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A Recombinant Allosteric Lectin Antagonist of HIV-1 Envelope gp120 Interactions

Karyn McFadden,[1] Simon Cocklin,[1] Hosahudya Gopi,[1] Sabine Baxter,[1] Sandya Ajith,[1] Naheed Mahmood,[2] Robin Shattock,[2] and Irwin Chaiken[1]*

[1]*Department of Biochemistry and Molecular Biology, Drexel University College of Medicine, Philadelphia, Pennsylvania 19102*

[2]*Division of Molecular Medicine, Department of Cellular and Molecular Medicine, St. George's University of London, London SW17 0RE, United Kingdom*

ABSTRACT **The first, critical stage of HIV-1 infection is fusion of viral and host cellular membranes initiated by a viral envelope glycoprotein gp120. We evaluated the potential to form a chimeric protein entry inhibitor that combines the action of two gp120-targeting molecules, an allosteric peptide inhibitor 12p1 and a higher affinity carbohydrate-binding protein cyanovirin (CVN). In initial mixing experiments, we demonstrated that the inhibitors do not interfere with each other and instead show functional synergy in inhibiting viral cell infection. Based on this, we created a chimera, termed L5, with 12p1 fused to the C-terminal domain of CVN through a linker of five penta-peptide repeats. L5 revealed the same broad specificity as CVN for gp120 from a variety of clades and tropisms. By comparison to CVN, the L5 chimera exhibited substantially increased inhibition of gp120 binding to receptor CD4, coreceptor surrogate mAb 17b and gp120 antibody F105. These binding inhibition effects by the chimera reflected both the high affinity of the CVN domain and the allosteric action of the 12p1 domain. The results open up the possibility to form high potency chimeras, as well as noncovalent mixtures, as leads for HIV-1 envelope antagonism that can overcome potency limits and potential virus mutational resistance for either 12p1 or CVN alone. Proteins 2007;67:617–629.** © 2007 Wiley-Liss, Inc.

Key words: HIV; gp120; cyanovirin; allosteric; microbicides; chimera

INTRODUCTION

There were an estimated 40.3 million people infected with HIV-1 in 2005, with close to five million people becoming newly infected. AIDS is affecting an increasing number of women worldwide particularly in developing countries where transmission occurs primarily through heterosexual intercourse. In sub-Saharan Africa 76% of young people infected with AIDS are women[1] reflecting social and biological vulnerabilities.[2] A discrete, female-controlled method of preventing HIV-1 infection is of paramount importance in the battle against AIDS. Since an effective vaccine is still years from development, topical microbicides have gained increased attention.[3–5] Topical microbicides are vaginally or rectally-applied compounds designed to inactivate HIV-1 and prevent infection. Ideally, they should be effective, safe, inexpensive, and easy to administer.[3]

Advances in our understanding of the mechanism of HIV-1 entry and infection have led to the development of microbicides that can target HIV-1 without harming the body's natural defense system.[4,6–8] The initial, critical step of HIV-1 infection is its entry through the fusion of the viral membrane with the membrane of either a T-cell or macrophage.[9,10] The fusion process is initiated by the viral envelope glycoprotein, gp120, and can be triggered by interaction of gp120 with the T-cell antigen receptor CD4 glycoprotein.[11–13] CD4 induces conformational changes in gp120 that are postulated to promote subsequent steps in the fusion process, such as coreceptor binding and dissociation of gp120 from gp41.[14,15] Several seven-transmembrane chemokine receptors, mainly CCR5 and CXR4, have been identified as obligate coreceptors for viral entry into the host cell.[16–19] Blocking the binding of CD4 with gp120 or preventing the CD4-induced conformational isomerization that promotes coreceptor binding and viral cell fusion could have potential value for the prevention and treatment of HIV-1 infection and AIDS.

Abbreviations AIDS, acquired immunodeficiency syndrome; BSA, bovine serum albumin; CVN, cyanovirin-N; *E. coli Escherichia coli*; EDTA, ethylenediamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; Gly_4Ser, $glycine_4serine$; gp120, HIV-1 envelope glycoprotein 120; HIV, human immunodeficiency virus; HRP, horseradish peroxidase; IPTG, isopropyl-1-thio-β-D-galactopyranoside; man, oligomannose; OPD, o-phenylenediamine; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PBST, PBS containing 0.1% Tween-20; RU, response unit; sCD4, recombinant, soluble CD4 receptor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SIV, simian immunodeficiency virus; SPR, surface plasmon resonance.

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*Correspondence to: Irwin Chaiken, Drexel University College of Medicine, 11102 New College Building, MS No. 497, 245 N, 15th St., Philadelphia, PA 19102. E-mail: ichaiken@drexelmed.edu

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One candidate topical microbicide is cyanovirin-N (CVN), an 11 kDa protein, isolated from the cyanobacteria *Nostoc ellipsosporum*[20] that was found to target the envelope complex of HIV-1, CVN has been demonstrated to have antiviral activity against a number of enveloped viruses, including HIV-1, SIV, FIV,[20] HIV-2,[21] influenza,[22] Ebola Zaire,[23–25] Marburg,[24] and hepatitis C.[26] It was recently tested against HIV-1 strains belonging to A, B, C, D, A/E, F, G, and O and was found to consistently have antiviral activity against all strains.[21] Recombinant CVN blocked HIV-1_{BaL} infection of human ectocervical explants[27] with no cytotoxic effects. Gel formulations of CVN applied rectally to male macaques protected against challenge by the SIV/HIV-1 virus SHIV89.6P.[28] Further demonstration of *in vivo* efficacy was shown in a vaginal challenge model with female macaques.[27] The macaques were treated with a vaginal gel containing CVN and challenged with SHIV89.6P. Under the challenge conditions of this assay, all placebo-treated and untreated controls (8 of 8) became infected, while 15 of 18 CVN treated macaques were not infected. CVN showed no clinically adverse effects in these *in vivo* assays.

The broad range of viruses inhibited by CVN can be attributed to the binding specificity of the protein. It has been demonstrated that CVN interacts with the carbohydrate moieties of glycoproteins on the surface of the viruses that it inhibits.[29,30] A large body of work has been completed on identifying the nature of carbohydrate epitopes on the envelope protein of HIV-1.[31–33] CVN specifically interacts with high mannose glycosylation sites of the envelope complex via terminal Manα(1-2)manα moieties on Man-8 and Man-9 glycans.[29,31,33–37] The high resolution structure of CVN has been solved by both X-ray crystallography[35,38,39] and nuclear magnetic resonance spectroscopy.[40,41] These studies have identified two carbohydrate binding sites on the CVN molecule.[34,42,43] One of these appears to have higher affinity but lower selectivity to disacharrides than the other.[44]

In spite of the advantages of CVN as an entry inhibitor, there are potential limitations. Recently, resistant strains of HIV-1 emerged after incubation with elevated doses of CVN as determined by *in vitro* experiments.[21,37] These viruses contained deletions of several high mannose glycosylation sites. Prolonged treatment with CVN could result in the emergence of strains of HIV-1 with either deletions in the high mannose residues, or other changes in the glycan shield[45] that would enable it to effectively evade CVN treatment while retaining a significant amount of protective glycosylation. In addition, CVN has been found to exert mitogenic activity on peripheral blood mononuclear cells (PBMC) at subtoxic concentrations.[21] Limitations of CVN could be overcome by combining this protein with a second inhibitory agent that binds to a different interaction site in the same molecular target, gp120, and synergizes its antagonist activity with that of CVN, in effect reducing the dose of CVN required.

As a potential synergy partner, we considered another potential microbicide candidate, the linear peptide 12p1 (RINNIPWSEAMM). This peptide was initially isolated from a phage display library and found to inhibit interaction of HIV-1 gp120 with both CD4 and a CCR5 surrogate, mAb 17b.[46] There is a direct interaction of 12p1 with the protein portion of gp120 that occurs with a 1:1 binding stoichiometry.[47] The peptide was shown to inhibit the binding of monomeric HIV-1_{YU2} gp120 to both sCD4 and 17b at IC_{50} values of 1.1 and 1.6 μM, respectively as determined by surface plasmon resonance (SPR) analysis.[47] This dual inhibition is a key feature of the action of 12p1. Peptide 12p1 also inhibited binding of these ligands to trimeric envelope glycoproteins, blocked the binding of gp120 to the native coreceptor CCR5,[47] and specifically inhibited HIV-1 infection of target cells *in vitro*. Analyses of sCD4 saturation of monomeric gp120 in the presence or absence of a fixed concentration of peptide suggest that 12p1 suppression of CD4 binding to gp120 is due to allosteric inhibitory effects rather than competitive inhibition of CD4 binding. This has been confirmed using isothermal calorimetry (ITC) where 12p1 was found to lower the affinity of sCD4 and 17b for gp120, consistent with a noncompetitive mechanism (Gopi et al., 2006, manuscript in preparation). Screening a panel of gp120 point mutants that exhibit weakened inhibition by 12p1, three residues were identified that were postulated to constitute a potential binding footprint for the peptide. Consistent with the hypothesis of noncompetitive mechanism of inhibition, the location of the amino acids mapped to a region adjacent to, but distinguishable from, the CD4 binding site. Additionally, 12p1 was unable to inhibit binding of sCD4 to a gp120 mutant, HIV-$1_{YU2(S375W)}$, which is believed to resemble the CD4-induced conformation of gp120. The results obtained to date strongly suggest that 12p1 preferentially binds gp120 prior to engagement of CD4, and alters the conformational state of gp120 to a form that has suppressed interactions with receptor ligands (CD4 and CCR5/CXCR4) that are generally believed crucial for viral entry.

One method for increasing the efficacy of a compound while reducing potential mitogenic or cytotoxic effects is through the creation of chimeric proteins. Immunofusins or Fc fusins contain the Fc region of an antibody fused to a target molecule and have been found to have advantageous biological properties.[48] Other methods include linking an antigen with a stimulatory cytokine[49] or fusing a cytokine to an antibody that targets a specific cell type.[50] The chimera approach is being used to create new cancer therapies with increased potency and reduced cytotoxicity.[51–54] Fusion proteins are also being used to treat sepsis[55] and autoimmune disease.[56,57] Chimeric proteins and immunofusins targeting HIV-1 have also been created. As a means of increasing the avidity and half life of CD4, CD4-Ig chimera were created[58,59] as well as dodecameric[60] and tetrameric[61] versions. CD4 chimera have also been created in which the D1D2 domain of CD4 was fused to coreceptor binding antibodies 17b[62] or CG10.[63] These complexes showed stronger binding and enhanced neutralization to some but not all clades of HIV-1. Recently a chimera was created, which contains a single chain version of HIV-1_{BaL} gp120 sequence linked to CD4

via an immunoglobulin γ subtype 1 hinge region. This protein mimics the complex that binds to CCR5 in HIV-1 infection. It blocks HIV-1 binding to CCR5 and neutralizes R5 but not X4 viruses.[64] However, one drawback is that it could potentially lead to viral switching to the more virulent X4 strain.

In this article, we describe the creation of a recombinant multifunctional chimera of CVN and 12p1. The ability of CVN to retain its antiviral ability after treatment with compounds such as denaturants, detergents, and extremes of temperature[20] makes it a strong candidate as a fusion partner. Chimeras of CVN and 12p1 offer a potential device for targeting gp120 at two discrete sites, by two different modes of inhibition, and with increasing potency versus either component alone. A chimera would combine the high affinity suppression of viral activity by CVN with the allosteric suppression of viral envelope interactions by 12p1. Furthermore, since the binding sites for CVN and 12p1 both reside within gp120 but at sterically separate locations, the combination of these two agents as a covalent chimera may increase the ability to overcome resistance mutations at individual binding sites as compared with either component alone.

MATERIALS AND METHODS

Reagents and Proteins

The following reagents were obtained from the NIH AIDS Reference and Reagent Program, Division of AIDS, NIAID: HIV-1_{BaL} gp120, HIV-1_{SF162} gp120, HIV-$1_{90CM235}$ gp120 from Protein Sciences, Corporation, HIV-1 gp120 monoclonal antibody (2G12) from Dr. Hermann Katinger; HIV-1 gp120 monoclonal antibody (F105) from Dr. Marshall Posner and Dr. Lisa Cavacini. HIV-$1_{93MW959}$ gp120, HIV-$1_{92UG21-9}$ gp120, HIV-$1_{92US715}$ gp120, and SIV_{PBj2-8} HIV-1 gp120 were gifts from Jim Arthos at NIAID. AN1 was provided by Jim Mullins. Monoclonal antibody 17b was obtained from Strategic Biosolutions. CVN plasmid and anti-CVN polyclonal antibodies were a gift from Biosyn.

Viral Synergy Studies

Doubling dilutions of each drug (12p1 or CVN), alone or in fixed combinations, were incubated with HIV-1_{BaL} (R5) for 1 h in 100 μL volume prior to the addition of PM-1 cells (100 μL at 0.50×10^6 cells/mL). Dilutions and cells were in RPMI supplemented with 10% fetal calf serum, penicillin-streptomycin, and glutamine. Cells were incubated for 7 days and viral replication determined by gp120 ELISA. Each condition was performed in sextuplet and the mean used to calculate IC_{50} values. Analysis of combined effects was also carried out using the median effect principle developed by Chou and Talalay,[65,66] and using the computer program CalcuSyn, (BioSoft).

Construction of CVN-12p1 Chimerae

The L5 chimera was constructed using standard recombinant DNA techniques. CVN was amplified from a plasmid template using the following primers: forward primer 5′pelb: 5′-gcgcatatgaaatacctgctgccgaccgctgctgctggtctgctgctcctcgctgcccagccggcgatggcccttggtaaattctcccagacctg-3′ and the reverse primer: 5′-gcgggatccttcgtatttcagggta-3′ to insert a pel b secretory sequence and eliminate a stop codon. CVN was ligated into pET30b+ vector. The linker: 5′-ggatccggtggcggagggtcgggcggaggtggaagcggaggtggaagcggaggtggcggtagtggtggaggcggatcc-3′; 12p1: 5′ggatccggtggcggagggtctcggattaacaatatcccgtggtcggaggcgatgatgaagctt-3′; or scrambled 12p1: 5′-ggatccggtggcggagggtcttggcggattatgatgatcccgtcggaggcgaacaataagctt-3′ cassettes were synthesized as separate oligonucleotides (Invitrogen) and ligated in frame with the C-terminal domain of CVN. The entire construct was amplified with the forward primer 5′pelb (described above) and the reverse primer: 5′-ggaattcaagcttttattaatgatgatgatgatgcaggtggcggtagtggtggagg-cggatcc-3′. The construct was sequenced by the University of Pennsylvania DNA Sequencing Facility prior to transformation into BL21 (DE3) strain of *Escherichia coli*.

Expression and Purification of CVN and CVN-12p1

BL21 Codon Plus (DE3) RIL competent cells (Stratagene) containing either CVN or the chimera were grown in Superbroth supplemented with 1 m*M* $MgSO_4$, 0.5% glucose, and 25 μg/mL kanamycin in a 37°C shaking incubator until the absorbance at 600 nm read 1.2. Protein expression was induced with 1 m*M* IPTG for 2 h at 37°C. The cells were then centrifuged at 3000 rpm for 15 min and the resulting pellet was resuspended in 100 m*M* Tris, 1 m*M* EDTA, and 20% sucrose. This was mixed for 1 h at 4°C followed by resuspension in cold water, which was stirred overnight at 4°C. The lysates were centrifuged at 10,000 rpm for thirty 30 min and the supernatant sterile filtered and dialyzed into 50 m*M* NaH_2PO_4, 300 m*M* NaCl, and 10 m*M* imidazole pH 8.0. The extracts were then adsorbed onto NiNTA agarose beads (Qiagen) and washed with three column volumes of 50 m*M* NaH_2PO_4, 300 m*M* NaCl, and 20 m*M* imidazole pH 8.0. The protein was eluted with 50 m*M* NaH_2PO_4, 300 m*M* NaCl, and 250 m*M* imidazole pH 8.0.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 4–20% polyacrylamide gel under reducing conditions (1% β-mercaptoethanol). Proteins were detected with Simply Blue Coomassie Stain (Invitrogen). A western blot was performed to confirm the presence of both the CVN and 12p1 domains. For immunoblot assays, proteins were transferred to nitrocellulose. The proteins were detected with a 1:10,000 dilution of polyclonal rabbit anti-CVN or anti-His followed by an appropriate secondary antibody. The membranes were incubated with chemilluminescent substrate (Amersham) and exposed to film. All proteins were quantified based on their absorbance at 280 nm.

YU2 gp120 and sCD4 Production

Recombinant HIV_{YU-2} gp120 from HIV-1 was produced in Schneider 2 (S2) *Drosophila* cells under the control of the metallothionein promoter as previously described.[67]

In brief, the cells were grown in Insect-Xpress medium (Cambrex) supplemented with 10 m*M* L-glutamine and 1% antibiotic/antimycotic in shaker flasks at 28°C. Protein expression was induced with 750 μ*M* copper sulfate until the viability as determined by trypan blue was 70%. The supernatant was fractionated on an F105 mAb affinity column, washed extensively, and eluted with 100 m*M* glycine-HCl, pH 2.1. The samples were neutralized with 1*M* Tris, pH 8.0 buffer immediately after elution. Fractions containing gp120 were pooled and dialyzed over night at 4°C against Dulbecco's phosphate buffered saline.

CHO-ST4.2 cells, which secrete the full extracellular domain of CD4, were obtained from Dr. Dan Littman through the AIDS Research and Reference program Division of AIDS, NIAID. They were grown in a hollow fiber bioreactor (FiberCell Systems) in HiQ CDM4CHO media (Hyclone) supplemented with 4 mM L-glutamine, 300 n*M* methotrexate, and 1% antibiotic/antimycotic. Supernatant from the CHO cells was diluted 10-fold in cold 50 m*M* MES/50 m*M* NaCl pH 6.0 and passed through a 0.2 μ*M* sterile filter. They were equilibrated with 50 m*M* MES/50 m*M* NaCl pH 6.0 at 4°C, and fractions collected from a 50 m*M* MES/500 m*M* NaCl pH 6.0 gradient. Fractions were dialyzed into 50 m*M* bis-tris propane pH 6.0, adjusted to pH 9.0 and loaded onto a Q-column equilibrated with bis-tris propane pH 9.0. The column flow through containing purified sCD4 was pooled and dialyzed into DPBS overnight at 4°C. All proteins were analyzed by SDS-PAGE/Coomassie stain and judged to be of greater than 95% purity.

Biacore Assays

Surface plasmon resonance studies were performed on a Biacore 3000 (Biacore, Uppsala, Sweden). Immobilization of ligands (HIV-1_{YU-2} gp120) to CM5 dextran chip was performed following standard amine coupling procedure according to the manufacturer's specification. Briefly, carboxyl groups on the sensor surface were activated by injection of a 50 μL of a solution of 0.2*M* EDC and 0.05*M* NHS at a flow rate of 5 μL min^{-1}. Next, the protein ligand in 10 m*M* sodium acetate (pH 5.0) buffer was passed over the chip surface at a flow rate of 5 μL min^{-1} until the desired number of response units for immobilized protein was reached. After unreacted protein had been washed out, excess active ester groups on the sensor surface were capped by an injection of 60 μL of 1*M* ethanolamine (pH 8.0) at a flow rate of 5 μL min^{-1}. A reference surface with the IL5 antibody 2E3 immobilized was generated under the same conditions and was used to correct for instrument and buffer artifacts.

Direct binding of CVN, L5, and 12p1 to gp120 variants

Analysis of direct binding to HIV_{YU-2} gp120 was achieved by passing 12.5–500 n*M* CVN or L5 over a 50 RU gp120 surface at a flow rate of 100 μL min^{-1} for 1 min association and 2 min dissociation. Specific regeneration of the surfaces between injections was achieved by 6 s pulses of 50 m*M* NaOH until the baseline returned to the value before exposure. Direct binding of 12p1 to gp120 was conducted as previously described.[47]

Data analysis

Data analysis was performed using BIAEvaluation 4.0 software (Biacore, Upsalla, Sweden). The responses of a buffer injection and responses from a reference flow cell (containing mAb 2E3) were subtracted to account for nonspecific binding. The association and dissociation were fitted locally to a simple 1:1 binding model with a parameter included for mass transport. The average kinetic parameters (association [k_a] and dissociation [k_d] rates) generated from a minimum of three data sets were used to define equilibrium association (K_A) and dissociation constants (K_D).

ELISA Assays

Ninety-six well plates (Corning) were coated with either 100 ng of the indicated gp120 or BSA overnight at 4°C. The wells were washed three times with 1× PBS-T (PBS, 0.1%Tween) and non-specific binding blocked with 1% (wt/vol) BSA in PBS. This was followed by three washes with PBST and incubation with the indicated doses of either CVN or the chimera for 1 h at room temperature. The wells were washed and incubated with a 1/1000 dilution of rabbit anti-CVN polyclonal antibody. After a 1 h incubation, the wells were washed and incubated with a 1/2500 dilution of anti-rabbit HRP (Amersham) for 1 h at room temperature. The wells were washed three times and the extent of binding was then determined by OPD (o-phenylenediamine-Sigma) and absorbance measured at 450 nm. Experiments were in triplicate and corrected for nonspecific binding to a BSA surface.

Antibody inhibition assays were performed as described above but with either 0.5 μg mL^{-1} dilution of F105, 50 μg mL^{-1} dilution of 2G12, or 2 μg mL^{-1} 17b added to the CVN or chimera dilutions. The extent of CVN binding was detected with rabbit anti-CVN primary antibody.

For CD4 competition assays, 2 ng μL^{-1} YU2-gp120 or BSA was adsorbed onto a 96 well plate. Serial dilutions of either CVN or the chimera were added for 30 min followed by the addition of 2 μg mL^{-1} sCD4 for 30 min. The wells were washed and then incubated with anti-CD4 (ARP356-NIBSC UK) for 1 h at room temperature. This was followed by washing and the addition of antimouse HRP conjugated secondary antibody. The extent of binding was then determined by OPD (o-phenylenediamine-Sigma) as described earlier.

Data analysis of ELISA competition

The extent of inhibition of CD4 or mAb binding to gp120 was calculated as a percent of the uninhibited interaction. The percent binding versus inhibitor concentration data were fitted to a nonlinear method that

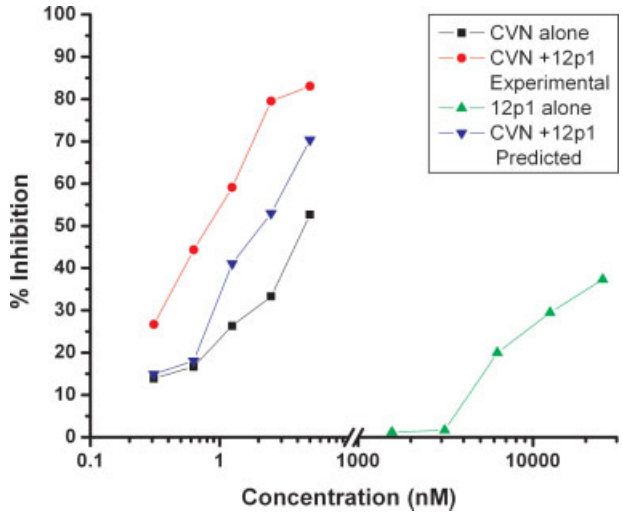

Figure 1. Synergy in inhibition of cell infection with 12p1 and CVN. HIV-1_{BaL} infection of PM-1 cells was determined as a function of dose of CVN alone (black squares), 12p1 alone (green triangles), and mixtures of the two (red circles). For the mixtures, the lowest dose of CVN was mixed with the lowest dose of 12p1, next higher dose of each mixed for the next higher concentration, and so on. Data for mixtures are plotted as a function of the concentration of CVN in the mixture. Also shown is the dose response curve of the mixture predicted if the effects of 12p1 and CVN were simply additive (blue triangles). These data show that mixing 12p1 with CVN does not interfere with the latter and instead synergizes to give a greater inhibition activity than expected from additive effects of the components.

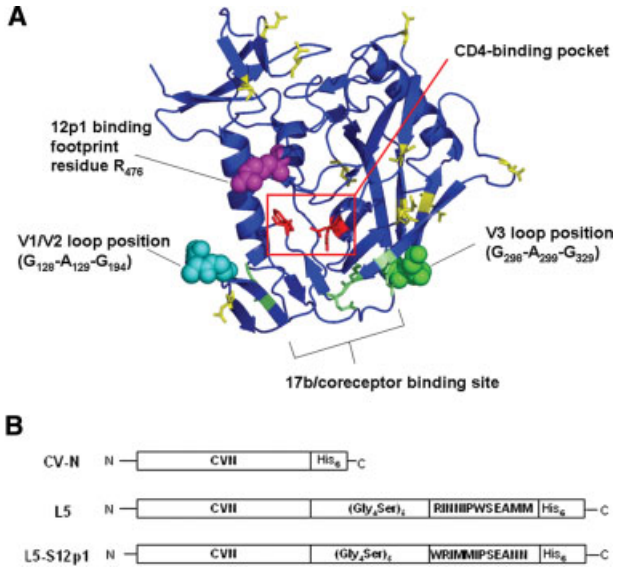

Figure 2. Construction of CVN chimerae: (**A**). The crystal structure[69] shows the HIV-1_{YU2} gp120 core denoting various ligand binding sites. The figure is shown in ribbon format and colored on the basis of secondary structure: red for alpha helix, blue for β-sheet, green for turns and white for irregularly ordered. Labeled are the potential glycosylation sites[70] that could be sites of CVN binding are shown as green ball and stick. The three residues found important for 12p1 binding (Lys92, Glu102, and Arg476)[47] are in space filling representations. (**B**) Schematic representation of the constructs used in this paper (not drawn to scale). All constructs contain a pel b secretory sequence and a hexa-histidine tag for purification. The L5 chimera contains the peptide 12p1 (RINNIPWSEAMM) c-terminal to the CVN domain with an intervening linker of five repeats of Gly_4Ser. The construct L5-S12p1 contains the scrambled peptide (WRIMMIPSEANN) c-terminal to the CVN domain and contains a linker of five repeats of Gly_4Ser.

utilized a four parameter fitting function that is part of the BIAevaluation software. In this method, the inhibition curve was converted into a calibration curve by the use of the 4-parameter equation,

$$R = R_{\text{high}} - \frac{(R_{\text{high}} - R_{\text{low}})}{1 + \left(\frac{Conc}{A_1}\right)^{A_2}} \quad (1)$$

where R is percent binding at inhibitor concentration "conc," R_{high} is the percent binding with no competitor and R_{low} is percent binding at highest inhibitor concentrations, A_2 is a fitting parameter and A_1 is the desired value IC_{50}. Data were plotted using Origin 7 software as percent binding to gp120 with the uninhibited reaction set to 100%.

RESULTS

Viral Synergy Analysis

Although it is known that CVN binds to high mannose groups on the heavily glycosylated gp120, the specific binding site in the envelope protein is unknown. Given this uncertainty, we initially tested for the ability of CVN and 12p1 to bind to gp120 simultaneously. This was done by ensuring lack of interference with each other and inhibition of viral infection. These assays were performed by the incubation of the indicated doses of CVN and 12p1 alone or in combination with HIV-1_{BaL} for 1 h prior to the addition of PM-1 cells. Viral replication was determined by gp120 ELISA after a 7 day incubation. Dose response curves from the mean of three independent experiments are given in Figure 1. From these and additional data, a synergy index for the combination was found to be in the 0.3–0.5 range, compared with a scale of 0.1–0.3 for strong synergism, 0.3–0.7 for synergism, 0.7–0.85 for moderate synergism, 0.85–0.9 for slight synergism, and 1.10–1.20 for nearly additive. Even though the synergism shown by these results is modest, it must be borne in mind that CVN has a far greater affinity than 12p1, so we considered the magnitude of the effect in noncovalent mixtures using 12p1 to be quite encouraging. Graphical evidence for how 12p1 can boost the activity of CVN can be seen in Figure 1(left) by comparing the values of % inhibition observed for CVN at the two lowest concentrations either alone (black squares) or with CVN at the same two lowest concentrations but with the two lowest concentrations of 12p1 (16–31 μ*M*) added (red circles). Even though 12p1 at those latter concentrations shows only a marginal inhibitory effect on its own, the presence of 12p1 with CVN significantly boosts efficacy in the mixture by a factor of close to twofold. These results indicate that not only can CVN and 12p1 bind gp120 simultaneously, but intriguingly appear to synergistically enhance viral inhibition. This lends credence to the feasibility of achieving effi-

cacy-enhancing effects by combining the two compounds in a chimeric protein.

Design and Purification of the CVN-12p1 Chimera

We sought to create a chimeric protein with the high affinity binding of CVN and the unique allosteric inhibition of 12p1. The design of the peptide sequence linking the 12p1 and CVN domains of the chimera was complicated by the lack of structural definition of the 12p1 binding site, the greater than 1:1 stoichiometry of the interaction of CVN with gp120 and lack of information of the exact glycosylation pattern of the insect derived HIV_{YU-2} gp120 used in our study [Fig. 2(A)].[47,68] Therefore, we initially chose a 25 amino acid linker as a starting interdomain spacer based upon the Gly_4Ser linker repeat commonly used in bridging scFvs.[71] We designed a construct in which the N-terminal domain of 12p1 would be linked to the C-terminal domain of CVN via a long flexible linker of five Gly_4Ser repeats [Fig. 2(B)]. Based on secondary structure prediction of the amount of extended strand regions of this sequence, the length of this linker region was estimated to be in the range of 30–75 Å. By using a combination of N-glycosylation site prediction of the HIV_{YU-2} gp120 and R476 as a marker of 12p binding epitope, this linker region was deemed appropriate because of the presence of potential CVN binding sites at distances of 30 Å from R476 using the available crystal structure of the HIV-1_{YU-2} gp120 core.[69,72] The chimera, designated L5, contains a hexa-histidine tag on its C-terminus both for ease of purification and for confirming the accessibility of the C-terminal 12p1 domain. We also designed a chimera in which the 12p1 sequence was scrambled, rendering it nonfunctional,[47] (unpublished observations). The latter construct was derived from the L5 chimera and was identical to it in all aspects except the sequence of the 12p1 component.

CVN, L5, and L5 with the scrambled 12p1 (L5-S12p1) were expressed in *E. coli* using the induction conditions optimized in Colleluori et al.[73] All constructs were expressed in the periplasmic fraction, isolated by osmotic shock and purified over a NiNTA column. The high degree of homogeneity of all purified expressed proteins was determined by Coomassie stain [Fig. 3(A)]. A western blot was performed to confirm the presence of both the CVN and 12p1 domains. Proteins were transferred to nitrocellulose and probed with either an antibody against CVN [Fig. 3(B)] or the hexa-histidine tag [Fig. 3(C)].

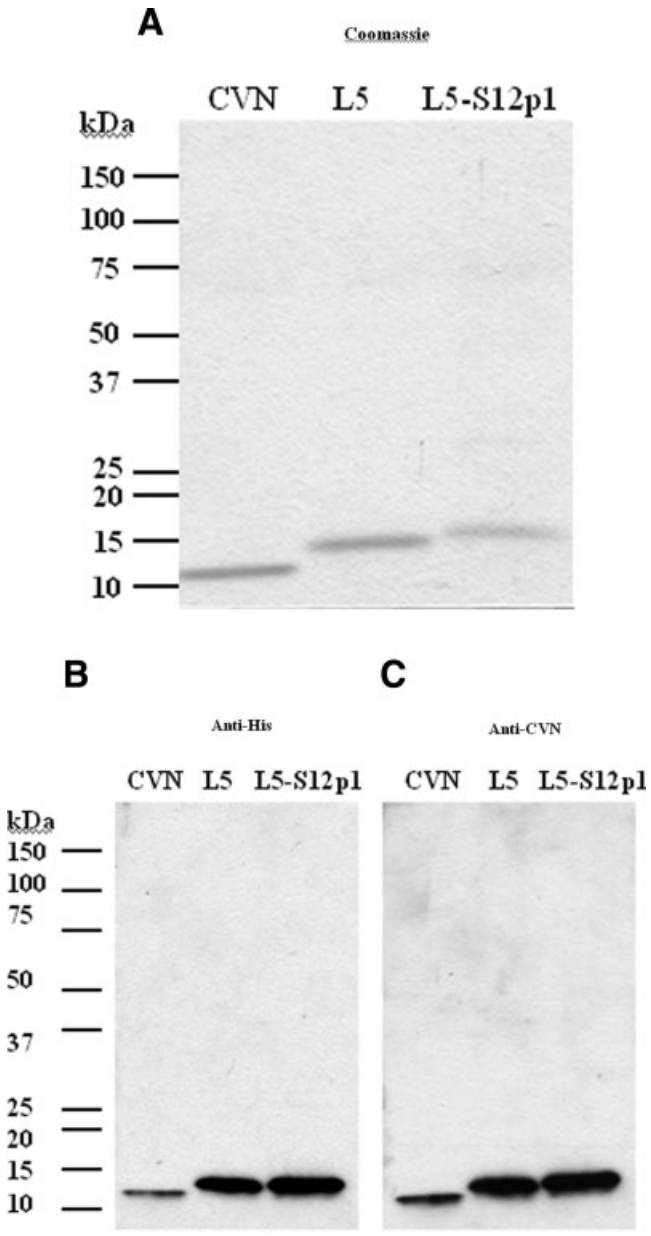

Fig. 3. Expression and purity of chimeras. All constructs were expressed in BL21(DE3) codon optimized *E. coli*, induced with 1 m*M* IPTG for 2 h after which the bacteria were then pelleted and the protein isolated through osmotic shock. Lysates were purified over a Nickel-NTA column and eluted with 250 m*M* imidazole. The arrowhead indicates the position of the CVN band (11 kDa) and the asterisk indicates the position of the chimerae (15 kDa). Samples were separated on a 4–20% polyacrylamide gel under reducing conditions and detected by Coomassie stain (**A**) or western blot with anti-His (**B**) or anti-CVN (**C**).

Direct Binding of CVN and L5 Chimera to gp120

A key need in the development of an agent for use as a microbicide is the ability to bind to and inhibit the great majority of forms of a particular pathogen. CVN itself has the ability to bind to and inhibit a wide variety of different strains of HIV-1 both primary and lab adapted isolates irrespective of tropism.[20,74] We therefore tested the chimera to ensure its retention of broad binding specificity for gp120s from a variety of strains of HIV-1 of diverse clades and tropisms. These included HIV-1_{SF162} (Clade B), HIV-$1_{93MW959}$ (Clade C), HIV-$1_{92UG021-9}$ (Clade D), HIV-$1_{92US715}$ (Clade B), SIV_{PBj2-8} from Jim Arthos at NIAID, AN1 (an ancestral clade B HIV provided by Jim Mullins,[75]), HIV-1_{BaL} (Clade B), HIV-$1_{90CM243}$ (circulating recombinant CRF01-AE obtained from Protein Sciences Corporation via the NIAID AIDS Reference and Reagent Program). Both the L5 chimera and CVN behaved equiva-

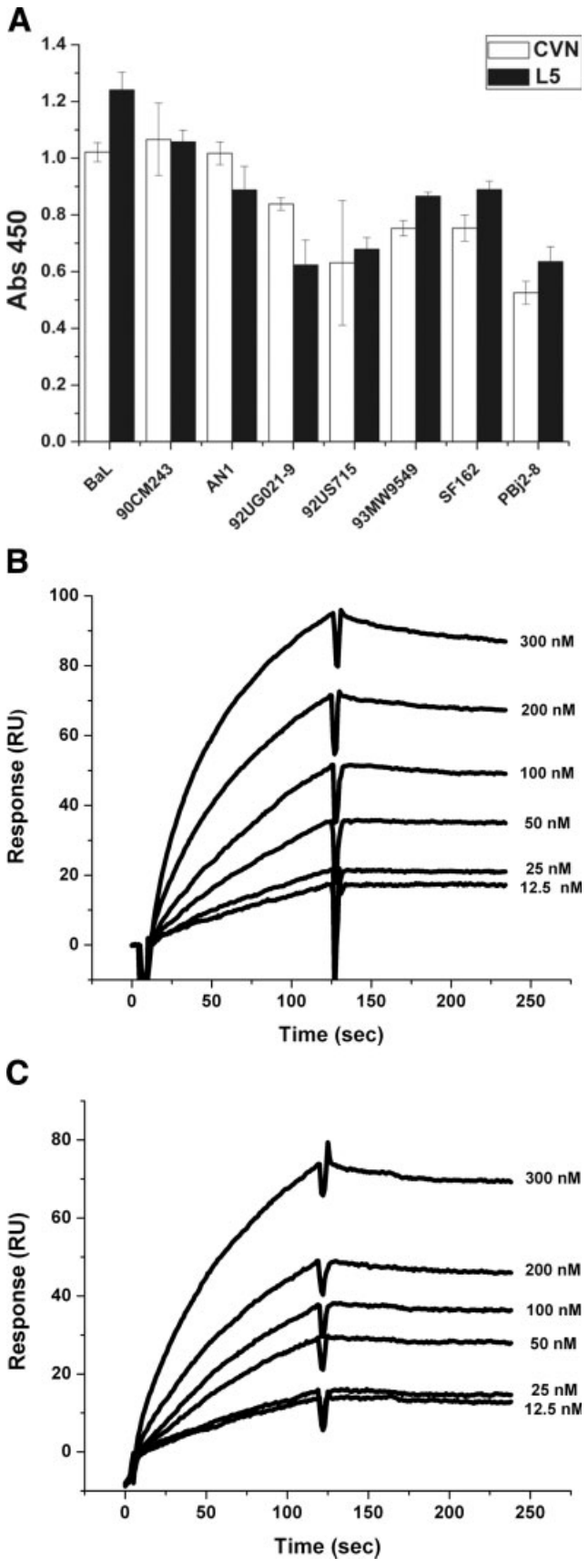

TABLE I. Kinetic Parameters for Direct Binding to HIV-1_{YU2} gp120 as Determined by Surface Plasmon Resonance (Biacore)

Protein	k_a (1/Ms)	k_d (1/s)	K_D (μM)
CVN[a]	1.79 $e^5 \pm 0.3$	3.3 $e^{-4} \pm 1.2$	0.004 ± 0.001
L5[a]	3.75 $e^5 \pm 1.3$	6.73 $e^{-4} \pm 3$	0.004 ± 0.002
12p1[b]	1.36 e^4	0.715	5.27

[a]Kinetic parameters determined by BIAevaluation of the association and dissociation fitted locally to a 1:1 binding model. Results reflect six concentrations run in triplicate after the subtraction of nonspecific binding.
[b]Kinetic parameters determined by BIAevaluation of the association and dissociation fitted globally to a 1:1 binding model. Results reflect six concentrations after the subtraction of nonspecific binding. Data are in agreement with previously published data.[47]

lently with all strains tested [Fig. 4(A)]. Only minor differences were observed in the amount of CVN or L5 that bound to a particular gp120. These differences could be attributed to strain specific differences in glycosylation or to cell-type specific glycosylation depending on the overproduction system used. The diverse HIV-1 variant specificity of the chimera, as CVN, hints at its potential for the development of a topical microbicide against stains of HIV-1, in particular Clades A and C, that afflict sub-Saharan Africa and other areas of high AIDS occurrence.

Given the ELISA-based confirmation of broad binding specificity of L5 to diverse forms of gp120, we quantified L5 binding using SPR biosensor analysis for the prototype case of YU2 gp120. Briefly, concentrations of 12.5–500 nM CVN or L5 were passed over channels in the sensor chip containing either gp120 or a reference protein. The response from the reference surface was subtracted from the response of CVN and L5 to gp120 to yield net sensorgrams [Fig. 4(B,C)]. The sensorgrams report increases and decreases in refractive index at the sensor surface that are caused by corresponding increases and decreases in mass buildup, upon association and dissociation, respectively, of mobile analyte (CVN or L5) to immobilized ligand (gp120). The equilibrium dissociation constant for CVN has been reported to be 5–50 nM[68] as compared with 3.6 μM for 12p1.[47] Based on these affinities one would predict that the binding of L5 would be primarily directed by the CVN domain of the chimera. Data analysis was performed using BIAevaluation 4.0 software (Biacore, Upsalla, Sweden) with nonspecific binding to

Fig. 4. Concentration-dependent binding of L5 chimera to gp120. (**A**) ELISA assay of CVN or the L5 chimera binding to gp120 of a variety of clades and tropisms. In ELISA experiments, 100 ng of the indicated gp120 was adsorbed onto an ELISA plate followed by the addition of 50 nM of either CVN (white bars) or the L5 chimera (black bars). The extent of binding was detected with a polyclonal antibody against CVN. Bars represent triplicate determination after the subtraction of nonspecific binding to BSA. Error bars indicate one standard deviation. A kinetic analysis of the interaction of gp120 with CVN or L5 was perfomed using a Biacore biosensor. Representative sensorgrams of serial dilutions of either CVN (**B**) or L5 (**C**) over a sensor chip immobilized HIV-1_{YU2}-gp120 are shown. All experiments were done in triplicate with background binding to a nonspecific reference subtracted out.

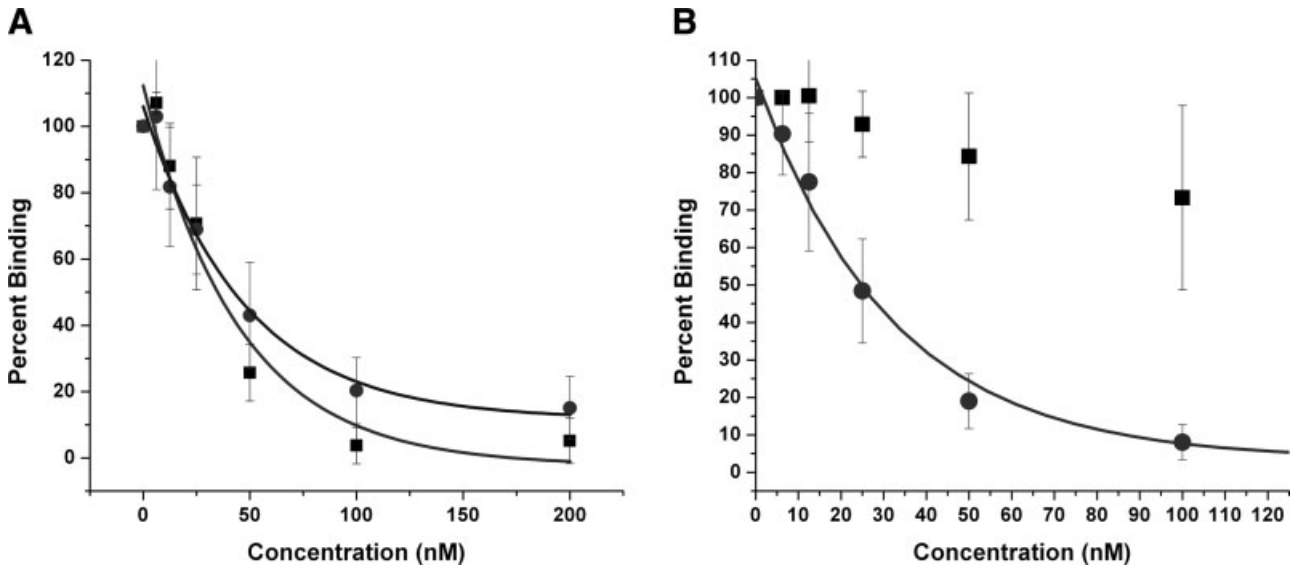

Fig. 5. L5 competition of the YU2 strain of glycoprotein 120 (gp120) binding to monoclonal antibody (mAb) F105 (Left) and 2G12 (Right). Monomeric HIV-1_{YU2} gp120 was adsorbed onto ELISA plates overnight prior to blocking with 1%BSA. Increasing doses of CVN (squares) or L5 (circles) was combined with a 0.5 μg mL^{-1} dilution of F105, a CD4 binding site antibody (**A**) or 50 μg mL^{-1} 2G12, which binds to high mannose glycosylation sites on gp120 (**B**) was added for 1 h at room temperature. The amount of F105 or 2G12 bound was detected. Results above are represented as percent of antibody bound after reactions were subtracting for nonspecific binding to BSA.

TABLE II. IC_{50} Values in n*M* of the Competition of HIV-1_{YU2} gp120 Interactions with Protein Ligands

Protein	CD4	17b	2G12	F105
CVN	>100	—	32.4 ± 3.4[a]	—
L5	16.5 ± 1.6[a]	25.5 ± 5[a]	34 ± 6[a]	23 ± 1.3[a]
L5-S12p1	>100	—	ND	ND

ND, not detemined. Entries with "—" indicate that IC50 values were not estimated, since the inhibition did not reach at least 50%.
[a]Values were calculated based on Eq. (1) described in Materials and Methods.

the IL5 mAb 2E3 subtracted out. Experimental data were fitted separately to a Langmuir 1:1 binding model with a parameter included for mass transport. As expected, both CVN and L5 exhibited similar kinetic constants with apparent equilibrium dissociation constants of 4 n*M* (summarized in Table I).

Effect of L5 Chimera on the Interactions of gp120 with mAb F105 and 2G12

Although the two components of the chimeric L5 protein, CVN and 12p1, share a common target, gp120, they display markedly different inhibition profiles. The peptide 12p1 has been demonstrated to inhibit the interactions of gp120 with a number of different ligands.[47] For example, 12p1 has an inhibitory effect on the binding of gp120 to antibody F105,[47] with an IC_{50} of 3.57 μ*M* (Cocklin, unpublished observations) whereas CVN has little effect.[76] In contrast, CVN has been demonstrated to disrupt the interaction of soluble monomeric gp120 with the antibody 2G12, an antibody that like CVN binds carbohydrate groups but has minimal effects on other antibodies against gp120.[76] 12p1 has been demonstrated to have little effect on the interaction of gp120 with 2G12.[47] We exploited the above differences to evaluate whether or not both domains of the chimera are functional in L5. An ELISA-based inhibition assay was conducted using F105 as an indicator of the functionality of the 12p1 domain, and mAb 2G12 as an indicator of the CVN domain. As shown in Figure 5, the chimera selectively inhibited the F105-gp120 interaction, whereas CVN did not. This demonstrated the functionality of the 12p1 domain of the chimera. Strikingly, the suppression of F105 binding was found to occur with an IC_{50} of 23 n*M* (Table II). This argued that the 12p1 domain binding was potentiated in the chimera due to the simultaneous binding of the CVN domain. Both proteins inhibited gp120–2G12 interaction, showing the functionality of the CVN domain in both cases.

Effect of the L5 Chimera on gp120 Interactions with sCD4 and 17b

We further evaluated the potential for unique binding properties of L5 to receptor site ligands related to HIV-1 entry. CVN has been shown to inhibit the interaction of cell-associated CD4 with gp120 but has only limited effect upon the interaction between soluble CD4 (sCD4) and gp120.[76,77] In contrast, 12p1 can suppress the interaction of sCD4 with gp120 with an IC_{50} value in the micromolar range.[47] We assayed for the ability of the L5 chimera to inhibit CD4 binding to gp120. HIV-1_{YU-2} gp120 was immobilized onto a 96 well ELISA plate overnight. The plate was blocked, and increasing doses of CVN, L5, or L5-S12p1 combined with 2 μg mL^{-1} sCD4 were added. The extent of binding was detected with a polyclonal antibody against CD4. The data are shown in Figure 6(A) as per-

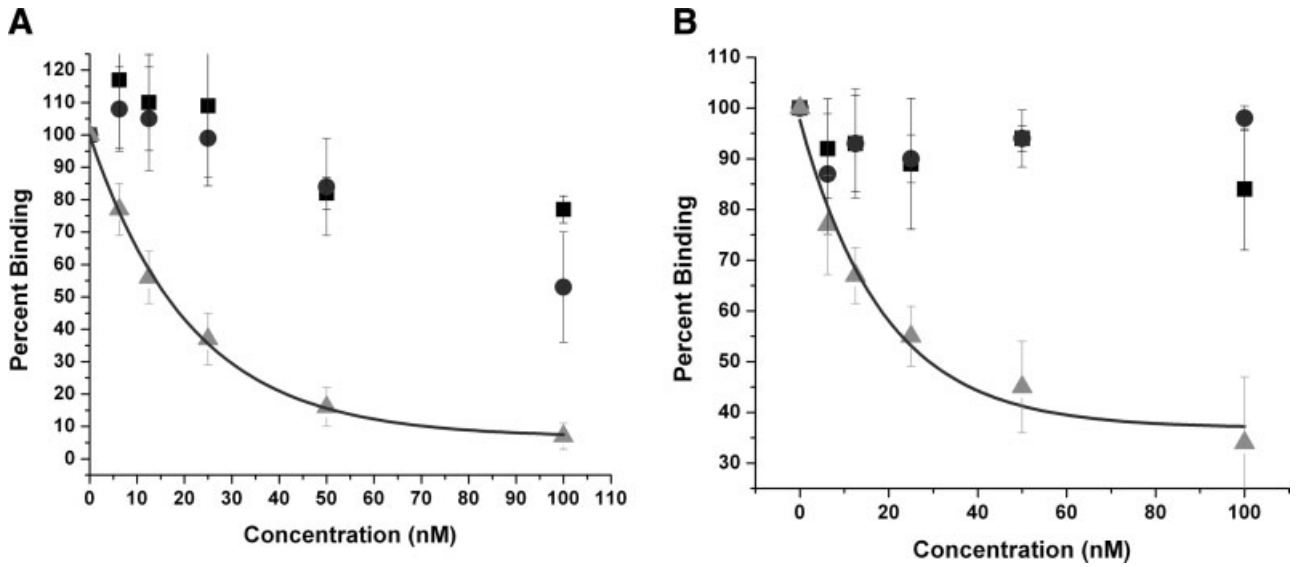

Fig. 6. L5 inhibition of the YU2 strain of gp120 binding to its receptor, soluble CD4 (sCD4) or the coreceptor surrogate, monoclonal antibody 17b. (**A**) Monomeric HIV-1_{YU2} gp120 was adsorbed onto ELISA plates prior to the addition of increasing doses of CVN (squares), L5 (circles), or L5-S12p1(triangles) incubated with 2 μg mL^{-1} soluble CD4 (sCD4). The binding of sCD4 to gp120 was detected with an antibody directed against sCD4. The data is represented as percent bound after nonspecific binding to BSA was subtracted out. (**B**) Monomeric HIV-1_{YU2} gp120 was adsorbed onto ELISA plates prior to the addition of increasing doses of CVN, L5 or L5-S12p1 incubated with 2 μg mL^{-1} 17b. The binding of 17b to gp120 was detected with an anti-human HRP secondary antibody. The data is represented as percent bound after nonspecific binding to BSA was subtracted out.

cent of uninhibited after subtracting nonspecific binding to BSA. Calculated values for IC_{50} for these data are given in Table II. The data demonstrate that the L5 chimera can inhibit CD4-gp120 interaction more potently than CVN alone. This enhanced inhibition is lost when the 12p1 domain of the chimera is scrambled. Taken together, the data demonstrate that the L5 chimera can inhibit the CD4-gp120 interaction more potently than CVN alone, and that this enhanced inhibition requires the specific 12p1 sequence in the chimera.

The monoclonal antibody 17b binds to an epitope that is present in unliganded gp120, further exposed upon CD4-binding and overlaps with the coreceptor binding site.[78–80] This has led to its use as a surrogate for coreceptor binding site.[81,47] CVN cannot inhibit the gp120-17b interaction[76] while 12p1 inhibits this interaction with an IC_{50} value in the micromolar range.[47] We assayed for the ability of the L5 chimera to inhibit gp120-17b binding using ELISA. The L5 chimera inhibits the interaction between 17b and gp120 while CVN and L5-S12p1 had limited effect [Fig. 6(B)]. The calculated value for IC_{50} is given in Table II. The inability of the L5-S12p1 chimera to inhibit this interaction indicates that the greatly enhanced 17b binding antagonism function is imparted specifically by the 12p1 domain and is not due to either a contaminant or nonspecific interaction of the linker domain. As with the inhibitory effects with sCD4 and F105, the inhibition of 17b occurs at a dose that reflects the high affinity binding of the CVN domain of the chimera.

Overall, the L5 chimera inhibits both the sCD4 and 17b interaction with gp120 at doses at which neither CVN nor 12p1 has an effect. These results demonstrate that both domains of the chimera are able to bind gp120 simultaneously. The chimera has the unique inhibitory properties of 12p1 with the high affinity binding of CVN.

DISCUSSION

The most dominant means of transmission of HIV and spread of AIDS worldwide is by heterosexual intercourse. Microbicides, compounds that could be used in vaginal and rectal formulations, are increasingly seen as an urgent goal to stop transmission. One compound currently in preclinical trials is the lectin, CVN, which binds to high mannose residues on the surface of the highly glycosylated HIV-1 gp120,[29,30] a protein that mediates viral entry. Although CVN shows great promise, there are potential limitations including the emergence of resistance mutations with altered glycosylation sites[21,45] and the mitogenic effect that it has on PBMCs at subtoxic doses.[21] One method of altering proteins to improve their efficacy and minimize toxicity is through the creation of chimeras. The theory behind chimeric proteins is to recombinantly combine two synergistic or complementary proteins to create a bi-functional protein with enhanced efficacy. In this work we tested the potential to combine CVN and allosteric dual antagonist 12p1 into a chimeric fusion inhibitor. In a noncovalent mixing experiment, we found that 12p1 and CVN showed evidence of synergistic action (Fig. 1), lending credence to the potential usefulness of a CVN:12p1 chimera as an inhibitor of HIV-1 entry.

We designed, expressed, and evaluated a chimeric protein, L5, which contains CVN and 12p1 domains covalently connected via a flexible linker (Fig. 2). The L5 chimera was produced in *E. coli* at levels similar to those of CVN alone. It was stable over prolonged periods and multiple freeze-thaw cycles. The chimera was found to bind to gp120 with affinity and broad specificity similar to these properties exhibited by CVN alone as determined by both ELISA and SPR assays (Fig. 4). This indicates that CVN is able to tolerate the addition of a long polypeptide tail onto its C-terminal domain with no disruption of its ability to bind gp120 with high affinity. We did not expect a significant enhancing effect of the 12p1 domain on the overall gp120 binding affinity of the L5 chimera because of the overwhelming (3 order of magnitude) dominance of the CVN component over the 12p1 domain for gp120 binding. The chimera nonetheless exhibited important new gp120-inhibiting actions that could pave the way for more potent HIV-1 entry inhibitors.

The profile of inhibitory properties of the L5 chimera, obtained by ELISA competition assays for a set of gp120 protein ligands, revealed that the chimera was able to combine interaction-inhibiting activities unique to the 12p1 domain with the much stronger affinity and slow dissociation rate of the CVN domain. This was evident in the inhibition of binding to CD4, 17b, F105, and 2G12 (Figs. 5 and 6). The L5 chimera is able to inhibit gp120 binding of all of these ligands with nanomolar IC_{50} efficacies, in stark contrast to the much weaker inhibition activities of CVN alone for all ligands except 2G12. This pattern of inhibition is lost when the 12p1 domain is scrambled. These observations demonstrate that the two domains of the chimera can bind at the same time to gp120, resulting in a high affinity, allosteric inhibitor that has the potential to suppress both host cell receptor interactions of the viral envelope.

The results of this work argue strongly that both the CVN and 12p1 domains of the CVN/12p1 chimera are able to bridge the 12p1 binding site and glycosylation sites in gp120. CVN domain binding is concluded from the ability of the chimera to bind at a CVN like affinity and its ability to inhibit 2G12 binding [Fig. 5(A)], while 12p1 binding is concluded from the competitive activity of the chimera on 17b, CD4 and F105 binding. Current advances in our understanding of the CVN[21,37] and 12p1[47] binding site based on escape mutants led us to speculate on the mode of action of the chimera. Calculations based on the Arg476 binding site of 12p1 indicate that the approximately 40 Å linker could span the distance to one of several proximal glycosylation sites. This would allow simultaneous binding of both chimera domains. The significantly slower dissociation of the CVN domain would have the effect of tethering the 12p1 domain in close proximity to its binding site. This would allow for the 12p1-mediated allosteric effect to occur at significantly lower concentrations than predicted for 12p1 alone, and indeed, we did observe this increased potency [Figs. 5(B) and 6(A,B)]. We consider it possible that the carbohydrate site alternatives occupied by the current chimera construct may not be the site that would lead to highest overall affinity of the chimera but instead is the site accessible given the linker length and domain compositions imposed here. A further complicating feature in interpreting mechanism is that CVN alone has been found to bind to gp120 with up to 5:1 stoichiometry.[68] Whether this is occurring by CVN aggregation or multisite binding is not known. Nonetheless, direct binding data using Biacore have shown that L5 also can bind with multiple stoichiometry to YU2 gp120. Hence, we cannot rule out the possibility of multisite binding with L5, though we would envision that only one L5 at a time would bridge a glycosylation site with 12p1 binding site.

We believe that the enhanced inhibitory activities of the chimera will lead to the development of an entry inhibitor that is more potent than CVN alone. Chimeras of the L5 family show the ability to inhibit HIV-1 binding to both host cell receptors of the virus, namely CD4 and coreceptors CCR5 and CXCR4. The HIV-1 envelope exhibits significant conformational plasticity that offers the virus the ability to mutate its surface in many nonconserved regions to resist inhibitor binding. Resisting two inhibitors simultaneously likely will be more difficult for the virus, and hence dual site inhibitors should be less vulnerable to resistance mutations. Furthermore, both the 12p1 and CVN exhibit the ability to inhibit diverse HIV-1 variants. The multi-enveloped specificity of the CVN moiety is shown in Figure 4, and ability to inhibit diverse gp120 variants has recently been observed for variants of the 12p1 peptide (Cocklin et al., 2006, manuscript in preparation).

While the results so far are promising, there is still substantial opportunity to improve the design of the chimera structurally to optimize the functional outcome of viral antagonism. The L5 chimera embodies a linker that was used as a first test because of current uncertainties of locations of the binding sites of the CVN and 12p1 components. This leaves open the possibility to improve the linker, by either variations in length[82,83] or changes in rigidity,[84] to improve the dual binding presentation of the 12p1 and CVN domains. The opportunity also exists to optimize the functional affinity of the 12p1 and CVN domains themselves. This is likely to be particularly true for the 12p1 domain, for which recent chemical modification studies have shown that variant forms of 12p1 with nanomolar K_D affinities for gp120 are possible.[85]

A major challenge that remains is to optimize the efficacy of the chimera to enable effective inhibition of viral entry. In the molecular investigations reported here using ELISA, the HIV-1 Env protein target was the gp120 monomer expressed in insect cells. Similar synergizing inhibition activities were also confirmed in SPR competition assays using gp120 monomers. In contrast, the ultimate target for viral inhibition is the HIV-1 Env trimer formed in human cells. The binding properties to this latter target may well be different and require adjustments to the structural composition of the 12p1-CVN chimera. A reflection of this caution is the low micromolar affinity observed for 12p1 alone in binding assays[47] versus the

somewhat greater IC_{50} observed in cell infection assay (Fig. 1). Nonetheless, preliminary results show that the L5 chimera has the capacity to inhibit HIV-1_{BaL} infection of p4-CCR5 cells (Krebs et al., unpublished results), showing that the chimera can access the viral spike. This establishes an important starting point for future studies to optimize chimera activity in increasingly biological environments.

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