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Analysis of the Interaction between the HIV-Inactivating Protein Cyanovirin-N and Soluble Forms of the Envelope Glycoproteins gp120 and gp41

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ABSTRACT

The novel virucidal protein cyanovirin-N (CV-N) binds with equally high affinity to soluble forms of either H9 cell-produced or recombinant glycosylated HIV-1 gp120 (sgp120) or gp160 (sgp160). Fluorescence polarization studies showed that CV-N is also capable of binding to the glycosylated ectodomain of the HIV-envelope protein gp41 (sgp41) (as well as SIV glycoprotein 32), albeit with considerably lower affinity than the sgp120/CV-N interaction. Pretreatment of CV-N with either sgp120 or sgp41 abrogated the neutralizing activity of CV-N against intact, infectious HIV-1 virions. Isothermal calorimetry and optical biosensor binding studies showed that CV-N bound to recombinant sgp120 with a K_d value ranging from 2 to 45 nM and to sgp41 with a K_d value of 606 nM; furthermore, they indicated an approximate 5:1 stoichiometry for CV-N binding to sgp120 and a 1:1 stoichiometry for CV-N binding to sgp41. Circular dichroism studies additionally illuminated the binding of CV-N with both sgp120 and sgp41, providing the first direct evidence that conformational changes are a consequence of CV-N interactions with both HIV-1 envelope glycoproteins.

The HIV viral envelope glycoproteins (Env) have been studied intensely in an effort to understand their specific interactions with cellular proteins that afford virus entry into cells and to determine how they help HIV evade host immune response (Chan and Kim, 1998; Wyatt and Sodroski, 1998). Present knowledge of Env indicates that HIV codes for the production of the envelope protein precursor complex gp160, which is glycosylated in the endoplasmic reticulum and cleaved in the Golgi apparatus to generate the mature Env glycoproteins gp120 and gp41 (for reviews, see Freed and Martin, 1995; Luciw, 1996; Wyatt and Sodroski, 1998). The gp120 and gp41 then associate into an oligomeric structure (most likely a trimer) in which gp41 is predominantly integrated into the viral membrane and gp120 is exposed on the outside of the viral particle. Both gp120 and gp41 take part in the viral fusion process, with gp120 responsible for the initial association with cellular surface proteins (both CD4 and chemokine receptors), after which gp41 undergoes conformational changes and integrates into the target cell membrane by means of a "fusion peptide" (Chan and Kim, 1998).

Cyanovirin-N (CV-N), a unique 101-amino-acid protein originally isolated from the cyanobacterium *Nostoc ellipsosporum* (Boyd et al., 1997; Gustafson et al., 1997), inactivates diverse macrophage-tropic, T-lymphocyte-tropic, and macrophage- and T-lymphocyte-tropic strains of HIV-1 as well as HIV-2, simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), and certain other enveloped viruses (Boyd et al., 1997; Dey et al., 2000). CV-N also prevents the in vitro fusion of HIV-infected and noninfected cells and inhibits cell-cell transmission of infection (Boyd et al., 1997). A series of previous studies has yielded convergent results indicating that the viral surface envelope glycoprotein gp120

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ABBREVIATIONS: Env, HIV envelope glycoproteins; gp120, glycoprotein 120 from HIV; gp41, glycoprotein 41 from HIV; gp160, glycoprotein 160 from HIV; CV-N, cyanovirin-N; ELISA, enzyme-linked immunosorbent assay; SIV, simian immunodeficiency virus, FIV, feline immunodeficiency virus; sCD4, soluble CD4; gp32, glycoprotein 32 from SIV; CD, circular dichroism; BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene; TPBS, PBS augmented with 0.05% Tween 20; ITC, isothermal titration calorimetry; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide.

is a molecular target of CV-N (Boyd et al., 1997; Gustafson et al., 1997; Mori et al., 1997; Mariner et al., 1998; Esser et al., 1999; Dey et al., 2000). CV-N is a promising candidate for development as a topical microbicide to prevent sexual transmission of HIV infection (Boyd et al., 1997; Esser et al., 1999; Dey et al., 2000); the development of a woman-controlled vaginal microbicide is now widely viewed as an urgent international priority.

Initial enzyme-linked immunosorbent assay (ELISA) studies from our laboratory indicated that CV-N bound specifically to a soluble form of glycosylated gp120 (sgp120), as well as to the functionally analogous SIV proteins sgp130 and sgp140, but did not bind to the soluble form of the cellular receptor CD4 (sCD4) or to a battery of other reference proteins (Boyd et al., 1997). Later observations with a series of CV-N mutant proteins suggested that although gp120 binding by CV-N was essential for antiviral activity, it was not necessarily sufficient (Mori et al., 1997). The present study analyzes several aspects of CV-N interactions with HIV-1 envelope glycoproteins, including: 1) a comparison of the binding of CV-N to sgp120 and sgp160 (from H9 cells) and recombinant sgp120 and sgp160 (produced in a baculovirus system); 2) the binding of CV-N to both SIV sgp32 (produced in HUT 78 cells) and recombinant HIV sgp41 (produced in yeast); 3) ELISA and fluorescence polarization studies of the relative binding affinities of sgp120 and sgp41 for CV-N; 4) isothermal calorimetric studies of the interaction of CV-N with sgp120 and sgp41; 5) optical biosensor studies of the binding interaction between CV-N and both HIV-1_{JRFL} and HIV-$1_{89.6}$ gp120s; 6) circular dichroism (CD) studies of the conformational changes associated with the binding of CV-N to either sgp120 or sgp41; and 7) the effect of prebinding CV-N with either sgp120 or sgp41 on its in vitro anti-HIV activity.

Materials and Methods

Proteins. HIV-1_{IIIB} sgp120 and sgp160 (produced in H9 cells) and recombinant HIV-1_{IIIB} sgp120 and sgp160 (fully glycosylated, produced in a baculovirus expression system) were purchased from Advanced Biotechnologies Inc. (Columbia, MD). Recombinant HIV-1_{HXB2} sgp41 ectodomain (amino acids 546–682, fully glycosylated, produced in *Pichia pastoris*, referred to hereafter as sgp41) was also purchased from Advanced Biotechnologies. Nonglycosylated HIV-1_{IIIB} sgp41 ectodomain (amino acids 571–651, produced in *Escherichia coli*) and nonglycosylated sgp120 were purchased from Intracel Corp. (Issaquah, WA). SIV_{MNE} sgp32 (produced in HUT 78 cells) was the gift of R. Sowder and L. Henderson (SAIC-Frederick, NCI-FCRDC, Frederick, MD). Purified recombinant CV-N was produced as reported previously (Boyd et al., 1997; Mori et al., 1998). BODIPY-labeled CV-N was prepared by reacting free amino groups on CV-N with 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene ($BODIPY_{503/512}$) using succinimidyl-ester coupling according to the manufacturer's protocols (Molecular Probes, Eugene, OR).

ELISA Protocols. Env proteins at a concentration of 1 μg/ml or control solutions of 1% BSA in 100-μl aliquots of PBS were bound to individual wells of 96-well protein-binding assay plates (Immobilon; Nunc, Naperville, CT) by incubation for 2 h at room temperature. Thereafter, the plates were washed three times with PBS augmented with 0.05% Tween 20 (TPBS), then blocked by the addition of 200 μl/well of a solution of 1% BSA (Sigma, St. Louis, MO) in PBS, followed by overnight incubation at 4°C and a further wash with TPBS. Dilutions of CV-N were then added to triplicate wells and allowed to incubate for 1 h, followed by a TPBS wash, followed by addition and 1 h incubation with 100 μl of a 1:1000 dilution of rabbit-anti-CV-N polyclonal antibodies in each well. Subsequently, 100 μl of a 1:1000 dilution of goat-anti-rabbit secondary antibodies ligated to alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) was added to each well and allowed to incubate for 1 h, followed by a TPBS wash and incubation with alkaline phosphatase substrate buffer (10% diethanolamine, 17 mM *p*-nitrophenylphosphate, 1 mM $MgCl_2$, pH 9.2). Absorbance was measured at 405 nm for each well and BSA control absorbance levels were subtracted.

The time-dependence of CV-N binding to the Env proteins was examined using ELISA assays as above, except that CV-N was added at a fixed concentration of 100 ng/well at various times before washing with TPBS and application of the anti-CV-N antibody.

Fluorescence Polarization Studies. Fluorescence polarization studies were performed on a FPM-1 fluorescence polarization detector (Jolley Consulting and Research Inc., Grayslake, IL). Each experiment was initiated by the addition of BODIPY-CV-N to fluorescence polarization buffer (Pan Vera, Madison, WI), resulting in a final concentration of 9 nM BODIPY-CV-N, then immediate transfer of 1 ml of this solution to individual tubes and measurement of fluorescence polarization at 30-s intervals in the presence or absence of added Env proteins (45 nM recombinant HIV-1_{HxB2} sgp41, 9 nM recombinant HIV-1_{IIIB} sgp120 or 31 nM SIV_{MNE} sgp32). As control samples, nonglycosylated gp120 and gp41 were used to confirm the baseline levels of polarization for addition of noninteracting protein. To estimate the relative binding strength of sgp41/CV-N and sgp120/CV-N interactions, BODIPY-CV-N solutions were prepared as before, followed by the addition of sgp41 or sgp120. After sufficient time for equilibration (≥5 min), unlabeled CV-N was added to the solution at various concentrations (0.18 μM, 2.3 μM, and 9.1 μM), followed by polarization measurement.

Effects of Pretreatment of CV-N with sgp120 or sgp41 on the Antiviral Activity of CV-N. CEM-SS cells (Nara et al., 1987) were maintained in RPMI 1640 media without phenol red and supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD), 2 mM L-glutamine (BioWhittaker), and 50 μg/ml gentamicin (BioWhittaker) (complete medium). Serial dilutions of either recombinant sgp120 or sgp41 (Advanced Biotechnologies Inc., Columbia, MD) were added to the wells of a 96-well plate containing 5×10^3 CEM-SS cells/well and 2 nM CV-N and were allowed to incubate for 15 min. Next, 50 μl of HIV-1_{RF} virus stock was added to each test well and the plate was allowed to incubate for 7 days at 37°C. Cell viability was estimated by the XTT-tetrazolium assay as described previously (Gulakowski et al., 1991). To accurately assess the results, 24 replicates were used for treatment conditions with HIV and 12 replicates for uninfected control cells. Averages of replicate wells were expressed as a percentage of the untreated cell control. The 95% confidence limits were calculated for the replicate points ($\alpha = 0.05$).

Isothermal Calorimetry. All experiments were carried out using an Omega titration calorimeter, at 25°C. In a typical experiment, 10-μl aliquots of CV-N were injected into 1.396 ml of either an sgp120 or an sgp41 protein solution, rapidly mixed at 400 rpm, and allowed 5 min of equilibration time between injections. Heats of reaction were determined by integration of the observed peaks. All isotherms were corrected for the heat of mixing and/or dilution by subtraction of the isotherm obtained after injection of CV-N into buffer in the absence of either glycoprotein. All protein solutions were constituted in 50 mM sodium phosphate, 0.2 M NaCl buffer containing 0.02% NaN_3, and 0.02% Triton X-100, pH 7.5.

In all experiments, the concentrations of CV-N, sgp120, and sgp41 were adjusted to 138 μM, 2.86 μM, and 3.7 μM, respectively. Concentrations were determined by amino acid analysis. The isotherms, corrected for dilution/buffer effects, were fit using the Origin ITC Analysis software according to manufacturer's protocols. A nonlinear fit of the titration data yielded a binding isotherm from which the values for enthalpy, stoichiometry, and the binding constant were extrapolated.

Optical Biosensor Binding Assays. Interaction analyses were performed on a BIA3000 optical biosensor (BIACORE Inc.). Immobilization of ligands (CVN and 17b) to CM5 sensor chips was performed following the standard amine coupling procedure according

to the manufacturer's specification. All experimental conditions (i.e., CV-N immobilization level <500RU; flow rate, 30 μl/min) were optimized to reduce the diffusion-effect to minimal or none. Briefly, carboxyl groups on the sensor chip surface were activated by injection of 35 μl of a solution containing 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and 0.05 M *N*-hydroxysuccinamide at a flow rate of 5 μl/min. Next, a protein ligand at a concentration of ~50 ng/ml in pH 4.5, 10 mM NaOAc buffer, was flowed over the chip surface at 25°C at a rate of 5 μl/min until the desired response units of reacted protein was reached. Then, after washing out unreacted protein, excess active ester groups on the sensor surface were capped by the injection of 35 μl of 1 M ethanolamine, pH 8.0, at a flow rate of 5 μl/min. A reference surface with BSA immobilized was generated at the same time under the same conditions and was used as background to correct instrument and buffer artifacts.

Binding experiments were performed at 25°C in PBS buffer, pH 7.4, with 0.005% Tween 20. PBS contains 10 mM phosphate and 150 mM NaCl. For the direct binding of sgp120 to immobilized CVN, association was measured by passing sgp120 solutions over the CVN surface at a flow rate of 30 μl/min for 3 min or 50 μl/min for 1 min. Dissociation of bound sgp120 was monitored while washing the surface with buffer for 3 min. Remaining analytes were removed in the surface regeneration step with three 15-s injections of 5 mM NaOH in 200 mM NaCl solution at a flow rate of 100 μl/min. When JRFL sgp120 protein was captured on the 17b surface, association was measured by passing CVN solutions over the sgp120–17b surface at a flow rate of 30 μl/min for 1.5 min. After 2 min of dissociation, the surfaces were regenerated by 10 mM HCl.

CD Studies. CD spectra were obtained with a Jasco J-720 spectropolarimeter scanning from 290 to 190 nm at a scan rate of 10 nm/min. A 1.0-mm path length optical cell was used. Spectra were obtained at room temperature with 1.0 nm bandwidth, and a typical averaging time of 0.25 s/step. Sample spectra of 2.5 μM sgp120, 6 μM sgp41, and CV-N (11.4 μM and 13.9 μM) were measured individually. All spectra were corrected for buffer effects by subtracting the spectra of 25 mM sodium phosphate buffer, pH 7.0, from each sample spectrum. The corrected spectra were normalized and the mean residue ellipticity conversion was performed according to the Jasco J-700 software protocol. Mean residue molar concentrations (C_r) of sgp120, sgp41, and CV-N were calculated according to the equation $C_r = n \cdot C_p$, where n is the number of amino acid residues in a sample protein and C_p is the molar concentration of the protein. Values for n used in the calculations were 486, 146, and 101 for sgp120, sgp41, and CV-N, respectively.

In the sgp120/CV-N binding experiments, the spectrum of a 2.5 μM sgp120/11.4 μM CV-N mixture was measured. For the sgp41/CV-N binding, a 6 μM sgp41/13.9 μM CV-N mixture was measured. The experimental binding spectra of all the mixtures were buffer-corrected and converted to mean residue molar ellipticity using the method previously published by Lawless et al. (1996), assuming an average protein length of 170 mean residues for the 2.5 μM sgp120/11.4 μM CV-N sample and of 116 residues for the 6 μM sgp41/13.9 μM CV-N sample. Theoretical noninteracting spectra were generated by summing the individual raw CD spectra of sgp120 and CV-N or of sgp41 and CV-N, then converting these to mean residue ellipticity plots, assuming the same average protein lengths used for the processing of the experimental binding spectra. To calculate the percentage of secondary structural elements of CV-N, sgp120, sgp41 and their mixtures, the k2d deconvolution software was employed (Andrade et al., 1993).

Results

CV-N Binding to sgp120 and sgp160. The binding of CV-N to HIV-1_{IIIB} sgp120 produced in H9 cells and recombinant glycosylated HIV-1_{IIIB} sgp120 (produced in a baculovirus system) was compared using an ELISA system in which the Env were used to capture CV-N. In this system, both sgp120s were able to capture CV-N with essentially equal efficiency (Fig. 1A). Similar results were obtained for sgp160, (likewise produced in H9 cells and a baculovirus system, results not shown). Because of these similar binding efficiencies, recombinant envelope proteins were used for all of the remaining studies.

CV-N Binding to sgp41, sgp120, and sgp160. ELISA studies were conducted to determine the relative affinity of CV-N for the HIV-1 Env proteins sgp120, sgp160, and sgp41. Each of the three Env proteins was bound to the wells of a 96-well plate followed by incubation with various concentrations of CV-N. Concurrent ELISA studies indicated that the time-dependence of the binding interaction was essentially equivalent for all three proteins (data not shown). Bound CV-N was visualized using polyclonal rabbit-anti-CV-N antibodies. CV-N bound to all three Env proteins in a concentration-dependent manner. The binding affinity of CV-N for the sgp41 appeared less than for sgp120 and sgp160 (Fig. 1B).

CV-N Binding to Recombinant Glycosylated versus Nonglycosylated sgp120 and sgp41. CV-N capture ELISAs were also performed to detect the binding of CV-N to recombinant, nonglycosylated (produced in *E. coli*) and recombinant, glycosylated HIV-1 sgp120 and sgp41. Neither of the nonglycosylated envelope proteins bound significantly to CV-N. This result confirms previously reported comparisons between CV-N binding to glycosylated and nonglycosylated sgp120 (Boyd et al., 1997). An example of the results with sgp41 is shown in Fig. 1C.

Time-Dependence of CV-N Binding to sgp120 and sgp41. An additional set of ELISA studies was conducted to compare the time-dependence of CV-N binding to either sgp120 or sgp41. Both glycoproteins showed time-dependent binding to CV-N, with approximately 90% of the CV-N bound within the first 5 min (Fig. 1D).

Fluorescence Polarization Studies of CV-N Binding to sgp120 and sgp41. The solid-phase ELISA results were confirmed by solution-phase fluorescence polarization studies. These experiments took advantage of the ability to detect protein-protein interactions by the increase in fluorescence polarization that results when a protein binds to a second protein labeled with an appropriate fluorescent probe (Sportsman et al., 1997). The binding of BODIPY-CV-N to sgp120 was detected by the increase in polarization, as illustrated in Fig. 2A, caused when the larger Env protein bound to the BODIPY-CV-N conjugate, thereby slowing its tumbling in solution. Fluorescence polarization results similar to those seen with the binding of sgp120 to CV-N were obtained for both recombinant sgp41-ectodomain and SIV sgp32 (Fig. 2B). The addition of nonglycosylated recombinant sgp120 or sgp41 did not, however, result in any increased polarization of BODIPY-CV-N, consistent with a lack of binding to CV-N (data not shown) and confirming the ELISA results with these proteins.

Competition for Binding to sgp41 and sgp120 by Unlabeled CV-N and BODIPY-CV-N. Because ELISA and fluorescence polarization studies indicated that both sgp41 and sgp120 bound to CV-N, further fluorescence polarization experiments were designed to assess the nature of the association between CV-N and the Env proteins. In these studies, BODIPY-CV-N was bound to either sgp41 or sgp120 and then

unlabeled CV-N was added to estimate the degree to which the BODIPY-CV-N was reversibly bound to the two viral proteins. A decrease in fluorescence polarization indicates that the unlabeled CV-N competed for binding with the BODIPY-CV-N. This experiment indicated that CV-N bound extremely tightly to sgp120, because addition of a 1000-fold molar excess of unlabeled CV-N decreased the polarization signal by only 30% (Fig. 2C). In contrast, addition of a 1000-fold molar excess of unlabeled CV-N to the sgp41/BODIPY-CV-N complex decreased the polarization signal to background levels (Fig. 2D). These results are consistent with a much stronger association between CV-N and sgp120 than between CV-N and sgp41.

Isothermal Calorimetric Studies of the Binding of CV-N to sgp120 and sgp41. The competition studies using fluorescence polarization indicated that both sgp120 and sgp41 bound CV-N with relatively high affinities. Isothermal titration calorimetry (ITC) was employed to further quantify the individual CV-N binding events of sgp120 and sgp41. ITC is a sensitive technique for measuring the thermodynamics of a binding event and has routinely been applied to both protein-protein and protein-ligand interaction studies (Ladbury and Chowdhry, 1996; Todd and Freire, 1999). The calorimetric technique measures small changes (on the order of microcals) in heat flow that occurs when proteins bind to ligands. The heat flow associated with binding is related to both the enthalpy and extent of reaction. From a single titration experiment, values for ΔH, the stoichiometry of the binding, and the binding constant (K_d), can be determined. From these parameters, ΔS, the entropic change that accompanies the binding, and ΔG, the Gibbs free energy of binding can be calculated.

A representative isotherm obtained for binding of CV-N to sgp120 is provided in Fig. 3. These results show a monotonic decrease in the exothermic heat of binding with each successive injection of CV-N until saturation of binding is achieved. It is also apparent from the isotherm that, at saturating levels of CV-N, perturbations of the baseline complicate the results. This disruption of a level baseline is probably the result of the formation of aggregates at saturating levels of CV-N. Such aggregates were seen in preliminary spectroscopic studies of CV-N/gp120 mixtures (results not shown). The formation of similar aggregates in ITC studies has been reported previously and has been shown not to interfere with

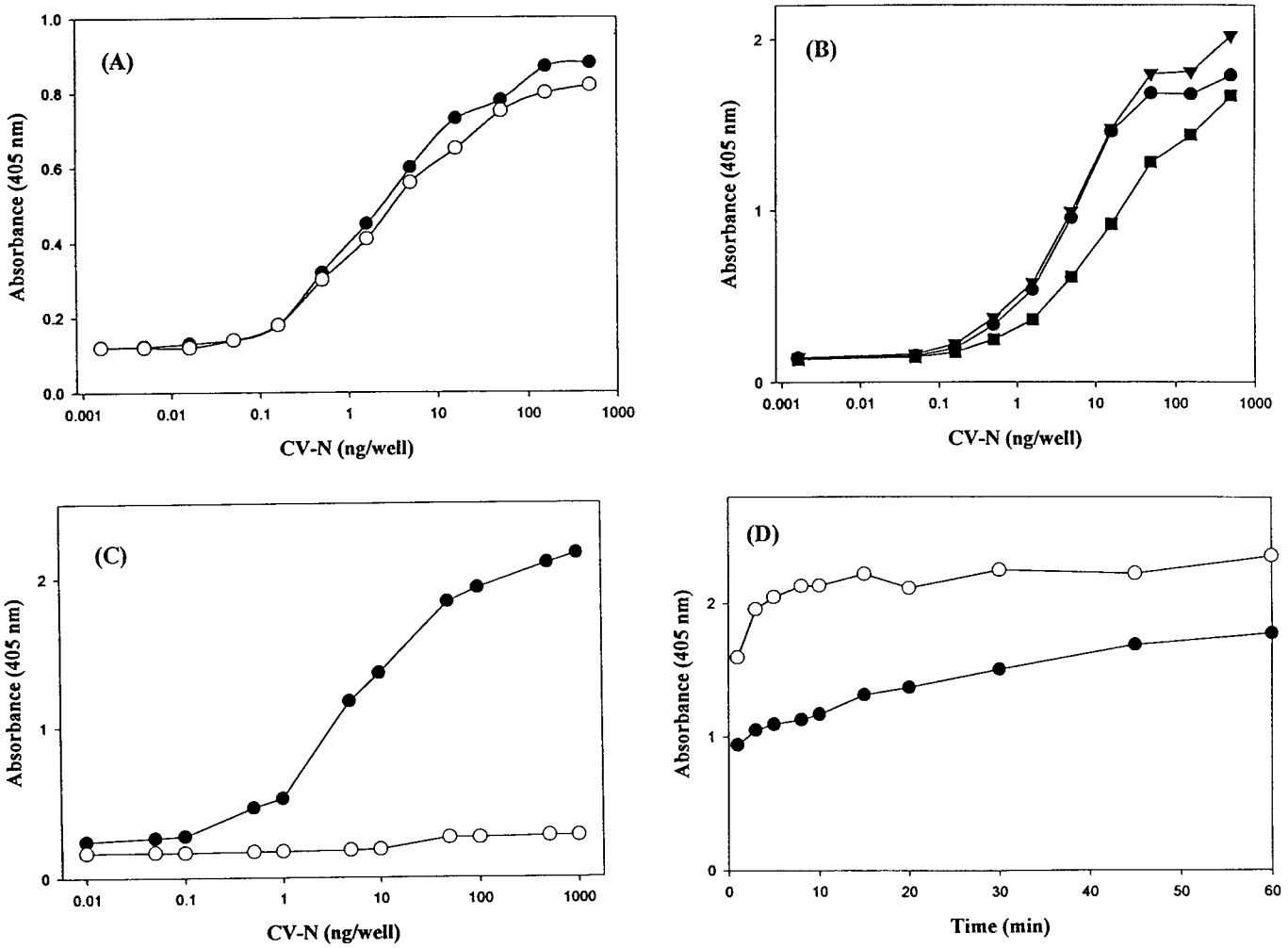


Fig. 1. ELISA studies of the binding of CV-N to Env. A, either H9 cell-produced (●) or recombinant sgp120 (○) (100 ng/well) was bound to an ELISA plate. B, either recombinant sgp120 (●), sgp160 (▼), or sgp41-ectodomain (■) (100 ng/well) was bound to an ELISA plate. C, either recombinant glycosylated (●) or recombinant nonglycosylated sgp41 (○) (100 ng/well) was bound to an ELISA plate. D, either sgp120 (●) or sgp41-ectodomain (○) (100 ng/well) was bound to an ELISA plate and then treated with 100 ng/well CV-N. In all cases, bound CV-N was determined by absorbance at 410 nm as described under *Materials and Methods*.

the interpretation of simple reversible binding phenomena (Dimick et al., 1999) The binding of CV-N to sgp41 resulted in a similar saturable binding isotherm but without aggregate formation (data not shown). The calculated thermodynamic parameters that describe binding between CV-N and both glycoproteins are shown in Table 1.

From the negative enthalpy values obtained for the CV-N binding to either sgp120 or sgp41, it was evident that polar and electrostatic interactions were predominant. The ΔG values were negative, indicating a spontaneous and favorable interaction of these glycoproteins with CV-N. HIV sgp120 exhibited a strong affinity for CV-N, resulting in a binding constant beyond the sensitivity level of the instrument ($K_d \leq$ 36.8 nM), although HIV sgp41 had at least a 15-fold lower affinity (K_d = 0.606 μM). The calculated stoichiometry of CV-N binding for sgp120 was 4.85, indicating that about 5 molecules of CV-N were bound per molecule of sgp120. The calculated stoichiometry of CV-N binding for sgp41 was 0.915, suggesting a 1:1 molecular complex between sgp41 and CV-N.

Optical Biosensor Studies of the Interaction of CV-N with HIV-1_{JRFL} and HIV-$1_{89.6}$ gp120. The sensorgram overlays of JRFL sgp120-CV-N and 89.6 sgp120-CVN binding are shown in Fig. 4A. The immobilization levels of CV-N were 430 RU and 225 RU for JRFL and 89.6 binding, respectively. Signal from the reference flow cell (with BSA immobilized) was subtracted to correct for bulk effect and nonspecific binding to the surface. The association and dissociation data were fit to one-to-one binding model separately. The kinetic constants from the curve-fitting analysis are summarized in Table 2. Comparing the kinetic constants, 89.6 sgp120 binds to CV-N with much faster on-rate (about 10-fold) and slightly slower off-rate (about 2-fold) than JRFL sgp120, which results in about a 25-fold difference in affinity.

To evaluate the binding ratio of sgp120 to CV-N when CV-N is in solution, we reversed the assay configuration with

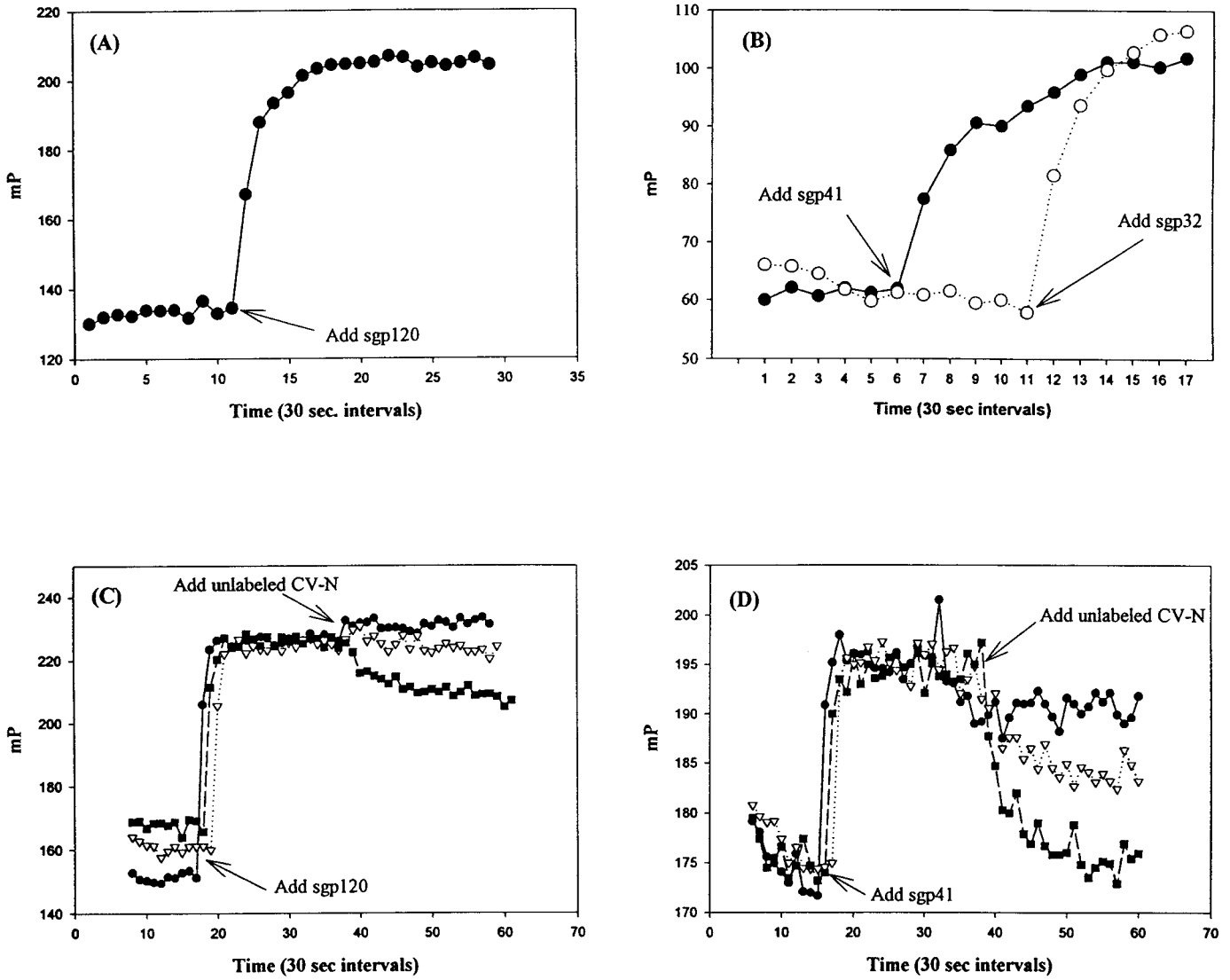


Fig. 2. Fluorescence polarization studies of the binding of CV-N to sgp120 and sgp41-ectodomain. BODIPY-labeled CV-N (9 nM) in assay buffer was treated with 9 nM sgp120 (A) and either 45 nM sgp41-ectodomain (●) or SIV sgp32 (○) (B) macromolecular binding to CV-N was determined by fluorescence polarization at 30-s intervals. BODIPY-labeled CV-N (9 nM) in assay buffer was treated with either 9 nM sgp120 (C) or 45 nM sgp41-ectodomain (D), unlabeled CV-N [either 0.2 μM (●), 2.3 μM (▽), or 9.1 μM (■)] was then added to the mixture and the polarization monitored at 30-s intervals.

JRFL sgp120 captured on the sensor surface through its antibody 17b. As shown in Fig. 4B, the average capture level of gp120 is 225RU. The molecular weight ratio of CV-N: sgp120 is 11:120. Because the biosensor response is proportional to the molecular weight, the maximum response of CV-N should be around 21RU if the stoichiometry of sgp120-CV-N binding is 1:1 and CV-N is in a monomeric form. However, the 50 nM CV-N solution gave R_{max} ~120. This clearly indicates that CV-N binds to sgp120 multivalently or as an oligomer. The estimated ratio from this biosensor assay is about sgp120: CV-N = 1:5. This is consistent with the ITC results (Fig. 3).

It should be noted that the off rates for the CV-N/gp120 interaction in the optical biosensor system differ from what

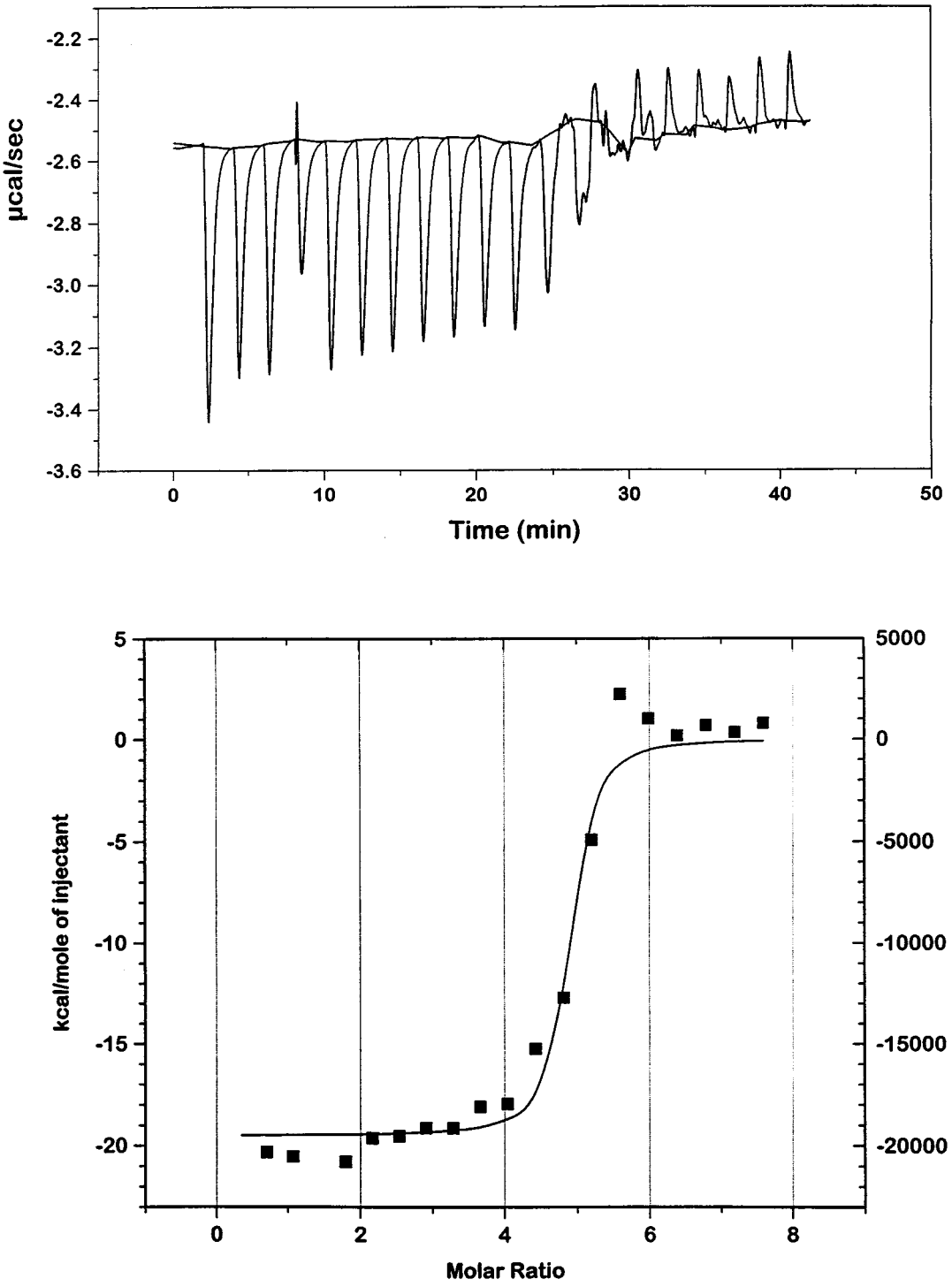


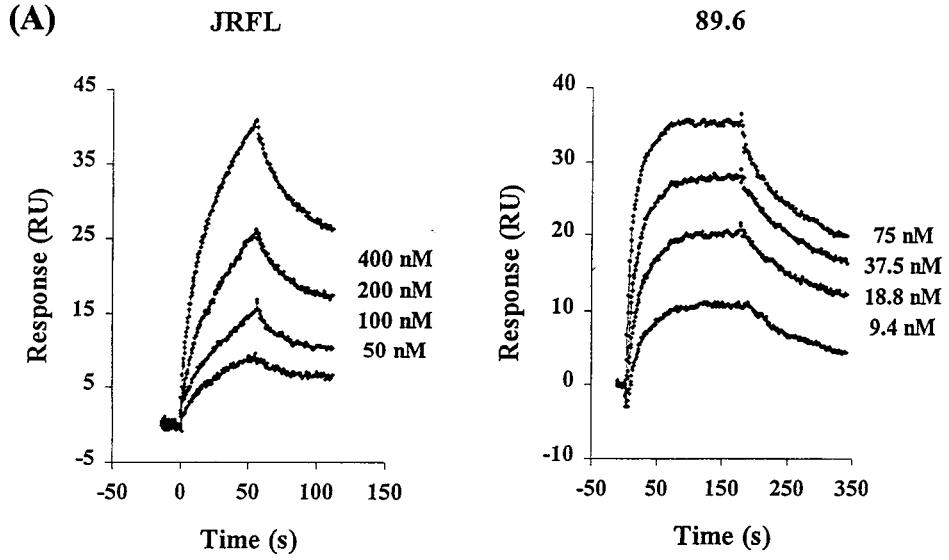
Fig. 3. Calorimetric titration of HIV recombinant sgp120 with CV-N. Top, instrument feedback (μcal/s) necessary to maintain a constant temperature (25°C) in the calorimeter cell as CV-N is periodically titrated into a rapidly mixing solution of 2.86 μM sgp120, pH 7.5, at 25°C. These results are then converted (bottom) to the binding isotherm. Saturation is achieved as the concentration of CV-N in the calorimeter cell reaches a high molar ratio relative to the amount of rgp120.

was expected based on the fluorescence polarization results (Fig. 2, C and D; much slower apparent off rate). The differences between the two are likely the result of the different nature of the experiments. In the optical biosensor system, one protein is attached to the biosensor, whereas in fluorescence polarization, both proteins are free in solution. As mentioned earlier (see ITC results), at saturating levels of CV-N (greater than 5-fold molar excess) the CV-N/gp120 complex begins to aggregate in solution. This aggregation could slow the off rate of CV-N in solution-phase experiments compared with solid-phase systems such as the Biacore system used here and could result in the apparent discrepancies.

TABLE 1
A comparison of the thermodynamic parameters determined for CV-N binding with sgp120 and sgp41

Protein	K_d	ΔH	ΔS	ΔG[a]	N (CV-N/protein)
	μM	*kcal/mol*	*eu*	*kcal/mol*	
sgp120	0.037 ± 0.003	−20.2 ± 0.420	−33.8	−10.1	4.85 ± 0.052
sgp41	0.606 ± 0.182	−14.3 ± 1.68	−19.5	−8.48	0.92 ± 0.090

[a] ΔG, −RTlnK; 298K.



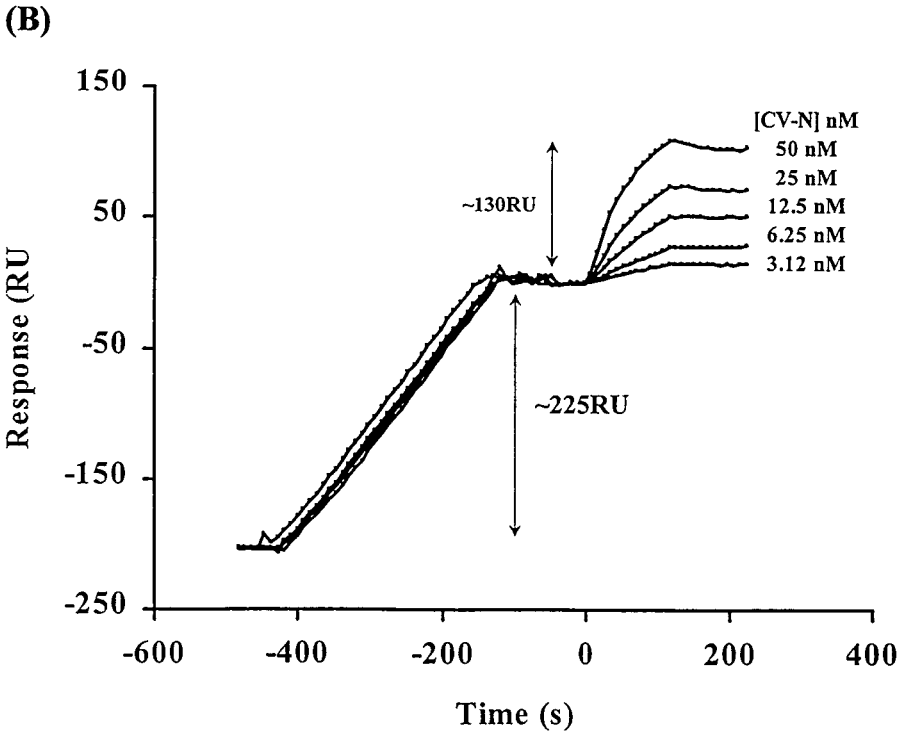


Fig. 4. Optical biosensor sensorgrams of the interaction of CV-N with HIV-1 JRFL and 89.6 gp120s. A, sensorgram overlays of gp120s binding to immobilized CV-N. Left, JRFL gp120 protein; right, 89.6 gp120 protein. Concentrations used are indicated on the graph. B, sensorgram overlays of CV-N binding to immobilized JRFL gp120. The capture of gp120 by immobilized 17b antibody was shown initially. The gp120 capture level was kept to ~225RU. The starting point of CV-N binding was then set as the zero point. The CV-N concentrations used are indicated.

CD Studies of CV-N/sgp120 and CV-N/sgp41 Interactions. CD spectrometry was employed to assess conformational changes that occur during a binding event of CV-N with either sgp120 or sgp41. Figure 5A illustrates the individual CD spectra of sgp120 and of the sgp120/CV-N binding experiment. If the two proteins did not interact with each other, no structural changes would result and the "theoretical, noninteracting" and "experimental" spectra (defined under *Materials and Methods*) would be identical. However, if two proteins did interact substantially, conformational changes in their structures would be detected, and in this case, the theoretical and the experimental spectra would be significantly different. Indeed as shown in Fig. 5A, when sgp120 and CV-N were combined, they interacted to produce evidence of a structural change. Figure 5B similarly presents the individual CD spectra of sgp41 and the spectra of the sgp41/CV-N binding experiment. Likewise in this instance, the theoretical noninteracting and the experimental spectra were markedly different, suggesting that a significant structural change had occurred as a result of interactions between the two proteins. In control CD experiments, performed by mixing CV-N and the human serum protein α-acid glycoprotein, the theoretical noninteracting spectrum and the experimental spectrum were essentially superimposable (data not shown), indicating that CV-N did not bind to this glycoprotein.

Abrogation of the Anti-HIV Activity of CV-N by Pretreatment with sgp41 and sgp120. Antiviral activity inhibition experiments were performed in which either exogenous sgp120 or sgp41 was preincubated with concentrations of CV-N (2 nM) that normally afforded complete protection of CEM-SS cells from infection by HIV-1_{RF}. If the binding of CV-N to either sgp120 or sgp41 uses the same binding site on CV-N that interacts with intact virions, then pretreatment of CV-N with exogenous sgp120, sgp41, or both, should diminish the neutralizing activity of CV-N and permit productive infection by HIV-1 in this assay. As gp120, and possibly gp41, might demonstrate protective activity against HIV-1 in this assay system, both were independently assayed to ensure that the concentrations used in the abrogation assay were not protective (data not shown). Figure 6A shows that exogenous gp120 eliminated the anti-HIV activity of CV-N in a concentration-dependent manner consistent with results of earlier experiments on sgp120/CV-N interactions (Boyd et al., 1997). Furthermore, in this assay system, the addition of exogenous sgp41 also eliminated the antiviral activity of 2 nM CV-N (Fig. 6B) although requiring considerably higher concentrations of sgp41 than for sgp120.

Discussion

CV-N irreversibly inactivates a broad spectrum of primary HIV-1 isolates, including both macrophage- and T-lymphocyte-tropic strains, which typically are resistant to neutralization by sCD4 and most known anti-gp120 monoclonal antibodies to either gp120 or CD4 (Boyd et al., 1997). Furthermore, CV-N seems to bind to gp120 in a manner that is distinct from either sCD4 or monoclonal antibodies (Boyd et al., 1997; Esser et al., 1999), and the association between CV-N and gp120 has been theorized as necessary but not sufficient for the antiviral activity of CV-N (Mori et al., 1997). Our present data, using a ELISA assay similar to that reported previously (Boyd et al., 1997; Esser et al., 1999) (Fig. 1, A and B) and a solution-phase fluorescence polarization system (Fig. 2A) are likewise consistent with gp120 as a molecular target of CV-N. Furthermore, optical biosensor assays (Fig. 4; Table 2) using the sgp120s of macrophage-tropic HIV-1_{JRFL} and macrophage- and T-lymphocyte-tropic HIV-$1_{89.6}$ show that the 89.6 sgp120 has a faster on-rate and consequent higher CV-N binding affinity (k_{on} = 2.14e6, K_d = 1.9 nM) than JRFL gp120 (k_{on} = 1.85e5, Kd = 45 nM). A previous report showed that CV-N inhibited the 89.6 HIV-1 strain more potently than the JRFL strain (Boyd et al., 1997). Taken together, these results support the notion that the interaction of CV-N to virus envelope protein gp120 is at least a major factor underlying the anti-HIV inhibitory activity of CV-N.

In addition, here we report binding interactions of CV-N with sgp41. ELISA results as well as fluorescence polarization studies initially revealed an association between CV-N and sgp41 (Fig. 1B) as well as between CV-N and the analogous SIV sgp32 (Fig. 2B). The interaction with sgp41 is similar to that between CV-N and sgp120 in that both initial binding events seem to be caused by electrostatic interactions. Furthermore, the negative ΔG values indicate spontaneous and favorable binding (Table 1). Both binding events also seem to have the same initial rates of reaction as indicated by ELISA assays (Fig. 1D). However, the interaction between CV-N and sgp41, although yielding a relatively strong binding constant (K_d = 606 nM), is distinctly weaker that the interaction between CV-N and sgp120 ($K_d \leq$ 37 nM) (Table 1; Fig. 2, C and D). Another notable difference between the interaction of sgp120 and sgp41 with CV-N is the apparent stoichiometry of the two interactions. Both isothermal calorimetric studies and optical biosensor assays indicated that approximately five CV-N molecules bound to each sgp120 molecule (Tables 1 and 2), whereas ITC experiments with sgp41 were consistent with only one CV-N molecule bound per gp41 molecule (Table 1).

The interaction between CV-N and Env seems to be glycosylation-dependent, because no significant binding of CV-N to nonglycosylated sgp120 or nonglycosylated sgp41 was detected (Fig. 1C). CV-N did not, however, show any difference in binding to recombinant, baculovirus-produced sgp120 compared with sgp120 produced in H9 cells (Fig. 1A). The glycosylation pattern of H9-cell-produced sgp120 has been previously investigated (Geyer et al., 1988; Mizuochi et al., 1990) and is significantly different from the glycosylation pattern on recombinant baculovirus-produced sgp120 (Yeh et al., 1993). In particular, baculovirus-produced sgp120 does not contain any *O*-linked oligosaccharides or any of the *N*-linked complex oligosaccharide chains found on H9-produced sgp120 but contains only high-mannose type *N*-linked oligosacharides (Yeh et al., 1993). Because CV-N shows no difference in its binding to either H9- or baculovirus-produced sgp120, the binding of CV-N to sgp120 does not seem to

TABLE 2
A comparison of the optical biosensor binding parameters determined for CV-N binding with HIV-1 JRFL and 89.6 sgp120s

Protein	k_{on}	k_{off}	K_d
	M/s	s	μM
JRFL sgp120	1.85e5 ± 0.1	8.3e−3 ± 0.1	0.045 ± 0.001
89.6 sgp120	2.14e6 ± 0.5	4.1e−3 ± 0.6	0.0019 ± 0.0001

specifically require either *O*-linked or *N*-linked complex oligosaccharide structures. It is important to note that in similar experiments CV-N did not bind to several other glycoproteins including α-acid glycoprotein, human serum albumin, horseradish peroxidase, and human IgGs (data not shown). Therefore, it is likely that, although the association of CV-N with Env glycoproteins is glycosylation dependent, CV-N does not bind nonspecifically to glycoproteins.

CV-N also showed a similar degree of binding to either SIV sgp32 or recombinant *P. pastoris*-produced sgp41-ectodomain

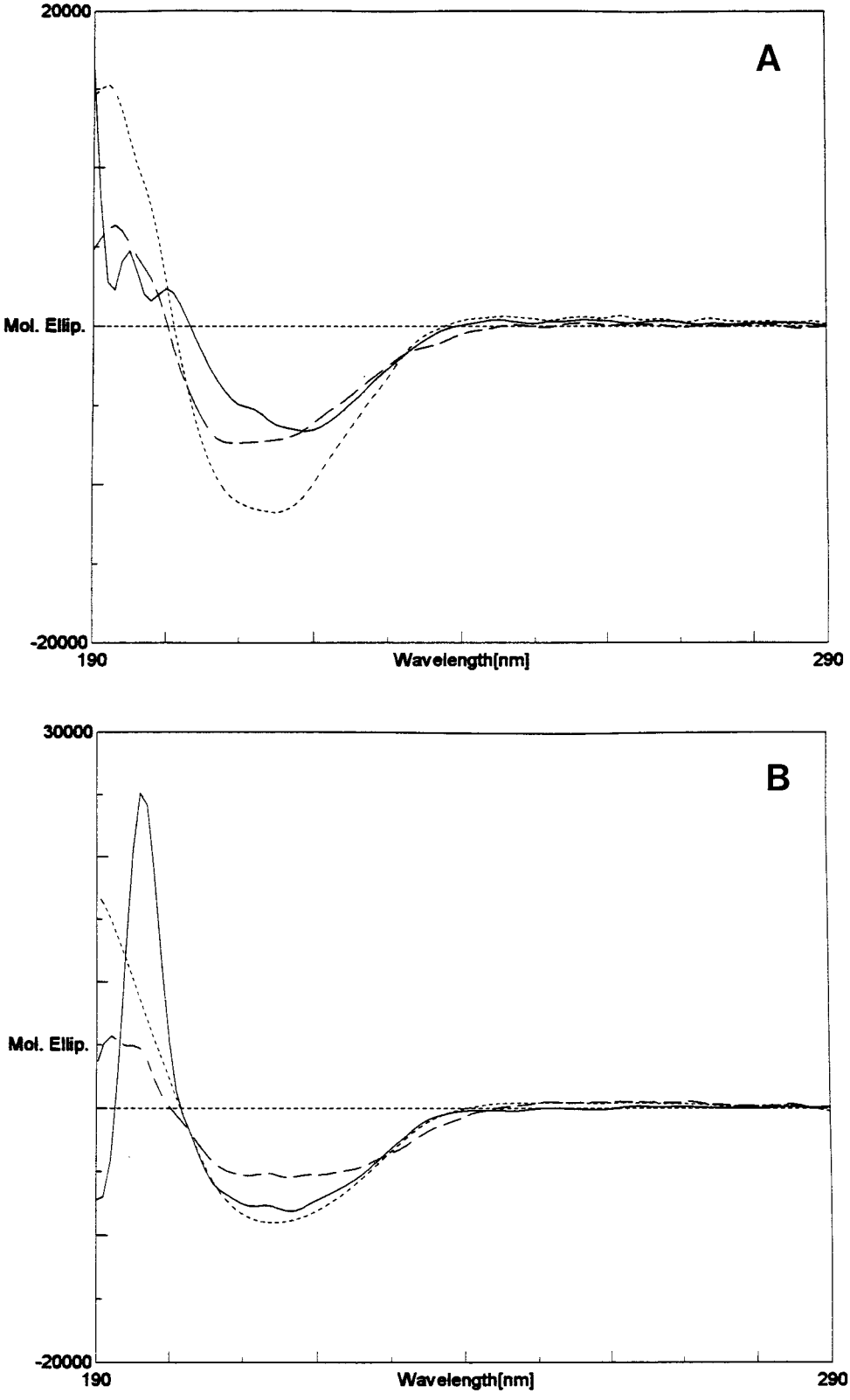


Fig. 5. CD spectra of the sgp120/CV-N and sgp41/CV-N complexes. A, dashed line, 2.5 μM recombinant sgp120; dotted line, theoretical noninteracting spectrum of a mixture of 2.5 μM recombinant sgp120/11.4 μM CV-N; solid line, experimental spectrum of a mixture of 2.5 μM recombinant sgp120/11.4 μM CV-N. B, dashed line, 6.0 μM recombinant sgp41; dotted line, theoretical noninteracting spectrum of a mixture of 6.0 μM recombinant sgp41/13.9 μM CV-N; solid line, experimental spectrum of a mixture of 6.0 μM recombinant sgp41/13.9 μM CV-N. Calculations for theoretical noninteracting spectra are detailed under *Materials and Methods*.

(Fig. 2B). Although the exact oligosaccharide structures of these two glycoproteins have not been determined, the similar thermodynamic binding parameters of the sgp120/CV-N and sgp41/CV-N binding events (Table 1) suggest that the sgp41/CV-N interaction may also have carbohydrate requirements similar to those for sgp120/CV-N binding.

CD spectrometry was used to further investigate the binding interaction between CV-N and both sgp120 and sgp41.

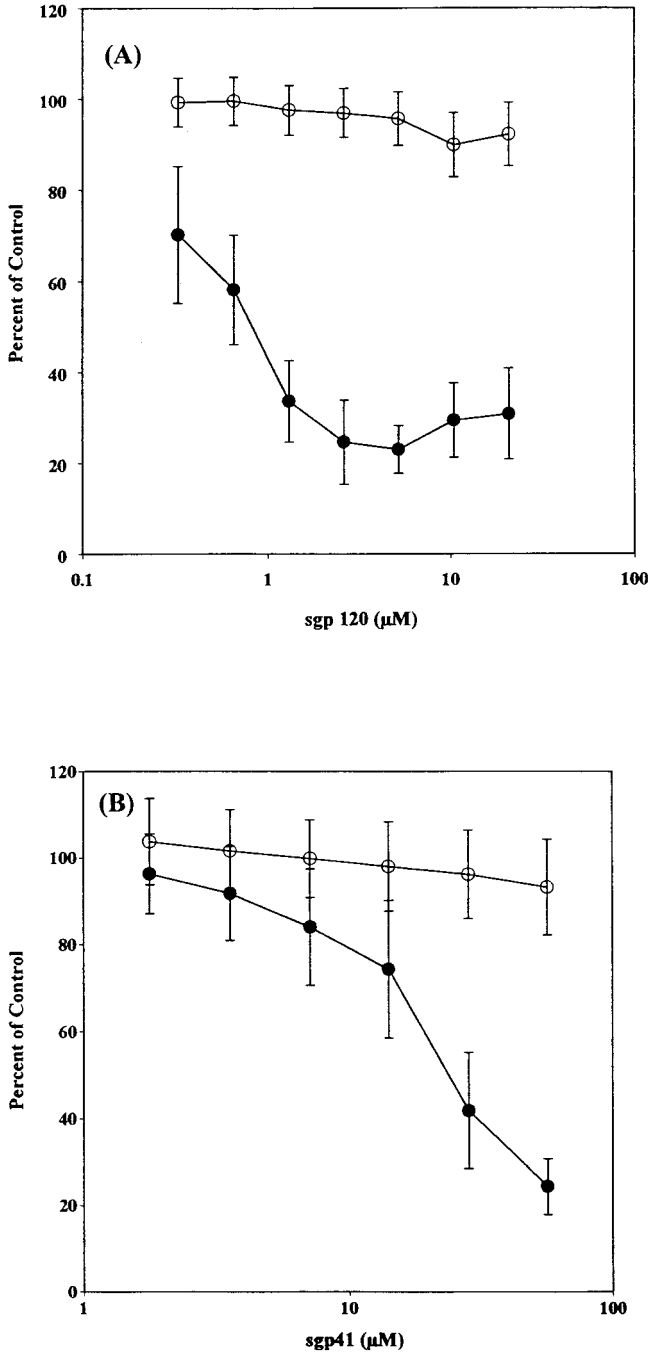


Fig. 6. Inhibition of the antiviral activity of CV-N by preincubation with either sgp120 or sgp41. The concentration-dependent effect of either sgp120 (A) or sgp41 (B) on CEM-SS cells treated with 2 nM CV-N (completely protective, 94.0 ± 8.3%). Closed symbols (●) indicate CEM-SS cells infected with HIV-1 $_{RF}$; open symbols (○) indicate uninfected CEM-SS cells. Error bars denote the 95% confidence limits of the observed values (α = 0.05).

When saturating concentrations of CV-N were added to sgp120, the CD data clearly showed that conformational changes had taken place as a consequence of the binding. A comparison of the theoretical noninteracting and experimental spectra (Fig. 5A) indicated that there was an approximate loss of 14% β-sheet and 2% α-helical structure in one or both of these proteins. Although conformational changes have previously been postulated to be a consequence of the CV-N/sgp120 binding interaction (Mariner et al., 1998, Esser et al., 1999), this is the first direct evidence that such structural changes do occur. Similar CD studies using sgp41 and saturating concentrations of CV-N also indicated significant conformational changes (Fig. 5B). In this case, the binding association resulted in an estimated loss of 9% β-sheet and 2% α-helical structure. Because both NMR (Bewley et al., 1998) and X-ray crystal studies (Yang et al., 1999) have shown that CV-N contains mostly β-sheet and the ectodomain of gp41 is predominantly α-helical in structure, this result, although not conclusive, implies that conformational changes within CV-N may accompany its binding with sgp41. It should be noted that in control CD experiments CV-N did not interact significantly with α-acid glycoprotein, further reinforcing previous ELISA studies (Boyd et al., 1997) and further indicating that CV-N did not bind indiscriminately to glycoproteins.

Previous gp120-specific monoclonal antibody studies have shown that CV-N does not alter epitope availability in either the V3 loop or the CD4 binding region of gp120 (Boyd et al., 1997; Esser et al., 1999), nor does it interfere with the normal conformational changes that occur when sgp120 binds to sCD4 (Esser et al., 1999). The only monoclonal antibody interaction with gp120 so far tested that is inhibited by CV-N pretreatment is that of the glycosylation-dependent antibody 2G12 (Esser et al., 1999). The 2G12 antibody is unique in that crystallization studies have suggested that it may bind in part to the putative "silent face" of gp120, which is heavily glycosylated and largely hidden immunologically (Kwong et al., 1998). These results suggest that binding of CV-N to gp120 does not affect those areas most readily engaged by the immune system. CV-N-induced changes in gp120 that potentially affect the "silent face" of gp120 might thereby provide a feasible means to explore further this little-understood region and might also provide a means to enhance the immunogenicity of the virus.

The biological relevance of the binding interaction between CV-N and HIV-1 envelope glycoproteins is further supported by anti-infectivity experiments showing that pretreatment of CV-N with virus-free sgp41 decreases the virucidal activity of CV-N in a concentration-dependent manner (Fig. 6B), similar to that found when CV-N is pretreated with sgp120 (Fig. 6A). It is interesting to note that the concentration of sgp41 required for complete abrogation of the antiviral activity of CV-N (IC_{50} = 24 μM) is significantly higher than the concentration of sgp120 required to obtain the same effect (IC_{50} = 0.9 μM). This may mirror the difference in relative binding affinities of sgp120 and sgp41 for CV-N observed in the initial ELISA experiments (Fig. 1) and ITC studies (Table 1).

There have been several reports of peptides derived from gp41, which are able to reversibly bind to gp41 and inhibit HIV infectivity (Wild et al., 1992, 1994; Judice et al., 1997; Silburn et al., 1998; Eckert et al., 1999). These peptides generally form α-helices in solution and mimic the structure

of the α-helices found in the gp41 fusogenic domain (Wild et al., 1994; Judice et al., 1997). The three-dimensional solution structure of CV-N as determined by NMR or crystallography contains mostly β-sheet (Bewley et al., 1998; Yang et al., 1999) and does not reveal any structural features similar to those reported for other sgp41-binding peptides. CV-N also apparently binds via electrostatic rather than hydrophobic interactions and interacts with a recombinant sgp41-ectodomain in a fusion active conformation (Weissenhorn et al., 1996) that is thought to be unlikely to interact with the α-helical peptides (Wild et al., 1994; Judice et al., 1997). It is therefore likely that CV-N interacts with gp41 in a manner that is distinct from that of the α-helical peptides.

In conclusion, CV-N interacts uniquely with both sgp120 and sgp41 in a highly specific, fully saturable, glycosylation-dependent manner. Indeed, it is conceivable that even a relatively lower affinity interaction of CV-N with gp41, when coupled with an unequivocally high affinity interaction of CV-N with gp120, could interfere with the dissociation and/or conformational changes of gp120 and gp41 normally required for the fusion process to proceed successfully. Furthermore, the detection of the interaction between CV-N and gp41 and the specific glycosylation-dependence of its binding to both gp41 and gp120, may help explain recent reports (Dey et al., 2000) that CV-N is active against certain enveloped, nonretroviruses that do not use gp120 or a functional equivalent for the cellular entry process.

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