120. Conversely, passive immunization with virus-neutralizing serum or neutralizing monoclonal antibodies prevented SHIV infection in rhesus macaques (9, 12). Thus both neutralizing and non-neutralizing antibodies to the HIV-1 envelope proteins are essential for achieving significant protection against HIV-1 infection. In addition, we may have to consider the antigenic variations in the envelope proteins of HIV-1 isolates from different parts of the world. One of the goals of this study was to assess the immunogenicity of the gp145 protein from a chronic Indian isolate of HIV-1 because these viral isolates form a group by themselves and have unique antigenic properties (32). As described, we have demonstrated the conformational integrity of trimeric gp145 from an Indian isolate by its reactivity with HIV-1-neutralizing human monoclonal antibodies and thus may have exposed epitopes for generating the corresponding antibodies. The envelope proteins from these isolates may expose additional uncharacterized epitopes to the immune system. The immunogenicity studies in rabbits showed that the 93IN101 gp145 is highly immunogenic and generates a strong response to the V2 and V3 domains of HIV-1 gp120. The rabbit sera also generated strong neutralizing antibodies against a number of clade B and African clade C viruses

**TABLE 3**
**Summary of neutralizing antibody titers for various HIV-1 strains by immunized rabbit serum**

| | | ID50 in TZM-bl cells[1] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MN.3 | MW965.26 | SHIV-1157ipEL-p | 246-F3_C10_2 | TRO.11 | CNE55 | CH119.10 | BJOX002000.03.2 | 25710-2.43 | X1632_S2_B10 | Ce703010217_B6 | Ce1176_A3 |
| | | Tier 1 Clade B | Tier 1 Clade C | Tier 1 Clade C | Tier 1 Clade AC | Tier 2 Clade B | Tier 2 CRF01_AE | Tier 2 CRF07_BC | Tier 2 CRF07_BC | Tier 2 Clade C | Tier 2 Clade G | Tier 2 Clade C | Tier 2 Clade C |
| Animal | Bleed Week | ID#1770 | ID#6411 | ID#1351 | ID#6451 | ID#772 | ID#5448 | ID#2502 | ID#2718 | ID#6366 | ID#2900 | ID#5512 | ID#6454 |
| C2507 | 0 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 |
| | 13 | 89 | 2307 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 28 |
| | 35 | 2240 | 2505 | 24 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 62 |
| C2508 | 0 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 20 |
| | 13 | 3937 | 6582 | 21 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 40 |
| | 35 | 2550 | 3686 | 34 | 23 | 25 | 27 | 24 | 22 | 34 | 26 | <20 | 83 |
| C2509 | 0 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 21 |
| | 13 | 85 | 2316 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 22 |
| | 35 | 3306 | 7199 | 105 | 23 | 23 | <20 | 24 | <20 | 30 | 28 | <20 | 57 |
| C2510 | 0 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 23 |
| | 13 | 2414 | 6220 | 41 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 23 |
| | 35 | 3146 | 6364 | 100 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | <20 | 45 |

[1]*Values are the sample dilution at which relative luminescence units (RLUs) were reduced 50% compared to virus control wells (no test sample).*

*20-1000* | 1000-5000 | >5000

(Tables 2 and 3). However, the Tier 1 neutralization is mainly due to the immune response against the V3 domain of gp120, similar to immunogenicity studies with other HIV-1 gp120 and gp140 (64, 65). The challenge is therefore to expand the breadth of the neutralization by generating antibodies against other conserved domains of the gp120-gp41 spikes on HIV-1 virions.

The 93IN101 gp145-immunized rabbits were boosted with a heterologous clade B HIV-1 Bal gp145. We observed an expanded but weak neutralization of Tier 2 viruses in the TZM-bl assay. This has not been reported before with other HIV-1 gp140s. Potent neutralization of HIV-1 Bal pseudovirus also was observed following boosting with Bal gp145, and this response was shown to be markedly higher than that observed in rabbits immunized with Bal gp145 only. These results suggest that 93IN101 gp145 might have primed epitopes involved in HIV-1 Bal neutralization. Together these data provide evidence that a combination of HIV-1 gp145s from different clades and more importantly different serotypes might generate broadly neutralizing antibodies. Also, as suggested by the RV144 trial, priming with viral vectors or DNA followed by immunization using a mixture of envelope proteins from different serotypes may be essential to boost the host protective immune response in vaccinees. The present study demonstrates that such epitopes are exposed in the 93IN101 gp145 but have only weak recognition by the immune system for generating broadly neutralizing antibodies, and this can be enhanced with an appropriate envelope protein from different serotypes. One of the possible approaches is to test a heterologous mixture of envelope proteins of HIV-1 in combination with a prime boost regimen in the vaccine strategy. In any event, the envelope proteins of HIV-1 are an essential component for future AIDS vaccine development. In view of the fact that many of these broadly neutralizing human monoclonal antibodies have undergone many somatic mutations from germ line sequences (66, 67), it is essential to identify envelope proteins that react with a broad spectrum of germ line sequences. The present study suggests that an appropriate Indian clade C envelope protein can be such a candidate in future HIV vaccine regimens.

## REFERENCES


1. Rosenberg, E. S., Billingsley, J. M., Caliendo, A. M., Boswell, S. L., Sax, P. E., Kalams, S. A., and Walker, B. D. (1997) Vigorous HIV-1-specific $CD4^+$ T cell responses associated with control of viremia. *Science* **278,** 1447–1450
2. Matano, T., Kobayashi, M., Igarashi, H., Takeda, A., Nakamura, H., Kano, M., Sugimoto, C., Mori, K., Iida, A., Hirata, T., Hasegawa, M., Yuasa, T., Miyazawa, M., Takahashi, Y., Yasunami, M., Kimura, A., O'Connor, D. H., Watkins, D. I., and Nagai, Y. (2004) Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial. *J. Exp. Med.* **199,** 1709–1718
3. Amara, R. R., Sharma, S., Patel, M., Smith, J. M., Chennareddi, L., Herndon, J. G., and Robinson, H. L. (2005) Studies on the cross-clade and cross-species conservation of HIV-1 Gag-specific CD8 and CD4 T cell responses elicited by a clade B DNA/MVA vaccine in macaques. *Virology* **334,** 124–133
4. Pitisuttithum, P., Gilbert, P., Gurwith, M., Heyward, W., Martin, M., van Griensven, F., Hu, D., Tappero, J. W., and Choopanya, K. (2006) Randomized, double-blind, placebo-controlled efficacy trial of a bivalent recombinant glycoprotein 120 HIV-1 vaccine among injection drug users in Bangkok, Thailand. *J. Infect. Dis.* **194,** 1661–1671
5. Gilbert, P. B., Peterson, M. L., Follmann, D., Hudgens, M. G., Francis, D. P., Gurwith, M., Heyward, W. L., Jobes, D. V., Popovic, V., Self, S. G., Sinangil, F., Burke, D., and Berman, P. W. (2005) Correlation between immunologic responses to a recombinant glycoprotein 120 vaccine and incidence of HIV-1 infection in a phase 3 HIV-1 preventive vaccine trial. *J. Infect. Dis.* **191,** 666–677
6. Walker, L. M., and Burton, D. R. (2010) Rational antibody-based HIV-1 vaccine design: current approaches and future directions. *Curr. Opin. Immunol.* **22,** 358–366
7. Kwong, P. D., Mascola, J. R., and Nabel, G. J. (2011) Rational design of vaccines to elicit broadly neutralizing antibodies to HIV-1. *Cold Spring Harb. Perspect. Med.* **1,** a007278
8. Stamatatos, L., Morris, L., Burton, D. R., and Mascola, J. R. (2009) Neutralizing antibodies generated during natural HIV-1 infection: good news for an HIV-1 vaccine? *Nat. Med.* **15,** 866–870
9. Mascola, J. R., Lewis, M. G., Stiegler, G., Harris, D., VanCott, T. C., Hayes, D., Louder, M. K., Brown, C. R., Sapan, C. V., Frankel, S. S., Lu, Y., Robb, M. L., Katinger, H., and Birx, D. L. (1999) Protection of macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. *J. Virol.* **73,** 4009–4018
10. Shibata, R., Igarashi, T., Haigwood, N., Buckler-White, A., Ogert, R., Ross, W., Willey, R., Cho, M. W., and Martin, M. A. (1999) Neutralizing antibody directed against the HIV-1 envelope glycoprotein can completely block HIV-1/SIV chimeric virus infections of macaque monkeys. *Nat. Med.* **5,** 204–210
11. Baba, T. W., Liska, V., Hofmann-Lehmann, R., Vlasak, J., Xu, W., Ayehunie, S., Cavacini, L. A., Posner, M. R., Katinger, H., Stiegler, G., Bernacky, B. J., Rizvi, T. A., Schmidt, R., Hill, L. R., Keeling, M. E., Lu, Y., Wright, J. E., Chou, T. C., and Ruprecht, R. M. (2000) Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. *Nat. Med.* **6,** 200–206
12. Mascola, J. R., Stiegler, G., VanCott, T. C., Katinger, H., Carpenter, C. B., Hanson, C. E., Beary, H., Hayes, D., Frankel, S. S., Birx, D. L., and Lewis, M. G. (2000) Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. *Nat. Med.* **6,** 207–210
13. Parren, P. W., Marx, P. A., Hessell, A. J., Luckay, A., Harouse, J., Cheng-Mayer, C., Moore, J. P., and Burton, D. R. (2001) Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization *in vitro*. *J. Virol.* **75,** 8340–8347
14. Mascola, J. R. (2003) Defining the protective antibody response for HIV-1. *Curr. Mol. Med.* **3,** 209–216
15. Hessell, A. J., Poignard, P., Hunter, M., Hangartner, L., Tehrani, D. M., Bleeker, W. K., Parren, P. W., Marx, P. A., and Burton, D. R. (2009) Effective, low-titer antibody protection against low-dose repeated mucosal SHIV challenge in macaques. *Nat. Med.* **15,** 951–954
16. Feng, Y., McKee, K., Tran, K., O'Dell, S., Schmidt, S. D., Phogat, A., Forsell, M. N., Karlsson Hedestam, G. B., Mascola, J. R., and Wyatt, R. T. (2012) Biochemically defined HIV-1 envelope glycoprotein variant immunogens display differential binding and neutralizing specificities to the CD4-binding site. *J. Biol. Chem.* **287,** 5673–5686
17. Bhattacharyya, S., Rajan, R. E., Swarupa, Y., Rathore, U., Verma, A., Udaykumar, R., and Varadarajan, R. (2010) Design of a non-glycosylated outer domain-derived HIV-1 gp120 immunogen that binds to CD4 and induces neutralizing antibodies. *J. Biol. Chem.* **285,** 27100–27110
18. McGuire, A. T., Hoot, S., Dreyer, A. M., Lippy, A., Stuart, A., Cohen, K. W., Jardine, J., Menis, S., Scheid, J. F., West, A. P., Schief, W. R., and Stamatatos, L. (2013) Engineering HIV envelope protein to activate germline B cell receptors of broadly neutralizing anti-CD4 binding site antibodies. *J. Exp. Med.* **210,** 655–663
19. Kovacs, J. M., Nkolola, J. P., Peng, H., Cheung, A., Perry, J., Miller, C. A., Seaman, M. S., Barouch, D. H., and Chen, B. (2012) HIV-1 envelope trimer elicits more potent neutralizing antibody responses than monomeric gp120. *Proc. Natl. Acad. Sci. U.S.A.* **109,** 12111–12116
20. Sanders, R. W., Derking, R., Cupo, A., Julien, J.-P., Yasmeen, A., de Val, N., Kim, H. J., Blattner, C., de la Peña, A. T., Korzun, J., Golabek, M., de Los Reyes, K., Ketas, T. J., van Gils, M. J., King, C. R., Wilson, I. A., Ward, A. B., Klasse, P. J., and Moore, J. P. (2013) A next-generation cleaved, soluble HIV-1 Env trimer, BG505 SOSIP.664 gp140, expresses multiple epitopes for broadly neutralizing but not non-neutralizing antibodies. *PLoS Pathog.* **9,** e1003618
21. Rerks-Ngarm, S., Pitisuttithum, P., Nitayaphan, S., Kaewkungwal, J., Chiu, J., Paris, R., Premsri, N., Namwat, C., de Souza, M., Adams, E., Benenson, M., Gurunathan, S., Tartaglia, J., McNeil, J. G., Francis, D. P., Stablein, D., Birx, D. L., Chunsuttiwat, S., Khamboonruang, C., Thongcharoen, P., Robb, M. L., Michael, N. L., Kunasol, P., and Kim, J. H. (2009) Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N. Engl. J. Med.* **361,** 2209–2220
22. Haynes, B. F., Gilbert, P. B., McElrath, M. J., Zolla-Pazner, S., Tomaras, G. D., Alam, S. M., Evans, D. T., Montefiori, D. C., Karnasuta, C., Sutthent, R., Liao, H.-X., DeVico, A. L., Lewis, G. K., Williams, C., Pinter, A., Fong, Y., Janes, H., DeCamp, A., Huang, Y., Rao, M., Billings, E., Karasavvas, N., Robb, M. L., Ngauy, V., de Souza, M. S., Paris, R., Ferrari, G., Bailer, R. T., Soderberg, K. A., Andrews, C., Berman, P. W., Frahm, N., De Rosa, S. C., Alpert, M. D., Yates, N. L., Shen, X., Koup, R. A., Pitisuttithum, P., Kaewkungwal, J., Nitayaphan, S., Rerks-Ngarm, S., Michael, N. L., Kim, J. H., Rosa, S. C. De, and Alam, M. (2012) Immune-correlates analysis of an HIV-1 vaccine efficacy trial. *N. Engl. J. Med.* **366,** 1275–1286
23. Gottardo, R., Bailer, R. T., Korber, B. T., Gnanakaran, S., Phillips, J., Shen, X., Tomaras, G. D., Turk, E., Imholte, G., Eckler, L., Wenschuh, H., Zerweck, J., Greene, K., Gao, H., Berman, P. W., Francis, D., Sinangil, F., Lee, C., Nitayaphan, S., Rerks-Ngarm, S., Kaewkungwal, J., Pitisuttithum, P., Tartaglia, J., Robb, M. L., Michael, N. L., Kim, J. H., Zolla-Pazner, S., Haynes, B. F., Mascola, J. R., Self, S., Gilbert, P., and Montefiori, D. C. (2013) Plasma IgG to linear epitopes in the V2 and V3 regions of HIV-1 gp120 correlate with a reduced risk of infection in the RV144 vaccine efficacy trial. *PLoS One* **8,** e75665
24. VanCott, T. C., Veit, S. C., Kalyanaraman, V., Earl, P., and Birx, D. L. (1995) Characterization of a soluble, oligomeric HIV-1 gp160 protein as a potential immunogen. *J. Immunol. Methods* **183,** 103–117
25. Selvarajah, S., Puffer, B., Pantophlet, R., Law, M., Doms, R. W., and Burton, D. R. (2005) Comparing antigenicity and immunogenicity of engineered gp120. *J. Virol.* **79,** 12148–12163
26. Kang, Y. K., Andjelic, S., Binley, J. M., Crooks, E. T., Franti, M., Iyer, S. P., Donovan, G. P., Dey, A. K., Zhu, P., Roux, K. H., Durso, R. J., Parsons, T. F., Maddon, P. J., Moore, J. P., and Olson, W. C. (2009) Structural and immunogenicity studies of a cleaved, stabilized envelope trimer derived from subtype A HIV-1. *Vaccine* **27,** 5120–5132
27. Kong, L., and Sattentau, Q. J. (2012) Antigenicity and immunogenicity in HIV-1 antibody-based vaccine design. *J. AIDS Clin. Res.* **S8,** 3
28. Visciano, M. L., Tagliamonte, M., Stewart-Jones, G., Heyndrickx, L., Vanham, G., Jansson, M., Fomsgaard, A., Grevstad, B., Ramaswamy, M., Buonaguro, F. M., Tornesello, M. L., Biswas, P., Scarlatti, G., and Buonaguro, L. (2013) Characterization of humoral responses to soluble trimeric HIV

gp140 from a clade A Ugandan field isolate. *J. Transl. Med.* **11,** 165
29. Liao, H.-X., Tsao, C.-Y., Alam, S. M., Muldoon, M., Vandergrift, N., Ma, B.-J., Lu, X., Sutherland, L. L., Scearce, R. M., Bowman, C., Parks, R., Chen, H., Blinn, J. H., Lapedes, A., Watson, S., Xia, S.-M., Foulger, A., Hahn, B. H., Shaw, G. M., Swanstrom, R., Montefiori, D. C., Gao, F., Haynes, B. F., and Korber, B. (2013) Antigenicity and immunogenicity of transmitted/founder, consensus, and chronic envelope glycoproteins of human immunodeficiency virus type 1. *J. Virol.* **87,** 4185–4201
30. Polonis, V., Wieczorek, L., Kalyanaraman, V., Matyas, G., Whitney, S., Williams, C., Tovanabutra, S., Sanders-buell, E., Wesberry, M., Ochsenbauer, C., Chenine, A., Rao, M., Tong, T., Alving, C., Cheng, H., Zollapazner, S., Michael, N., Vancott, T., and Marovich, M. (2012) Antigenicity and immunogenicity of a novel, acute HIV-1 Tanzanian Subtype C gp145 envelope protein for clinical development. *Retrovirology* **9,** Suppl. 2, P323
31. Dietrich, U., Grez, M., von Briesen, H., Panhans, B., Geissendörfer, M., Kühnel, H., Maniar, J., Mahambre, G., Becker, W. B., and Becker, M. L. (1993) HIV-1 strains from India are highly divergent from prototypic African and US/European strains, but are linked to a South African isolate. *AIDS* **7,** 23–27
32. Kulkarni, S. S., Lapedes, A., Tang, H., Gnanakaran, S., Daniels, M. G., Zhang, M., Bhattacharya, T., Li, M., Polonis, V. R., McCutchan, F. E., Morris, L., Ellenberger, D., Butera, S. T., Bollinger, R. C., Korber, B. T., Paranjape, R. S., and Montefiori, D. C. (2009) Highly complex neutralization determinants on a monophyletic lineage of newly transmitted subtype C HIV-1 Env clones from India. *Virology* **385,** 505–520
33. Shen, C., Craigo, J., Ding, M., Chen, Y., and Gupta, P. (2011) Origin and dynamics of HIV-1 subtype C infection in India. *PLoS One* **6,** e25956
34. Seaman, M. S., Janes, H., Hawkins, N., Grandpre, L. E., Devoy, C., Giri, A., Coffey, R. T., Harris, L., Wood, B., Daniels, M. G., Bhattacharya, T., Lapedes, A., Polonis, V. R., McCutchan, F. E., Gilbert, P. B., Self, S. G., Korber, B. T., Montefiori, D. C., and Mascola, J. R. (2010) Tiered categorization of a diverse panel of HIV-1 Env pseudoviruses for assessment of neutralizing antibodies. *J. Virol.* **84,** 1439–1452
35. Binley, J. M., Wrin, T., Korber, B., Zwick, M. B., Wang, M., Chappey, C., Stiegler, G., Kunert, R., Zolla-Pazner, S., Katinger, H., Petropoulos, C. J., and Burton, D. R. (2004) Comprehensive cross-clade neutralization analysis of a panel of anti-human immunodeficiency virus type 1 monoclonal antibodies. *J. Virol.* **78,** 13232–13252
36. Ringe, R., Thakar, M., and Bhattacharya, J. (2010) Variations in autologous neutralization and CD4 dependence of b12 resistant HIV-1 clade C env clones obtained at different time points from antiretroviral naïve Indian patients with recent infection. *Retrovirology* **7,** 76
37. Gharu, L., Ringe, R., and Bhattacharya, J. (2011) HIV-1 clade C envelopes obtained from late stage symptomatic Indian patients varied in their ability towards relative CD4 usages and sensitivity to CCR5 antagonist TAK-779. *Virus Res.* **158,** 216–224
38. Huang, J., Ofek, G., Laub, L., Louder, M. K., Doria-Rose, N. A., Longo, N. S., Imamichi, H., Bailer, R. T., Chakrabarti, B., Sharma, S. K., Alam, S. M., Wang, T., Yang, Y., Zhang, B., Migueles, S. A., Wyatt, R., Haynes, B. F., Kwong, P. D., Mascola, J. R., and Connors, M. (2012) Broad and potent neutralization of HIV-1 by a gp41-specific human antibody. *Nature* **491,** 406–412
39. Mochizuki, N., Otsuka, N., Matsuo, K., Shiino, T., Kojima, A., Kurata, T., Sakai, K., Yamamoto, N., Isomura, S., Dhole, T. N., Takebe, Y., Matsuda, M., and Tatsumi, M. (1999) An infectious DNA clone of HIV type 1 subtype C. *AIDS Res. Hum. Retroviruses* **15,** 1321–1324
40. Walker, L. M., Phogat, S. K., Chan-Hui, P.-Y., Wagner, D., Phung, P., Goss, J. L., Wrin, T., Simek, M. D., Fling, S., Mitcham, J. L., Lehrman, J. K., Priddy, F. H., Olsen, O. A., Frey, S. M., Hammond, P. W., Protocol G Principal Investigators, Kaminsky, S., Zamb, T., Moyle, M., Koff, W. C., Poignard, P., and Burton, D. R. (2009) Broad and potent neutralizing antibodies from an African donor reveal a new HIV-1 vaccine target. *Science* **326,** 285–289
41. Stiegler, G., Kunert, R., Purtscher, M., Wolbank, S., Voglauer, R., Steindl, F., and Katinger, H. (2001) A potent cross-clade neutralizing human monoclonal antibody against a novel epitope on gp41 of human immunodeficiency virus type 1. *AIDS Res. Hum. Retroviruses* **17,** 1757–1765
42. Purtscher, M., Trkola, A., Gruber, G., Buchacher, A., Predl, R., Steindl, F., Tauer, C., Berger, R., Barrett, N., and Jungbauer, A. (1994) A broadly neutralizing human monoclonal antibody against gp41 of human immunodeficiency virus type 1. *AIDS Res. Hum. Retroviruses* **10,** 1651–1658
43. Thali, M., Moore, J. P., Furman, C., Charles, M., Ho, D. D., Robinson, J., and Sodroski, J. (1993) Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding. *J. Virol.* **67,** 3978–3988
44. Buchacher, A., Predl, R., Strutzenberger, K., Steinfellner, W., Trkola, A., Purtscher, M., Gruber, G., Tauer, C., Steindl, F., and Jungbauer, A. (1994) Generation of human monoclonal antibodies against HIV-1 proteins; electrofusion and Epstein-Barr virus transformation for peripheral blood lymphocyte immortalization. *AIDS Res. Hum. Retroviruses* **10,** 359–369
45. Burton, D. R., Barbas, C. F., 3rd, Persson, M. A., Koenig, S., Chanock, R. M., and Lerner, R. A. (1991) A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. *Proc. Natl. Acad. Sci. U.S.A.* **88,** 10134–10137
46. Wu, X., Yang, Z.-Y., Li, Y., Hogerkorp, C.-M., Schief, W. R., Seaman, M. S., Zhou, T., Schmidt, S. D., Wu, L., Xu, L., Longo, N. S., McKee, K., O'Dell, S., Louder, M. K., Wycuff, D. L., Feng, Y., Nason, M., Doria-Rose, N., Connors, M., Kwong, P. D., Roederer, M., Wyatt, R. T., Nabel, G. J., and Mascola, J. R. (2010) Rational design of envelope identifies broadly neutralizing human monoclonal antibodies to HIV-1. *Science* **329,** 856–861
47. Diskin, R., Scheid, J. F., Marcovecchio, P. M., West, A. P., Jr., Klein, F., Gao, H., Gnanapragasam, P. N., Abadir, A., Seaman, M. S., Nussenzweig, M. C., and Bjorkman, P. J. (2011) Increasing the potency and breadth of an HIV antibody by using structure-based rational design. *Science* **334,** 1289–1293
48. Stamatatos, L., Lim, M., and Cheng-Mayer, C. (2000) Generation and structural analysis of soluble oligomeric gp140 envelope proteins derived from neutralization-resistant and neutralization-susceptible primary HIV type 1 isolates. *AIDS Res. Hum. Retroviruses* **16,** 981–994
49. Platt, E. J., Wehrly, K., Kuhmann, S. E., Chesebro, B., and Kabat, D. (1998) Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. *J. Virol.* **72,** 2855–2864
50. Brown, B. K., Darden, J. M., Tovanabutra, S., Oblander, T., Frost, J., Sanders-Buell, E., de Souza, M. S., Birx, D. L., McCutchan, F. E., and Polonis, V. R. (2005) Biologic and genetic characterization of a panel of 60 human immunodeficiency virus type 1 isolates, representing clades A, B, C, D, CRF01_AE, and CRF02_AG, for the development and assessment of candidate vaccines. *J. Virol.* **79,** 6089–6101
51. Chung, H. K., Suschak, J., Galmin, L., Rose, N., and Pal, R. (2010) Characterization of a SHIV162P3 variant evolved in an infected rhesus macaque with persistent plasma viremia. *Virus Res.* **151,** 229–234
52. Sellhorn, G., Kraft, Z., Caldwell, Z., Ellingson, K., Mineart, C., Seaman, M. S., Montefiori, D. C., Lagerquist, E., and Stamatatos, L. (2012) Engineering, expression, purification, and characterization of stable clade A/B recombinant soluble heterotrimeric gp140 proteins. *J. Virol.* **86,** 128–142
53. Chen, B., Zhou, G., Kim, M., Chishti, Y., Hussey, R. E., Ely, B., Skehel, J. J., Reinherz, E. L., Harrison, S. C., and Wiley, D. C. (2000) Expression, purification, and characterization of gp160e, the soluble, trimeric ectodomain of the simian immunodeficiency virus envelope glycoprotein, gp160. *J. Biol. Chem.* **275,** 34946–34953
54. Kraus, M. H., Parrish, N. F., Shaw, K. S., Decker, J. M., Keele, B. F., Salazar-Gonzalez, J. F., Grayson, T., McPherson, D. T., Ping, L. H., Anderson, J. A., Swanstrom, R., Williamson, C., Shaw, G. M., and Hahn, B. H. (2010) A rev1-vpu polymorphism unique to HIV-1 subtype A and C strains impairs envelope glycoprotein expression from rev-vpu-env cassettes and reduces virion infectivity in pseudotyping assays. *Virology* **397,** 346–357
55. Srivastava, I. K., Kan, E., Sun, Y., Sharma, V. A., Cisto, J., Burke, B., Lian, Y., Hilt, S., Biron, Z., Hartog, K., Stamatatos, L., Diaz-Avalos, R., Cheng, R. H., Ulmer, J. B., and Barnett, S. W. (2008) Comparative evaluation of trimeric envelope glycoproteins derived from subtype C and B HIV-1 R5 isolates. *Virology* **372,** 273–290
56. Thali, M., Olshevsky, U., Furman, C., Gabuzda, D., Posner, M., and Sodroski, J. (1991) Characterization of a discontinuous human immunodeficiency virus type 1 gp120 epitope recognized by a broadly reactive

neutralizing human monoclonal antibody. *J. Virol.* **65,** 6188–6193
57. Sanders, R. W., Venturi, M., Schiffner, L., Kalyanaraman, R., Katinger, H., Lloyd, K. O., Kwong, P. D., and Moore, J. P. (2002) The mannose-dependent epitope for neutralizing antibody 2G12 on human immunodeficiency virus type 1 glycoprotein gp120. *J. Virol.* **76,** 7293–7305
58. Davenport, T. M., Friend, D., Ellingson, K., Xu, H., Caldwell, Z., Sellhorn, G., Kraft, Z., Strong, R. K., and Stamatatos, L. (2011) Binding interactions between soluble HIV envelope glycoproteins and quaternary-structure-specific monoclonal antibodies PG9 and PG16. *J. Virol.* **85,** 7095–7107
59. Smith, D. H., Winters-Digiacinto, P., Mitiku, M., O'Rourke, S., Sinangil, F., Wrin, T., Montefiori, D. C., and Berman, P. W. (2010) Comparative immunogenicity of HIV-1 clade C envelope proteins for prime/boost studies. *PLoS One* **5,** e12076
60. Tong, T., Crooks, E. T., Osawa, K., Robinson, J. E., Barnes, M., Apetrei, C., and Binley, J. M. (2014) Multi-parameter exploration of HIV-1 virus-like particles as neutralizing antibody immunogens in guinea pigs, rabbits and macaques. *Virology* **456–457**, 55–69
61. Bontjer, I., Melchers, M., Tong, T., van Montfort, T., Eggink, D., Montefiori, D., Olson, W. C., Moore, J. P., Binley, J. M., Berkhout, B., and Sanders, R. W. (2013) Comparative immunogenicity of evolved V1V2-deleted HIV-1 envelope glycoprotein trimers. *PLoS One* **8,** e67484
62. Montefiori, D. C. (2005) Evaluating neutralizing antibodies against HIV, SIV, and SHIV in luciferase reporter gene assays. *Curr. Protoc. Immunol.* **Chapter 12,** Unit 12.11
63. deCamp, A., Hraber, P., Bailer, R. T., Seaman, M. S., Ochsenbauer, C., Kappes, J., Gottardo, R., Edlefsen, P., Self, S., Tang, H., Greene, K., Gao, H., Daniell, X., Sarzotti-Kelsoe, M., Gorny, M. K., Zolla-Pazner, S., LaBranche, C. C., Mascola, J. R., Korber, B. T., and Montefiori, D. C. (2014) Global panel of HIV-1 Env reference strains for standardized assessments of vaccine-elicited neutralizing antibodies. *J. Virol.* **88,** 2489–2507
64. Kim, M., Qiao, Z.-S., Montefiori, D. C., Haynes, B. F., Reinherz, E. L., and Liao, H.-X. (2005) Comparison of HIV Type 1 ADA gp120 monomers versus gp140 trimers as immunogens for the induction of neutralizing antibodies. *AIDS Res. Hum. Retroviruses* **21,** 58–67
65. Vaine, M., Wang, S., Crooks, E. T., Jiang, P., Montefiori, D. C., Binley, J., and Lu, S. (2008) Improved induction of antibodies against key neutralizing epitopes by human immunodeficiency virus type 1 gp120 DNA prime-protein boost vaccination compared to gp120 protein-only vaccination. *J. Virol.* **82,** 7369–7378
66. Klein, F., Diskin, R., Scheid, J. F., Gaebler, C., Mouquet, H., Georgiev, I. S., Pancera, M., Zhou, T., Incesu, R. B., Fu, B. Z., Gnanapragasam, P. N., Oliveira, T. Y., Seaman, M. S., Kwong, P. D., Bjorkman, P. J., and Nussenzweig, M. C. (2013) Somatic mutations of the immunoglobulin framework are generally required for broad and potent HIV-1 neutralization. *Cell* **153,** 126–138
67. Derdeyn, C. A., Moore, P. L., and Morris, L. (2014) Development of broadly neutralizing antibodies from autologous neutralizing antibody responses in HIV infection. *Curr. Opin. HIV AIDS* **9,** 210–216

# Antigenicity and Immunogenicity of a Trimeric Envelope Protein from an Indian Clade C HIV-1 Isolate

Rangasamy Sneha Priya, Menon Veena, Irene Kalisz, Stephen Whitney, Dhopeshwarkar Priyanka, Celia C. LaBranche, Mullapudi Sri Teja, David C. Montefiori, Ranajit Pal, Sundarasamy Mahalingam and Vaniambadi S. Kalyanaraman

*J. Biol. Chem. 2015, 290:9195-9208.*
*doi: 10.1074/jbc.M114.621185 originally published online February 17, 2015*