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Structural characteristics correlate with immune responses induced by HIV envelope glycoprotein vaccines

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Abstract

HIV envelope glycoprotein (Env) is the target for inducing neutralizing antibodies. Env is present on the virus surface as a trimer, and, upon binding to CD4, a cascade of events leads to structural rearrangement exposing the co-receptor binding site and entry into the CD4+ host target cells. We have designed monomeric and trimeric Env constructs with and without deletion of the variable loop 2 (ΔV2) from SF162, a subtype B primary isolate, and performed biophysical, biochemical and immunological studies to establish a potential structure–functional relationship. We expressed these Envs in CHO cells, purified the proteins to homogeneity and performed biophysical studies to define the binding properties to CD4, structural characteristics and exposure of epitopes recognized by b12 and CD4i mAb (17B) on both full-length and mutant HIV Env proteins. Parameters evaluated include oligomerization state, number and affinity of CD4 binding sites, enthalpy and entropy of the Env–CD4 interaction and affinity for b12 and 17b mAbs. We observed one CD4 binding site per monomer and three active CD4 binding sites per trimer. A 40-fold difference in affinity of the gp120 monomer vs. the o-gp140 trimer towards CD4 was observed (Kd = 58 nM and 1.5 nM, respectively), whereas only a 2-fold difference was observed for the V2 deleted Envs (Kd of gp120ΔV2 = 19 nM, Kd of o-gp140DV2 = 9.3 nM). Monomers had 3-fold higher affinity to the mAb 17b and at least 3-fold weaker affinity to b12 compared to trimers, with gp120DV2 having the weakest affinity for b12 (Kd = 446 nM). Affinity of CD4 binding correlated with proportion of the antibodies induced against the conformational epitopes by the corresponding Envs, and changes in mAb binding correlated with the induction of antibodies directed against linear epitopes. Furthermore, biophysical analysis reveals that the V2 deletion has broad structural implications in the monomer not shared by the trimer, and these changes are reflected in the quality of the immune responses induced in rabbits. These data suggest that biophysical characteristics of HIV Env, such as affinity for CD4, and exposure of important neutralizing epitopes, such as those recognized by b12 mAb, may be important predictors of its in vivo efficacy and may serve as important surrogate markers for screening Env structures as potential vaccine candidates.

Keywords: HIV; Env; gp120; gp120DV2; o-gp140; o-gp140DV2; CD4; b12; 17b; SPR; ITC; Vaccine

Introduction

AIDS continues to be a major health problem throughout the world, with approximately 40.3 million people infected with human immunodeficiency virus (HIV) and 25 million deaths due to AIDS recorded so far. In North America today, an estimated 1.2 million individuals are living with HIV, and 43,000 new primary infections occurred last year (UNAIDS/WHO, 2004). Moreover, the situation is continuously deteriorating as a result of rapid emergence of drug resistance against most of the effective anti-virals. Therefore, there is an urgent need for an effective anti-HIV vaccine that may be used alone as a prophylactic vaccine or in conjunction with effective anti-virals as a therapeutic vaccine.

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Historically, it has been difficult to induce neutralizing antibody responses against diverse primary HIV-1 isolates by utilizing monomeric HIV Env (i.e., gp120) (Barnett et al., 1997; Bou-Habib et al., 1994; Burton, 1997; Mascola et al., 1996; Moore et al., 1995; Moore and Ho, 1995; Moore and Sodroski, 1996; Parren et al., 1997, 1999; VanCott et al., 1995; Wentworth et al., 1994; Wrin et al., 1994; Wrin and Nunberg, 1994). Yet, primary isolate neutralizing antibodies are induced during the natural infection (Cao et al., 1995; Stamatos et al., 1998). Therefore, there are differences between the quality of antibodies induced during the infection and the quality of those induced through vaccination using gp120. It appears that this is not due to the absence of conserved neutralization epitopes on gp120 (Steimer et al., 1991). However, the level of exposure of these epitopes may be different on gp120 compared to their exposure on native Env trimers present on virion (Kwong et al., 1998; Moore et al., 1995; Parren et al., 1999; Sattentau and Moore, 1995; Stamatatos and Cheng-Mayer, 1995; Wyatt et al., 1998).

In general, compared to non-neutralizing monoclonal antibodies (mAb), neutralizing mAb such as b12, 2G12 and 2F5 appears to have stronger affinities for the native envelope (trimer) (Burton et al., 1994; Fouts et al., 1997; Roben et al., 1994; Sattentau and Moore, 1995; Sattentau et al., 1995; Sullivan et al., 1995; Trkola et al., 1996b), suggesting that the neutralizing epitopes recognized by these mAbs are better presented on native trimeric Env. Therefore, efforts have been made to purify HIV Env in trimeric conformation and evaluate its ability to induce primary isolate-neutralizing antibodies in animal models. Earlier studies suggested that HIV Env oligomers, consisting of gp120 and the ectodomain of gp41, might be superior to gp120 for inducing strong humoral responses directed toward conformational epitopes (Broder et al., 1994; Earl et al., 1994; Pinter et al., 1989; Putney et al., 1986; Richardson et al., 1996; VanCott et al., 1997). In addition, antibodies induced by oligomeric Env cross-reacted with HIV Envs of other subtypes and neutralized both T-cell-adapted (TCLA or X4) and selected primary HIV-1 isolates (R5) (Earl et al., 2001; VanCott et al., 1997; Yang et al., 2001). Recent evidence has demonstrated that oligomers may be superior to monomers at inducing primary isolate-neutralizing antibodies (Earl et al., 2001; VanCott et al., 1997; Yang et al., 2001), suggesting that trimeric HIV Env has the potential to be effective in inducing broadly cross-reactive neutralizing antibodies.

Little structural information is available on HIV Env to predict and design novel immunogens that may induce antibodies of appropriate specificity and protective efficacy (Pantaleo and Koup, 2004). Based on the available 3-D structure of gp120, it is evident that the heavily glycosylated face of Env is immunologically silent (Kwong et al., 1998), suggesting that the virus is using glycosylation for shielding critical functional epitopes. Furthermore, we and others have shown that glycosylation is needed for Env to attain functional conformation (Barr et al., 1987; Lasky et al., 1987; Li et al., 1993; McDougal et al., 1986; Papandreou et al., 1996; Putney et al., 1986; Sanders et al., 2000; Sattentau and Weiss, 1988; Scandella et al., 1993) since non-glycosylated Env (Env 2–3) does not bind to CD4 (Abrignani et al., 1990; Haigwood et al., 1990). Recent evidence from SIV and SHIV rhesus infection models suggests that the emergence of viruses resistant to antibody mediated neutralization is associated with modification of the glycosylation profiles and that resistance is, at least partially, due to the alteration in the glycosylation sites within Env (Back et al., 1994; Cheng-Mayer et al., 1999; Johnson and Desrosiers, 2002; Ly and Stamatatos, 2000; Lue et al., 2002; Narayan et al., 1999; Overbaugh and Rudensey, 1992; Overbaugh et al., 1991; Reitter et al., 1998; Rudensey et al., 1998). Therefore, the challenge is to minimize the level of glycosylation in order to enhance the exposure of functionally relevant epitopes while preserving the structure of Env.

We hypothesized that Env is more immunogenic in its native trimeric conformation and that, by introducing a V2 loop deletion, we may further enhance its immunogenicity or its ability to induce better neutralizing antibodies due to the exposure of functional epitopes (Cao et al., 1997; Stamatatos and Cheng-Mayer, 1998). Towards this end, we have demonstrated that the ΔV2 trimer derived from SF162 (o-gp140ΔV2) induced antibodies that neutralized 5 of 7 primary isolates tested (Barnett et al., 2001). Furthermore, rhesus macaques immunized with o-gp140ΔV2 SF162 in a DNA prime and protein boost regimen demonstrated blunting of the acute phase viremia upon challenge with pathogenic SHIV-$_{SF162P4}$. These animals were able to control their viremia with no sign of disease for at least 900 days after challenge (Barnett et al., 2001; Cherpelis et al., 2001a; Cherpelis et al., 2001b).

To increase our understanding of the structural properties of HIV Env and their impact on the quality and magnitude of antibody responses, we have performed detailed biophysical, biochemical and immunological characterization of Env. Here, we describe the purification and characterization of different Env variants and compared their properties such as affinity and thermodynamics of CD4 binding, epitope exposure and structural rearrangement to establish a structure–function relationship that may be useful in the design and development of effective Env-based HIV vaccine.

Results

Purification of HIV-1 SF162 envelope glycoproteins

Four different soluble HIV-1 Envs (Fig. 1A) from SF162, a subtype B isolate, were expressed as monomers (gp120 and gp120ΔV2) as well as trimers that included the gp120 and the extracellular parts of gp41 (o-gp140 and o-gp140ΔV2) in CHO cells and purified as described elsewhere in detail (Srivastava et al., 2002, 2003). The protease cleavage site between gp120 and gp41 was eliminated to stabilize Env in trimeric conformation as described earlier (Srivastava et al., 2003). Purity and identity of all four Envs were confirmed using SDS-PAGE (Fig. 1B), Western analysis (Fig. 1C) and qualitative CD4 binding (data not shown). Gel filtration and SDS-PAGE analyses confirmed that purity of all four proteins was >90%. Furthermore, we analyzed the glycosylation profiles of the purified Envs by digesting the purified Env with different glycosidases and

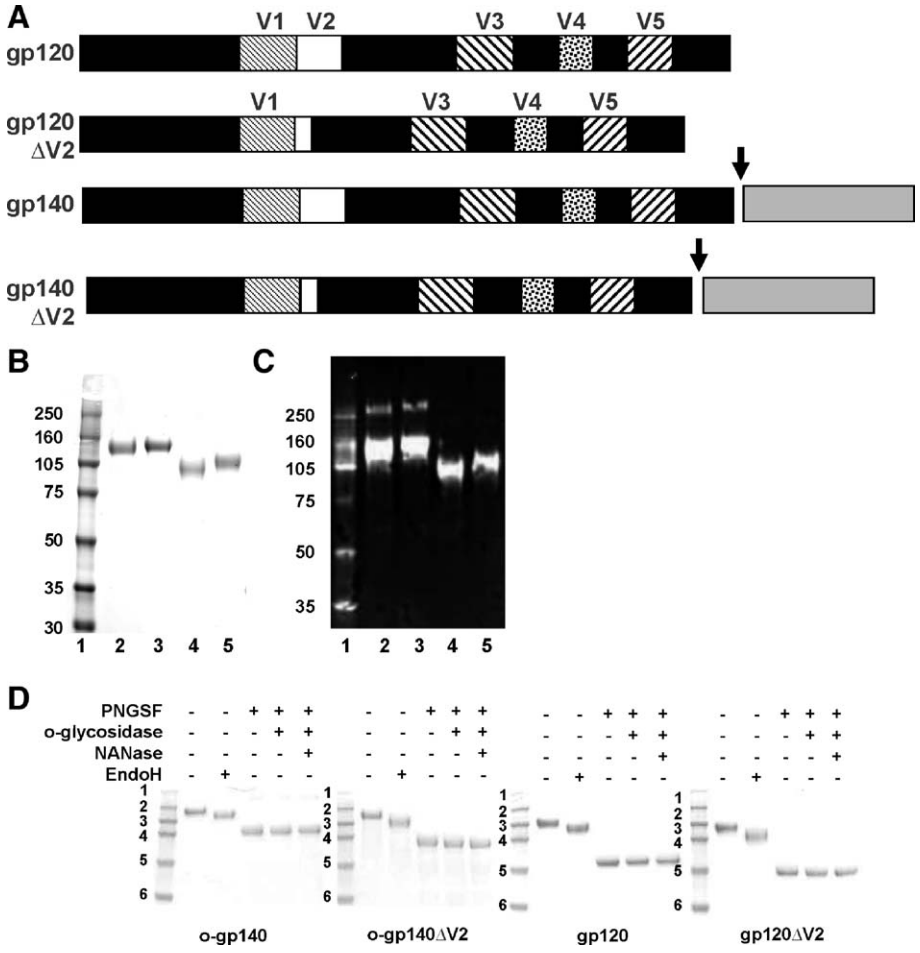

Fig. 1. (A) Schematic diagram of the SF162 envelope structures generated, purified and used in this study. All constructs contain the variable domains V1 and V3–V5, whereas only the full-length gp120 and full-length o-gp140 proteins contain the V2 loop. gp41, the transmembrane domain, and the intracellular domain were deleted to yield soluble versions of the envelope protein. For creating the trimers either with or without V2 loop, the primary and secondary protease cleavage sites were modified as described elsewhere (Srivastava et al., 2004). (B) The purified proteins were analyzed by SDS-PAGE, lane 1—molecular weight standards, lane 2—o-gp140ΔV2, lane 3—o-gp140, lane 4—gp120ΔV2, and lane 5—gp120. (C) Immunoprobing of purified proteins, lane 1—molecular weight standards, lane 2—o-gp140ΔV2, lane 3—o-gp140, lane 4—gp120ΔV2, and lane 5—gp120. (D) Glycosidic linkage analysis of purified proteins treated with different glycosidases, lanes 1, 7, 13 and 19 are molecular weight standards (1—250 kDa, 2—160 kDa, 3—105 kDa, 4—75 kDa, 5—50 kDa, 6—35 kDa).

demonstrated that all forms of Env have predominantly N-linked glycosylation (Fig. 1D). Endo H digestion of purified Envs revealed that different Envs had similar levels of terminal mannoses (Fig. 1D). Oligomerization state was assessed by size exclusion chromatography (SEC) (Fig. 2) and triple detector analysis (data not shown). All four Envs were highly homogenous, gp120 and gp120ΔV2, were >90% monomeric, while the trimers, o-gp140 and o-gp140ΔV2, were >95% trimeric.

Affinity of Env for CD4: direct binding

Affinity of CD4 binding to different Envs was determined using surface plasmon resonance (SPR). Flowing Env over a chip coupled to CD4 (Fig. 3) revealed a slow rate of association ($k_{on} = 10^4\ M^{-1}\ s^{-1}$), indicating a large conformational change in Env upon binding to CD4. By measuring the association and dissociation rates at several Env concentrations, we determined the apparent affinities of different Envs for CD4 (Table 1). gp120, o-gp140 and o-gp140ΔV2 yielded a similar magnitude of binding as reflected by response units (RU) at the same molar concentration (Figs. 3A, C, D). However, gp120ΔV2 showed a higher magnitude of binding to CD4 (higher RU) at the same concentration of analyte (Fig. 3B versus A). Trimeric o-gp140 bound to CD4 approximately 40 times tighter than the monomeric gp120, however, the difference between gp120ΔV2 and o-gp140ΔV2 is only 2-fold (Table 1). gp120ΔV2 yielded a Kd similar to that of gp120, however, compared to other Envs, the model did not fit the data well as evidenced by the high χ^2 value (Table 1) and systematic residuals (data not shown).

Affinity of Env for CD4: indirect binding

To confirm the apparent affinities obtained for Env to CD4 by SPR, we did the converse experiment by capturing the Env on chip and using CD4 as an analyte. The Kd values observed in this experiment for all the Env with the exception of gp120ΔV2 were in agreement to those observed in the reverse experiment (above) where Env was used as an analyte and CD4 was used as

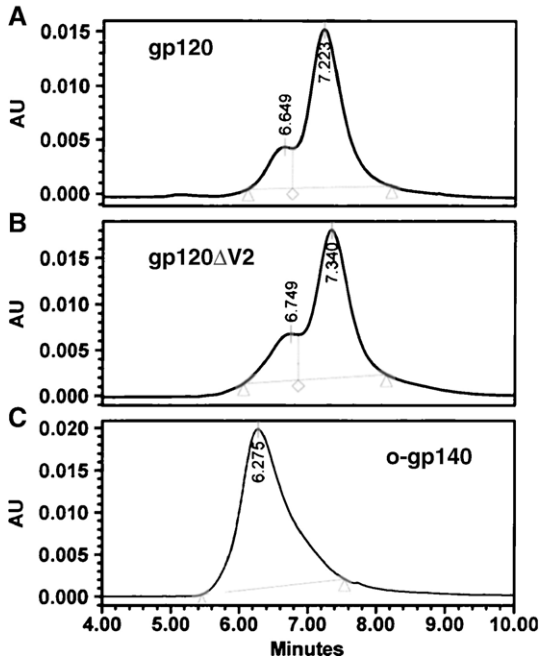

Fig. 2. Size exclusion profiles of Envs after the final purification step (A) gp120 SF162, (B) gp120SF162ΔV2 and (C) o-gp140SF162 demonstrating that all proteins are >90% homogenous and of the predicted size. The size exclusion profile for o-gp140ΔV2 is presented in panel 2 of Fig. 5A.

a ligand (Table 1). No binding was observed using gp120ΔV2 as a ligand (data not shown).

Thermodynamics of the Env–CD4 interaction

We used ITC to determine the stoichiometry and affinity of binding for Envs to CD4 and also to gain insight into the thermodynamics of the interaction in the aqueous state. The binding isotherms obtained (Fig. 4) were analyzed to determine affinity of association (Ka) as well as enthalpy (ΔH), entropy (reflected as $-T\Delta S$) and change in Gibbs free energy (ΔG) (Table 1).

High enthalpies favoring binding were measured for the Env–CD4 interactions (Table 1), suggesting that multiple binding interactions occur during complex formation. Large differences in the binding enthalpies for CD4 between different Envs were also observed. Interestingly, o-gp140 produced the lowest enthalpy favoring association (ΔH = −14 kcal/mol), whereas gp120ΔV2 produced the largest enthalpy favoring association (ΔH = −40 kcal/mol). gp120ΔV2 and o-gp140ΔV2 both produced a binding enthalpy ~4 kcal larger than their corresponding V2 intact Envs.

A large change in entropy opposing binding to CD4 for all the four Envs tested was observed (Table 1), suggesting that significant molecular rearrangement takes place during the binding phase and results in loss of structural freedom. When comparing the calculated Gibbs free energy, enthalpy–entropy compensation is observed as the CD4–envelope complexes yield interaction energies favoring association and differing by only 1 kcal/mol (Table 1).

The specific activity of gp120, o-gp140 and o-gp140ΔV2 was high (>80%) as reflected by the calculated active sites (*n* values). The gp120ΔV2 monomer appears to be 50% active in this assay (Table 1). Note that the number of active CD4 binding sites (*n* value) for the trimers cannot be determined by inspection of the *x* axis of the titration (Fig. 5) as the analysis software calculates the *x* axis as the molar ratio of titrant to ligand (Env to CD4 in this case—see Materials and methods).

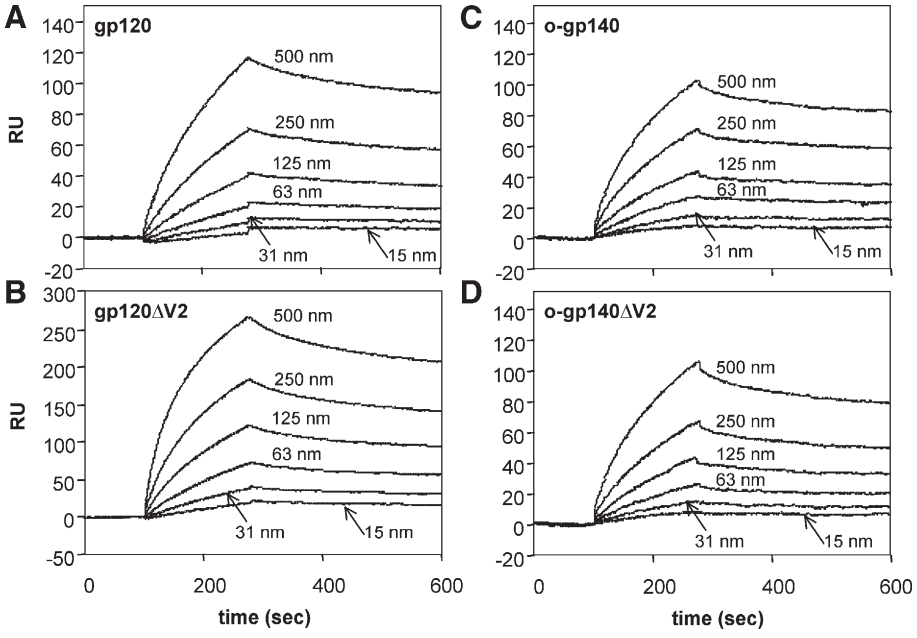

Fig. 3. CD4 binding of Env proteins using BIAcore: (A) gp120, (B) gp120ΔV2, (C) o-gp140 and (D) o-gp140ΔV2. All measurements were taken in duplicate or triplicate using 1000 RUs of four domain CD4 coupled to a CM4 sensor chip. The concentration ranges for HIV Env analytes were 5 to 1000 nM. Biosensor data were prepared by subtracting binding responses obtained from a BSA reference surface from the specific responses.

Table 1
Kinetic and thermodynamic parameters of Env–CD4 binding as determined by SPR and ITC

	SPR measurements									
	CD4 ligand, Env analyte			Env ligand, CD4 analyte		ITC measurements				
	Ka (1/μM)	Kd (nM)	χ^2	Ka (1/μM)	Kd (nM)	Ka (1/μM)	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)	ΔG (kcal/mol)	n
gp120	17	58	7.8	30	33	5.3 ± 0.4	−36 ± 0.5	26	−9	0.8 ± 0.01
gp120ΔV2	53	19	50.3	ND	ND	11.0 ± 1.4	−40 ± 0.9	30	−10	0.5 ± 0.01
o-gp140	667	1.5	1.5	238	4.2	4.2 ± 0.4	−14 ± 0.2	5	−9	2.9 ± 0.04
o-gp140ΔV2	108	9.3	9.3	106	9.4	2.1 ± 0.2	−18 ± 0.3	9	−9	3.3 ± 0.05

Affinity (Kd) was measured by analyzing multiple concentrations of analyte in SPR experiments and fitting the sensorgrams to one set of parameters. Affinity (Ka), enthalpy (ΔH), entropy (expressed as $-T\Delta S$) and number of CD4 binding sites (n) were determined by analyzing ITC titrations. All data reported were repeated in duplicate or triplicate.

Stoichiometry of CD4 binding to envelope proteins

We first determined the stoichiometry of binding of different Envs to CD4 using ITC. Initial analysis of the data revealed potential heterogeneity in the material. Therefore, we analyzed post-titrated ITC materials by size exclusion chromatography (SEC-HPLC) to determine the concentration of active vs. aggregated fraction of Env (data not shown). We observed that as much as 20% of the concentrated Envs protein was aggregated consistent with the n values observed, except in the case of the gp120ΔV2 monomer which yields an n value of 0.5 by ITC (Table 1), but appears to be at least 80% monomeric by post-titration SEC-HPLC (data not shown). After adjusting the active concentration of different Envs to reflect the active concentration determined by SEC-HPLC, the ITC data yielded appropriate number of CD4 binding sites (n) for each Env (n = 1 for gp120 and gp120ΔV2 and n = 3 for o-gp140 and o-gp140ΔV2).

We confirmed our results for stoichiometry of binding by SEC-HPLC using different ratios of Env to CD4. Chromatograms of a titration for o-gp140ΔV2 are shown in Fig. 5A. Using a molar excess of Env compared to CD4 yielded a dominant peak corresponding to the free trimer (panel 3). As additional CD4 was added, the retention time of the dominant peak shifted to a larger species corresponding to an Env–CD4 complex. At a molar ratio of 3 CD4 molecules:1 trimer, we did not observe any free CD4, indicating that all three CD4 binding sites in o-gp140ΔV2 were saturated. Titrations of CD4 against all other Envs yielded similar results, i.e., monomeric Envs (gp120 and gp120ΔV2) bound to a single CD4, whereas trimeric Envs (o-gp140 and o-gp140ΔV2) bound three CD4s (Fig. 5B). Reverse titrations where the CD4 concentration was kept constant confirmed the data obtained (data not shown).

Characterization of envelope proteins by monoclonal antibody binding

We monitored the exposure of the CD4 binding site and the CD4 inducible site in the different Envs using mAbs b12 (Kessler et al., 1997; Trkola et al., 1996a, 1998) and 17b (Thali et al., 1993). All four Envs bound to b12; data for gp120 (Fig. 6A) and o-gp140 (Fig. 6B) are shown. Kinetic data indicate that both trimers exhibited nanomolar binding to b12 (Table 2), consistent with previously reported results (Barbas et al., 1992). However, gp120 exhibited a 3-fold weaker affinity for b12 compared to o-gp140. The apparent affinity of gp120ΔV2 for b12 was 70-fold weaker compared to o-gp140ΔV2 (Table 2). gp120ΔV2 consistently yielded a two-fold higher RU value compared to gp120 at the same concentration (data not shown), however, this difference was not observed for o-gp140ΔV2.

All four Envs bound to 17b in the absence of CD4. Binding of gp120 and o-gp140 to 17b in presence and absence of CD4 is shown (Figs. 6C–F). The kinetic data indicate that both gp120 and gp120ΔV2 bind to 17b in the absence of CD4 with nanomolar affinity (Table 2). Interestingly, in the absence of CD4, both o-gp140 and o-gp140ΔV2 exhibit similar affinities for 17b but have roughly 3-fold weaker affinity compared to monomers (Table 2).

In the presence of CD4, the 17b epitope was up-regulated in all Envs (Figs. 6D, F); up-regulation was approximately 2-fold for gp120 and gp120ΔV2 (Figs. 6C, D) and was greater than 4-fold for o-gp140 and o-gp140ΔV2 (Figs. 6E, F). The affinities of Envs for 17b in the presence of CD4 could not be accurately determined due to the extremely high affinity of binding (Figs. 6D, F).

Immunogenicity studies

We evaluated these Envs for their ability to induce antibody responses in rabbits. Groups of rabbits were immunized in a DNA prime and protein boost regimen. All of the animals induced Env-specific antibodies after three DNA immunizations (data not shown), which were substantially enhanced after the protein boost (Fig. 7A). To assess the quality of these binding antibodies, we determined the proportion of antibodies directed against the CD4 binding site. Animals primed with o-gp140ΔV2 DNA followed by o-gp140ΔV2 protein boost induced the highest percentage of antibodies directed against the CD4 binding site (Fig. 7A).

We also determined the relative proportion of antibodies directed against total and linear epitopes. Animals primed with o-gp140 and o-gp140ΔV2 and boosted with o-

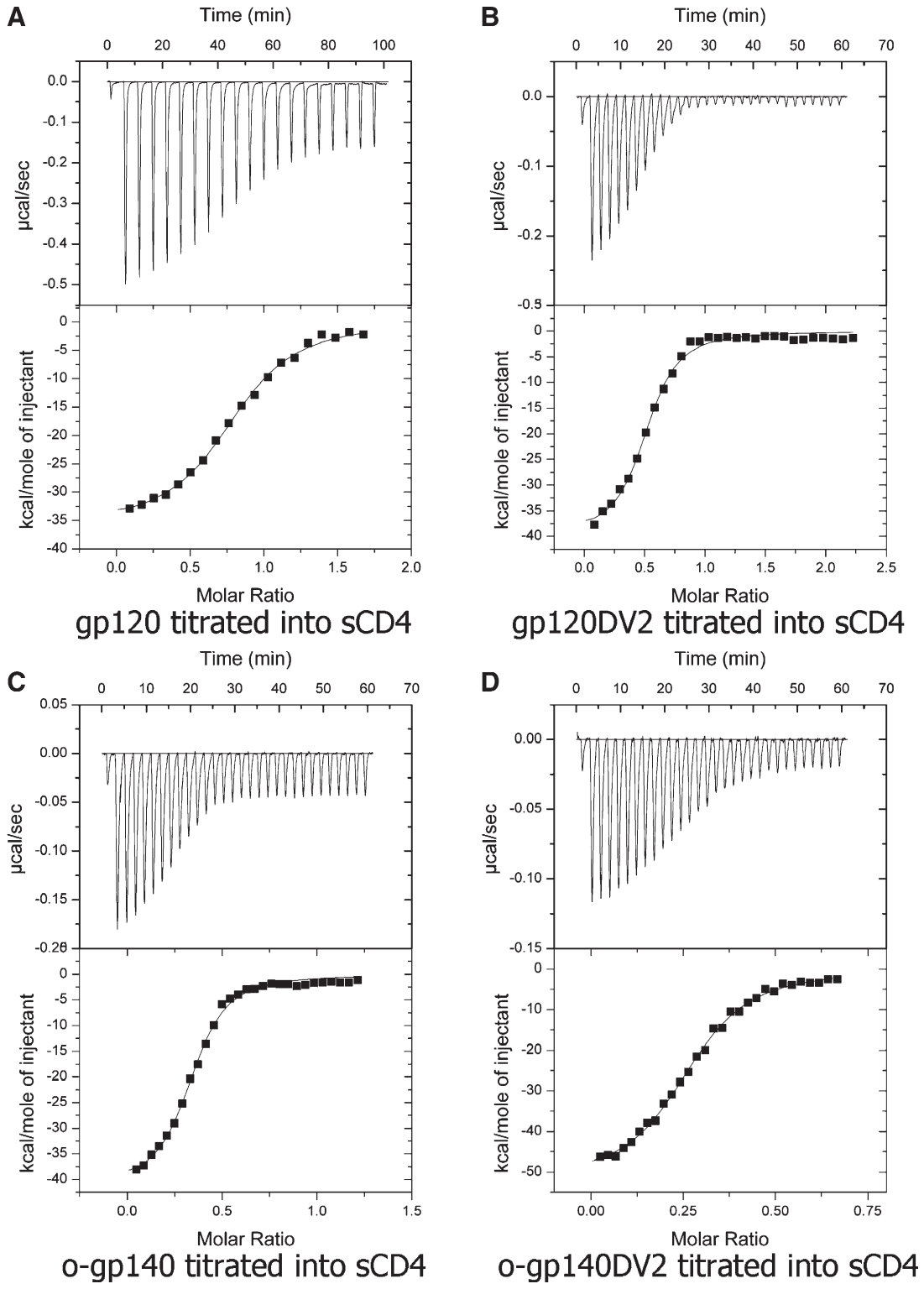

Fig. 4. Isothermal titration calorimetry (ITC) is used to measure the heat of the reaction between four-domain CD4 and Env in free solution: (A) gp120, (B) gp120ΔV2, (C) o-gp140 and (D) o-gp140ΔV2. All titrations were performed in duplicate with the heat of mixing and dilution subtracted from the final data. The top panels represent the raw data (power:time). The bottom panels represent integrated areas per mole of injected ligand (envelope) as a function of molar ratio. Note that, for the trimeric envelope proteins, the number of CD4 binding sites (n) cannot be determined by the x axis (see Materials and methods). The solid line represents the best nonlinear fit to the experimental data.

gp140ΔV2 induced a higher proportion of antibodies directed towards the conformational epitopes compared to either gp120 or gp120ΔV2 primed animals (Fig. 7B). The difference in titers against linear epitopes between the gp120ΔV2 monomer and o-gp140 and o-gp140ΔV2 was statistically significant ($P < 0.05$).

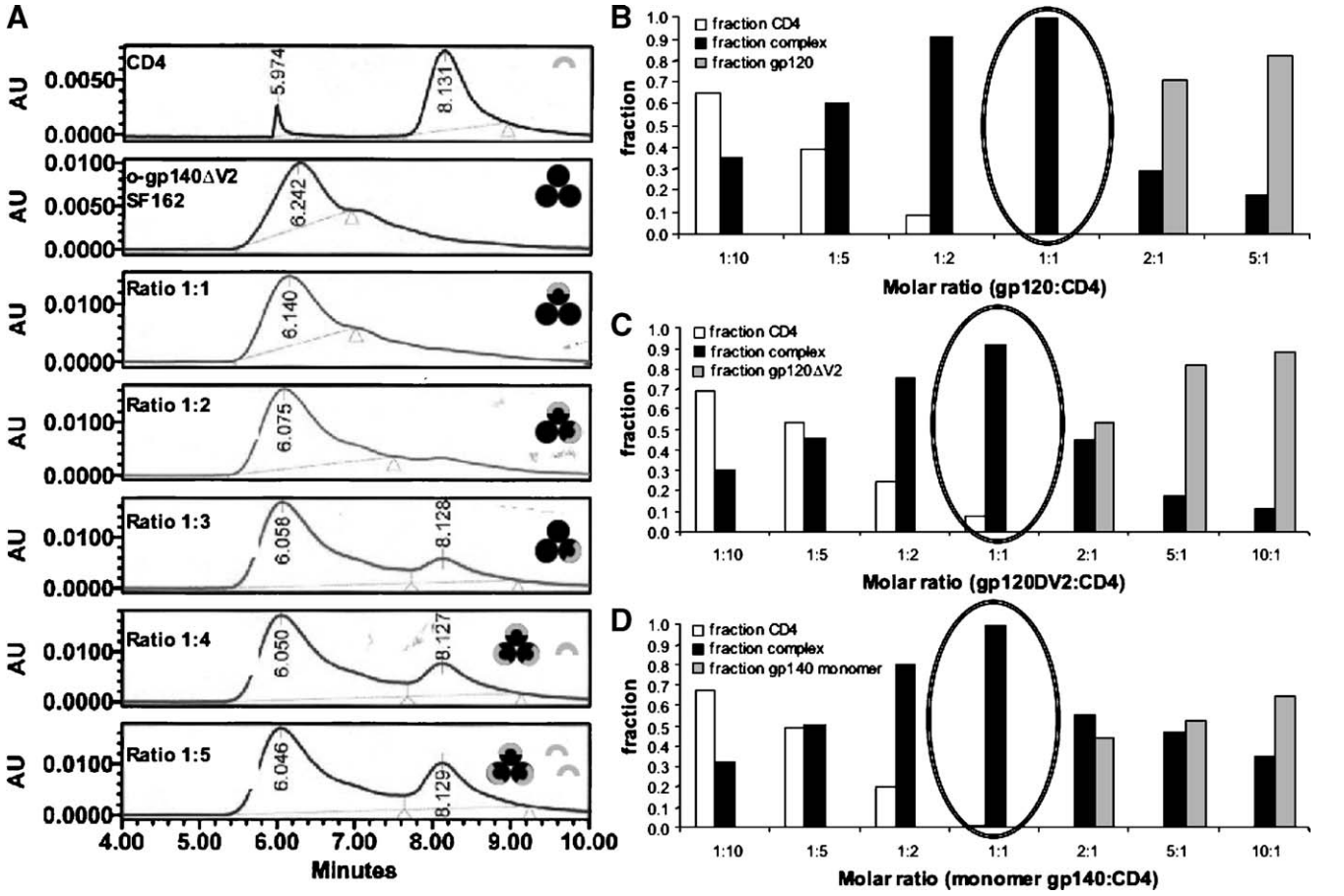

Fig. 5. Env/CD4 titration followed by size exclusion HPLC defines the number of active binding sites. (A) A typical profile of mixtures of o-gp140ΔV2SF162 to CD4 at different molar ratios at 25 °C. The interpretation of the data for different mixtures is presented by envelope trimers (circles) and sCD4 monomers (crescents). The trimeric protein continues to bind the titrant (CD4) until a molar ratio of 1:3 is observed, confirming that all three CD4 binding sites on the trimer are active and saturated. (B) Molar fractions of gp120, CD4 and the complex were calculated from peak area for each component from a chromatograph of gp120 titrated with a ten-fold concentration range of sCD4; (C) gp120ΔV2 titrated with a ten-fold concentration range of sCD4 and (D) o-gp140 titrated with a ten-fold concentration range of sCD4, with the molar fraction of trimer calculated as the number of potential CD4 binding sites.

To further analyze qualitative differences in the antibodies induced by different forms of Env, we determined the proportion of antibodies directed against linear V3 epitopes. Priming with gp120ΔV2 and boosting with o-gp140ΔV2 induced four-fold higher antibody titers to linear V3 epitopes compared to gp120 (Fig. 7C). In addition, priming with o-gp140ΔV2 and boosting with o-gp140ΔV2 induced the lowest responses against linear epitopes in the V3 loop. Avidity of antibodies induced by different Envs was measured (Fig. 7D). At 4 weeks post-final DNA immunization, the antibody avidities were relatively low for all groups. However, at 4 weeks post-final protein boost, the avidities were highest for o-gp140 and o-gp140ΔV2 primed and o-gp140ΔV2 boosted groups compared to gp120 and gp120ΔV2 primed and o-gp140ΔV2 boosted groups (Fig. 7D).

Discussion

Designing an immunogen for use as an HIV vaccine remains a challenge, in large part due to the lack of understanding of the attributes necessary to design an effective immunogen capable of inducing protective neutralizing antibody responses. As HIV Env is currently the best candidate for such a vaccine, we analyzed the biophysical, biochemical and immunological properties of different Envs and attempted to correlate structural data to immunogenicity to establish structure–function relationships.

The apparent affinity, kinetics and thermodynamics of the CD4–Env interaction were defined by SPR and ITC. The interaction between CD4 and the engineered Envs was of high affinity (nanomolar), with the trimers binding approximately 3-fold tighter to CD4 than their corresponding monomers as measured by SPR. In addition, there was strong favorable Gibbs free energy of binding for all four Envs as measured by ITC. Using Envs (gp120, o-gp140 or o-gp140ΔV2) either as a ligand or an analyte in SPR yielded similar affinities for the resulting complexes, thus confirming the results.

Similar to previous findings (Stites et al., 1997), we observed slow rates of association for Env to CD4 by SPR along with large entropic costs of association as measured by ITC. These data confirm that significant molecular rearrangements take place in Env upon binding to CD4 as observed in previous

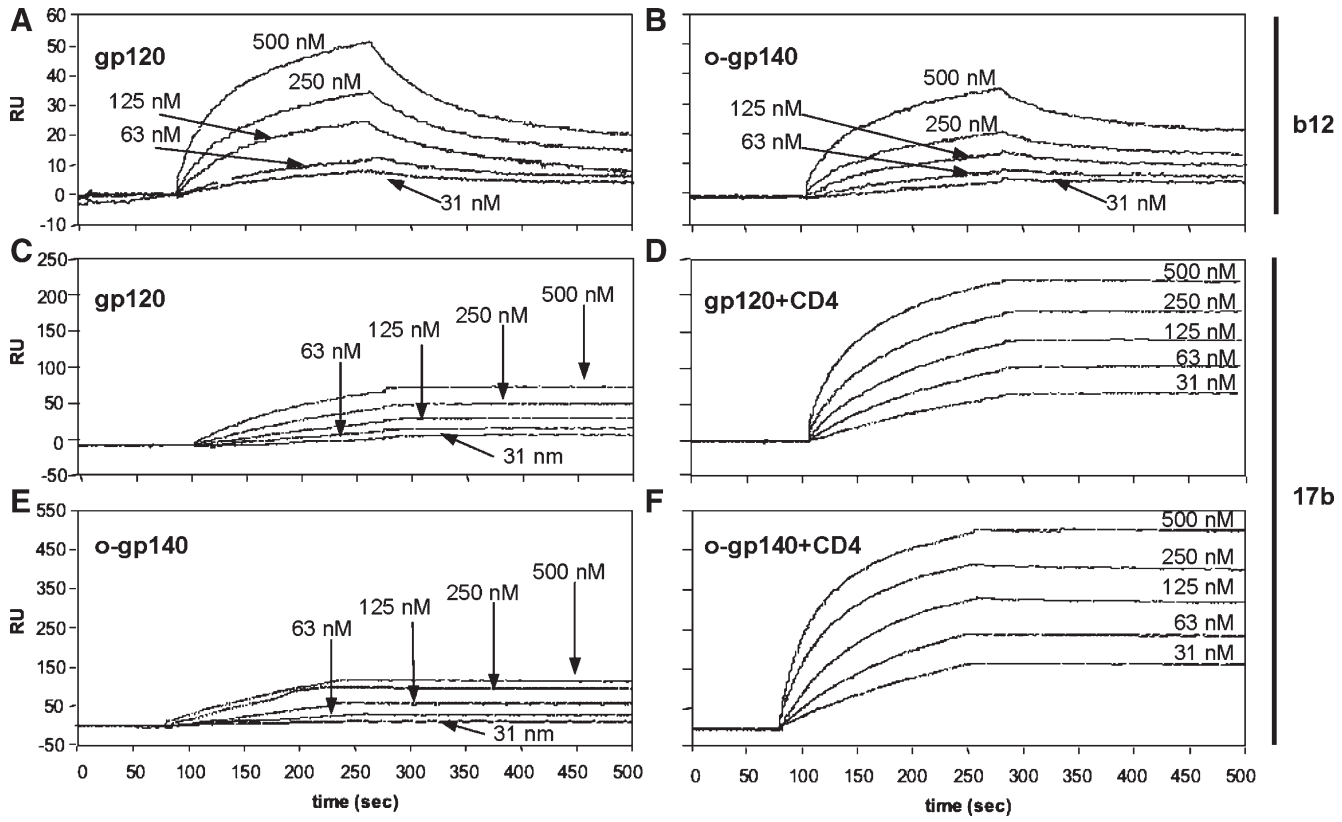

Fig. 6. Probing of Env structure with monoclonal antibodies. b12 binding observed by SPR for: (A) gp120 and (B) o-gp140. 17b binding for (C) gp120, (D) gp120–CD4 complex, (E) o-gp140, and (F) o-gp140–CD4 complexes. The concentration ranges for HIV Env were 5 to 1000 nM. As a negative control, we used a control surface coupled to 1000 RUs of BSA (Pierce). All measurements were taken in duplicate or triplicate.

studies. One of the striking observations is that apparent affinities measured by SPR, as well as enthalpies and Gibbs free energies calculated from ITC data for all the Envs were remarkably consistent with previous studies despite using gp120 from a different isolate, using four-domain rather than two-domain CD4 and performing experiments under different conditions (Myszka et al., 2000; Pancera et al., 2005). These data suggest that these biophysical techniques are quite robust and should be used in characterization of novel immunogens.

There is a wealth of data demonstrating that recombinant Env binds to CD4 (Kwong et al., 1998; Myszka et al., 2000; Sattentau and Weiss, 1988; Trkola et al., 1996a; Srivastava et al., 2002, 2003), however, there is no clear evidence demonstrating if three or fewer CD4 binding sites in a trimer are accessible for CD4. Molecular modeling of a trimeric Env based on the monomeric structure suggests that all three binding sites would be accessible to surface expressed CD4, and CD4: CD4 steric hindrance may not occur due to large spacing (~200 Å) between the receptor molecules (Kwong et al., 2000).

With the use of SEC-HPLC, we have demonstrated that each individual monomer in a trimer had an intact CD4 binding site regardless of the presence or absence of the V2 loop. The stoichiometry determined by ITC is in agreement with the data obtained in SEC-HPLC if we adjust the concentration of functional Env (non-aggregated material), thereby demonstrating the importance of post-ITC titration analysis of the material.

Large binding enthalpies that favor association are indicative of multiple binding interactions, whereas large entropies unfavorable to association are indicative of large structural rearrangements and a loss of structural freedom (Myszka et al., 2000). It has been suggested that the large structural rearrangement that Env undergoes upon CD4 binding may inhibit B cell recognition and induction of high affinity antibodies especially in the case of the unliganded Env (Chen et al., 2005; Kwong, 2005). Differences in enthalpies were observed especially between the monomers (gp120 and gp120ΔV2) and the trimers (o-gp140 and o-gp140ΔV2) (trimers yielding roughly 2-fold higher enthalpies favoring binding to CD4 than the corresponding monomers).

Table 2
SPR studies of envelope binding to b12 and 17b monoclonal antibodies

Proteins tested	SPR measurements			
	b12 ligand, Env analyte		17b ligand, Env analyte	
	Ka (1/μM)	Kd (nM)	Ka (1/μM)	Kd (nM)
gp120	39	26	512	2
gp120ΔV2	2	446	508	2
o-gp140	113	8.8	142	7.0
o-gp140ΔV2	159	6.3	135	7.4

For kinetic analysis, SPR sensorgrams were fitted simultaneously to a single-site conformational change-binding model by using a nonlinear data analysis program (Biaevaluation 4.01, Biacore AB, Uppsala, Sweden). All kinetic parameters reported were repeated in duplicate or triplicate.

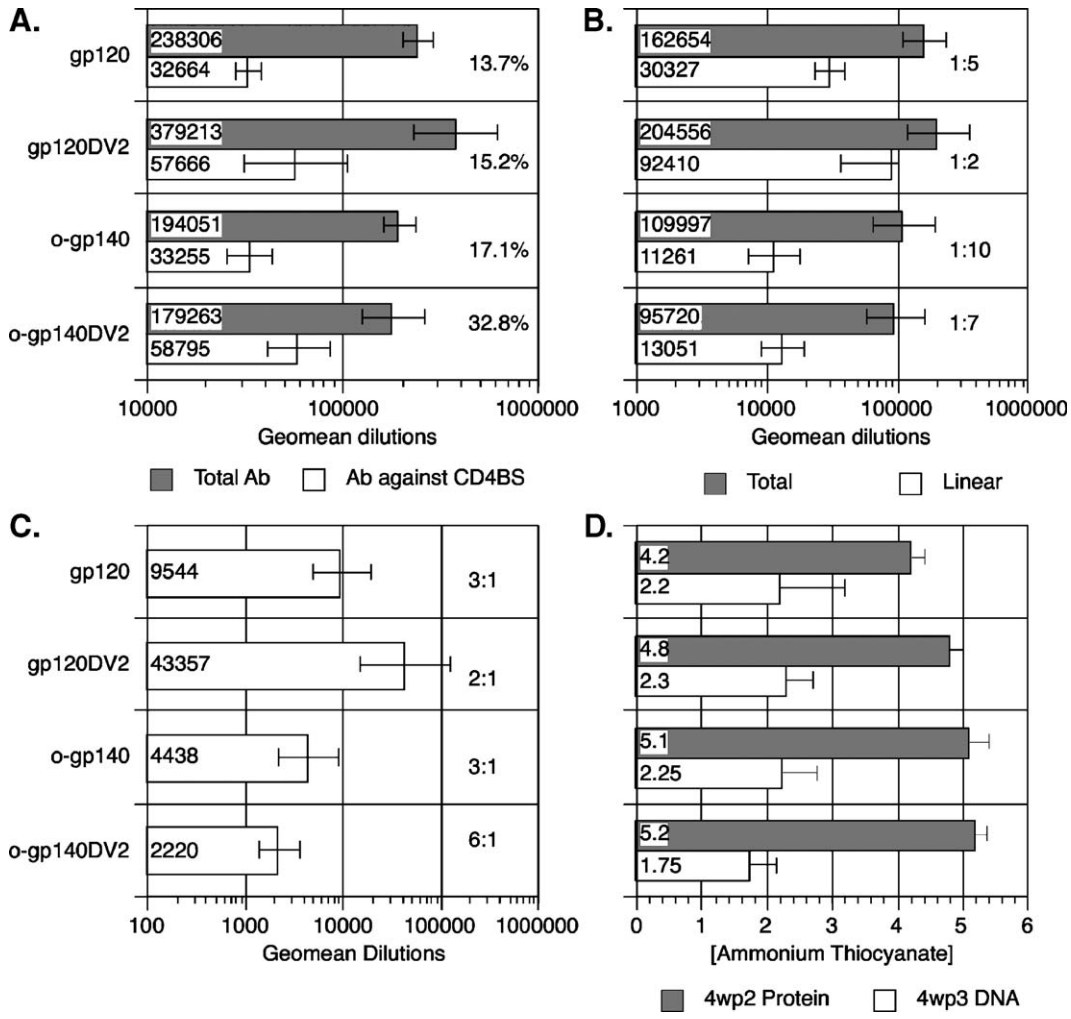

Fig. 7. Quantity and quality of antibodies induced in rabbits in prime boost regimen: (A) total anti-Env antibodies and percentage of antibodies directed against CD4 binding site 4 weeks post-second protein boost. (B) Comparison of proportion of antibodies directed towards total and linear epitopes induced by different Env. (C) Antibody responses directed against the linear V3 epitope and the ratio of antibodies directed against linear epitopes in V3 loop vs. all linear epitopes. (D) Avidity of antibodies induced by different immunogens in DNA prime and protein boost regimen.

Compensating the enthalpies, large entropies unfavorable to association were observed. A large entropic cost for the association of the gp120 monomer with CD4 is supported by modeling calculations (Hsu et al., 2005) and has been attributed to formation of intermolecular hydrogen bonds and hydrophobic contacts due to conformational rearrangement of the bridging sheet in gp120 upon binding (Hsu et al., 2005; Myszka et al., 2000). This model is supported by recent elucidation of the apo gp120 core structure (Chen et al., 2005) that suggests through large movements and rotations that the bridging sheet forms when Env is liganded to CD4. To our knowledge, entropic cost of structural rearrangement upon binding for the soluble trimeric Env has not previously been reported. It is noteworthy that, in the case of gp120ΔV2 and o-gp140ΔV2, the magnitude of entropy is greater, suggesting that in the context of the V2 loop deletion, o-gp140ΔV2 undergoes more structural rearrangement.

The trimers produced by using the trimerization domain in the gp41 in this study have all three CD4 binding sites intact and have a strong affinity for CD4. Pancera et al. (2005) found somewhat relatively weaker binding for an engineered soluble trimer expressed in *Drosophila* using GCN4 as a trimerization domain for gp120 subunits (Kd = 65 nM). Interestingly, unlike the trimeric proteins we constructed using a fused gp41 domain, the GCN4 linked trimers appeared to bind only two molecules of sCD4 unless multiple variable loops were deleted, suggesting that access to all three binding sites may be linked to the orientation of the monomers provided by gp41.

The affinities of Env for CD4 measured by ITC did not reflect the tighter binding of the trimers versus the monomers as observed by SPR. There are several potential explanations that may account for these differences: (i) differences in experimental conditions, in ITC the interaction takes place in aqueous phase, while in SPR Env protein is coupled to solid phase, and therefore more stable albeit with lesser degrees of freedom, (ii) aggregation of the envelope protein at high concentrations may play a role in affinity measurements in ITC and (iii) ITC is relatively limited at measuring higher affinity (nanomolar) interactions in the absence of competition. It is also important to note that multivalent binding of a

trimeric Env to CD4 is not likely to be observed in SPR experiments due to steric hindrance and/or orientation of the interacting partners, thus apparent affinities measured by SPR may be indicative of only one or two sites on a trimer despite all three sites being active in ITC.

Monoclonal antibodies were used to probe the exposure of important epitopes to elucidate the differences or lack of differences in the structure of different Envs using SPR. Data obtained using the b12 antibody indicated that this epitope was preserved and properly exposed on all Envs to a similar extent, with the exception of gp120ΔV2. Similar to the CD4 binding experiments, gp120ΔV2 yielded a much higher maximum association signal by SPR. However, the affinity of gp120ΔV2 for b12 was approximately 20-fold less compared to gp120. The affinity for the b12 antibody was at least 3-fold tighter for o-gp140 and o-gp140ΔV2 compared to gp120 and gp120ΔV2, possibly an indication of avidity or better exposure or preservation of this epitope on trimers compared to monomers.

All four proteins exhibited similar magnitudes of binding to 17b (after normalizing data for molecular weight) in the presence or absence of CD4. The trimeric proteins o-gp140 and o-gp140ΔV2 exhibited a 3-fold weaker affinity compared to corresponding monomers. It is possible that not all the CD4i epitopes are accessible in a trimer or the binding site may be altered by oligomerization. Both monomeric proteins gp120 and gp120ΔV2 up-regulated 17b binding by two-fold in the presence of CD4, whereas the trimeric proteins o-gp140 and o-gp140ΔV2 up-regulated the epitopes at least four-fold.

By normalizing the CD4 and b12 SPR data for molecular weight of different Env analytes, we observed 3-fold greater magnitude of association as reflected by higher RU for gp120 compared to o-gp140 and o-gp140ΔV2. These data suggest that 3-fold fewer trimer molecules can bind to the surface most likely due to steric hindrance caused by the larger size of the trimer. However, we observed a different behavior for gp120ΔV2 where normalized maximum RU levels for CD4 and b12 are 3-fold higher than for gp120 and nearly 10-fold higher than for the trimers. Additionally, data obtained for CD4 binding to gp120ΔV2 were difficult to fit to a single-site binding model suggesting either gp120ΔV2 represents a heterogeneous population of structures or its structure is altered compared to the other three Env structures. When we performed the reverse SPR experiment using gp120ΔV2 as a ligand (coupling to the solid phase) and CD4 as an analyte, no binding is observed in contrast to the other Env proteins. Furthermore, ITC titrations of gp120ΔV2 and CD4 suggest that approximately 20–50% of gp120ΔV2 is inactive. These data suggest that indeed the structure of gp120ΔV2 is altered.

These data taken together suggest that the V2 deletion in the monomer allows a functional albeit altered CD4 binding site. This deletion may also affect folding in other parts of the molecule, allowing increased number of molecules to bind in SPR analysis and a higher proportion of inactive molecules as measured by ITC. In contrast, CD4 binding measured by SPR and ITC, and b12 and 17b binding measured by SPR remained relatively unchanged in o-gp140ΔV2 relative to o-gp140. This suggests that, in the context of the trimer, the V2 deletion does not greatly alter the structure as it does in the context of the monomer. Interestingly, maximum normalized RU measured by SPR for 17b both in the presence and absence of CD4 appears to be unaffected by the V2 deletion for both monomer and trimer as they respond similarly to gp120 and o-gp140 in this assay. This suggests that the 17b epitope is rather unaffected by the V2 deletion compared to the CD4 binding site.

Priming with gp120ΔV2 induced a higher proportion of antibodies directed against all linear epitopes and linear epitopes in the V3 loop than priming with other Envs. For linear epitopes, the increase in titer was significant compared to antibodies induced by the trimers ($P < 0.05$). However, differences between gp120 and gp120ΔV2 were not significant ($P = 0.06$).

We understand that priming with different Envs and boosting all the animals with o-gp140ΔV2 protein is not ideal. However, it is interesting to note that, despite priming the animals with different Env DNA constructs and boosting them with the same (o-gp140ΔV2), we still observed a statistically significant difference in the anti-Env antibodies induced by different structures. We expect that priming and boosting the animals with matched constructs such as gp120ΔV2 would greatly enhance the qualitative differences observed compared to animals primed with gp120ΔV2 and boosted with o-gp140ΔV2.

Affinity of CD4 binding correlated with proportion of the antibodies induced against the conformational epitopes. Biophysical analysis of gp120ΔV2 suggests that it may be partially inactive or heterogeneous, consistent with induction of higher proportion of antibodies directed against linear, and other non-functional epitopes. Furthermore, biophysical analysis reveals that the V2 deletion has broad structural implications in the monomer not shared by the trimer, and these changes are reflected in the quality of the immune response. This study demonstrates that designed features of engineered immunogens should be examined using biophysical techniques as it may be relevant in predicting the in vivo efficacy and provide an important tool for characterizing and selecting an effective vaccine candidate for further evaluation in preclinical studies. This is the initial attempt to establish a correlation between biophysical properties and immunogenicity of a designed immunogen, further studies with optimized regimens and larger sample sizes will be needed to confirm these initial findings.

Materials and methods

Envelope plasmid construction

The sequences encoding the open reading frame of the ectodomain of the Env protein from the HIV-1 SF162 and HIV-1 SF162ΔV2 isolates were optimized for higher expression, as described earlier (Srivastava et al., 2002),

and constructed synthetically as a 2.1-kb *Eco*RI–*Xba*I DNA fragment. Human tissue plasminogen activator (TPA) signal sequence was added for efficient secretion. To stabilize the Env proteins in a trimeric conformation, we introduced a series of mutations in the primary and secondary protease cleavage sites in the Env polypeptides (Srivastava et al., 2003). The resulting Env expression cassettes (gp120SF162, gp120SF162ΔV2, gp140SF162 and gp140SF162ΔV2) were cloned into the pCMV3 expression vector for both transient expression and the development of stable CHO cell lines.

Development of stable CHO cell lines secreting HIV SF162 envelope proteins

Stable CHO cell lines secreting gp120SF162, gp120-ΔV2SF162, gp140SF162 and gp140SF162ΔV2 were derived by using DG-44 cells with a double deletion in the dihydrofolate reductase gene following the experimental protocol described elsewhere (Srivastava et al., 2003, 2002).

Production of HIV envelope proteins

The CHO cell clones producing the Env proteins of interest were bioreactor-adapted in low serum containing media, selected for the best clone for each protein and grown in 12 l production bioreactors. Bioreactors were monitored daily for cell density, pH, CO_2 and O_2 concentration. Collected media were concentrated 20-fold through a 100-kDa pore-size membrane filter and stored at −80 °C in the presence of 1 mM EDTA and 1 mM EGTA.

Purification of envelope proteins

All the envelope proteins were purified as described elsewhere in detail using a combination of lectin capture and ion exchange columns (Srivastava et al., 2003). During the purification process, fractions were analyzed by polyacrylamide gel electrophoresis (PAGE) both under reducing and denaturing and under native conditions following standard methods and also in a CD4 receptor-binding assay (Srivastava et al., 2002, 2003). Gels were stained with Coomassie brilliant blue or processed for immunoblotting. All the fractions containing Env monomer with and without V2 loop were pooled, concentrated and stored frozen at −80 °C. Peak fractions containing o-gp140SF162 and o-gp140 SF162ΔV2 were pooled, concentrated and fractionated on a 16 × 90 mm Superdex-200 column (Pharmacia) equilibrated with 10 mM NaCitrate plus 300 mM NaCl to separate monomer from trimer. The fractions containing Env protein in trimeric conformation were pooled, concentrated and kept frozen at −80 °C until used.

Surface plasmon resonance (SPR) assay

CD4 binding assay

Surface plasmon resonance assays were performed using a BIACORE 3000 optical biosensor system (Biacore AB, Uppsala, Sweden). To perform the kinetic study of the binding of Env to CD4, CD4 was immobilized using amine coupling onto CM5 sensor chip (BIAcore AB, Uppsala, Sweden) to attain 1000 response units (RUs). Association was assessed by flowing different Envs (5 to 1000 nM) over the chip at a flow rate of 25 μl/min. Signal from a reference cell coupled to BSA was subtracted from the specific responses. The association and dissociation phase data for double injections of five concentrations of analyte were fitted simultaneously to a single-site conformational change-binding model using Biaevaluation 4.01 (Biacore AB, Uppsala, Sweden).

The reverse experiment was performed by immobilizing Env proteins to CM5 chip using amine coupling reaction to attain 800–1200 RUs. The assay was performed as described above.

mAbs binding assay

To perform mAb binding to Env, b12 and 17b mAbs were immobilized using amine coupling onto CM5 sensor chip to attain 1000 RUs, and the assay was performed under the experimental conditions described as above.

Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry was performed on a VP-ITC (MicroCal LLC, Northampton, MA) using a 1.4 ml cell and a 0.25 ml syringe. Proteins were dialyzed extensively in PBS containing 0.005% Tween and concentrated using a stir cell (Millipore Inc.) prior to loading. Three to five micromolar CD4 was loaded in the cell, with 18- to 30-μM envelope protein in the syringe. Titrations were performed using 15 μl injections with 5-min delay between each injection. All runs were adjusted for heat of dilution and mixing by subtracting a blank run of envelope titrated into buffer only. Binding isotherms obtained were auto-fitted to a baseline using Origin® software (MicroCal LLC, Northampton, MA). Using a floating number of binding sites, isotherms were best fit to a single set of binding parameters. Fits were generated using the Origin® 7.0 software parameter "ligand in cell" option, thus generated figures for the trimers do not reflect the *n* values on the *x* axis as the software does not re-calculate the molar ratio on the figure but does reflect the change in the fit obtained (MicroCal LLC).

Size exclusion chromatography

All titrations were performed at 25 °C in PBS pH 7.4. Mixtures containing different ratios of Env and CD4 were prepared, incubated for 15 min at room temperature and injected onto a pre-equilibrated Bio Sil SEC 250 gel filtration HPLC column (Bio-Rad Laboratories). Samples were run in 20 mM NaH_2PO_4 and 400 mM ammonium sulfate (pH 6.0) at a flow rate of 1 ml/min, and absorbance was measured at 214 nm using a Photodiode Array Detector (Waters Corporation, Milford, California). Molar fraction was determined by integrating the corresponding peak area for each component of the mixture. 500 nM o-gp140ΔV2 was titrated with a range of 500 nM to 2.5 μM sCD4, 500 nM gp120 SF162 was titrated with a range of 5 nM to 2.5 μM sCD4, 500 nM gp120ΔV2SF162 titrated with

5 nM to 5 μM sCD4 and 500 nM o-gp140 was titrated with 150 nM to 15 μM sCD4.

Immunogenicity study

Age- and weight-matched New Zealand female rabbits (4 rabbits per group) were immunized intramuscularly with 1 mg of plasmid DNA corresponding to o-gp140, o-gp140ΔV2, gp120 and gp120ΔV2 at 0, 4 and 12 weeks using a biojector. On weeks 24 and 36, all the animals irrespective of prime were boosted with 50 μg of o-gp140ΔV2 protein in MF59 at two sites (250 μl each). These rabbits were bled regularly at 2-week intervals until the completion of the study, and serum was collected and analyzed for binding and neutralizing antibodies.

Measurement of antibody responses

Total antibody responses

To determine the total antibodies, ELISA was performed on serum samples collected every 2 weeks, as described previously (Srivastava et al., 2002).

Linear antibody responses

To determine the antibodies induced against the linear epitopes, HIV Env protein was denatured and reduced in 0.1% SDS and 10 mM DTT by boiling for 3–5 min. The ELISA plates were coated with 100 ng of the reduced and denatured protein and the assay performed as described elsewhere in detail (Srivastava et al., 2002).

Anti-peptide ELISAs

To determine the antibody responses directed against linear V3 epitopes, appropriate peptides derived from the V1, V2 and V3 loops were synthesized, reconstituted in PBS at pH 7.4 and used to coat the ELISA plates at 1 μg/well. All other steps were performed as described elsewhere in detail (Srivastava et al., 2002).

Determining the proportion of antibodies directed against CD4 binding site

To determine the proportion of antibodies directed against the CD4 binding site, the ELISA plates were coated with 100 ng of trimeric Env protein as described above. After blocking the non-specific binding sites, half the Env coated wells in each plate were incubated with 1 μg of sCD4 (four domain). The wells were washed, and all the sera samples were tested in duplicate (one without CD4 and other with CD4). All other steps were performed as described above. We have compared the ELISA titers obtained for individual serum sample in the presence and absence of CD4. The difference in titers in presence of CD4 was presented as % of antibodies directed against the CD4 binding site.

Antibody avidity index

Antibody avidity index determination was performed using an ammonium thiocyanate displacement (NH_4SCN) ELISA as described elsewhere (Srivastava et al., 2002).

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