Biochemical and Biological Characterization of a Dodecameric CD4-Ig Fusion Protein

IMPLICATIONS FOR THERAPEUTIC AND VACCINE STRATEGIES

Drug toxicities associated with HAART lend urgency to the development of new anti-HIV therapies. Inhibition of viral replication at the entry stage of the viral life cycle is an attractive strategy because it prevents de novo infection. Soluble CD4 (sCD4), the first drug in this class, failed to suppress viral replication in vivo. At least three factors contributed to this failure: sCD4 demonstrated poor neutralizing activity against most primary isolates of HIV in vitro; it demonstrated an intrinsic capacity to enhance viral replication at low concentrations; and it exhibited a relatively short half-life in vivo. Many anti-gp120 monoclonal antibodies, including neutralizing monoclonal antibodies also enhance viral replication at suboptimal concentrations. Advances in our understanding of the events leading up to viral entry suggest strategies by which this activity can be diminished. We hypothesized that by constructing a sCD4-based molecule that is large, binds multiple gp120s simultaneously, and is highly avid toward gp120, we could remove its capacity to enhance viral entry. Here we describe the construction of a polymeric CD4-IgG1 fusion protein. The hydrodynamic radius of this molecule is ~12 nm. It can bind at least 10 gp120 subunits with binding kinetics that suggest a highly avid interaction toward virion-associated envelope. This protein does not enhance viral replication at suboptimal concentrations. These observations may aid in the design of new therapeutics and vaccines.

The widespread use of highly active antiretroviral therapy has dramatically improved the clinical course for many individuals infected with HIV (1). However, toxicities associated with long term highly active antiretroviral therapy have put a high priority on the design and development of less toxic therapies. Among the “next generation” of antiviral inhibitors is T-20 (2, 3), a relatively nontoxic peptide that disrupts viral fusion thereby protecting CD4+ lymphocytes from de novo infection. In clinical trials, T-20 has been shown to reduce plasma viral load by up to 2 logs (4). These results demonstrate that the entry stage of the HIV replication cycle is a viable target for the development of new antiretroviral therapies.

Viral entry is a complex biochemical event that can be subdivided into at least three stages: receptor docking, viral-cell membrane fusion, and particle uptake (5). Receptor docking is a multistep process that begins with the gp120 component of a virion spike binding to the CD4 receptor on the target cell. Conformational changes in gp120 induced by gp120-CD4 interaction promote a high affinity interaction between gp120 and either CCR5 or CXCR4 cellular co-receptors. This is followed by gp41-mediated fusion of the viral and target cell membranes. Agents designed to block gp120-CD4, gp120-CCR5/CXCR4, or gp41/cell membrane interactions are in various stages of development (5). Several laboratories have constructed recombinant proteins that fuse the gp120 binding domain of CD4 to immunoglobulin constant domains (6–10). Unlike monomeric soluble CD4 (sCD4), these Ig fusion proteins are able to efficiently neutralize primary isolates of HIV in vitro. This increased capacity to neutralize probably results from increased avidity associated with the multivalent presentation of gp120 binding epitopes. Of note, a highly potent tetravalent CD4-Ig fusion protein termed Pro-542 is currently being evaluated in clinical trials (11). The strategy underlying these CD4-based therapies (i.e. blocking the interaction between gp120 and the CD4 receptor) encompasses advantages distinct from current highly active antiretroviral therapy regimens. In particular, such agents, by blocking de novo infection, may prevent the expansion of viral reservoirs. Monomeric sCD4 was one of the first reagents in this group to be tested clinically (12). Unfortunately, sCD4 failed to demonstrate significant antiviral activity in vivo (12). Among the problems inherent to sCD4 was its inability to efficiently neutralize primary isolates of HIV. The differential capacity of sCD4 to neutralize tissue culture laboratory-adapted strains versus many primary isolates is striking. In the initial report describing this difference, Ho and colleagues (13) found that the concentrations of sCD4 required to neutralize primary isolates in vitro were up to 1000-fold higher than those required to neutralize tissue culture laboratory-adapted strains.

Additional properties of sCD4 are likely to have contributed to its failure in the clinic. At low concentrations, sCD4 enhances the infectivity of most primary isolates (14–22). This property is of particular concern, because sCD4 exhibited an extremely short serum half-life (23). This unfavorable pharma-cokinetic property increases the likelihood that at sites of viral replication (i.e. lymphoid tissue), concentration gradients fa-
voring enhanced replication of HIV-1 could occur.

The precise molecular basis underlying sCD4-mediated enhancement of virus replication is unclear. In vitro, the relatively low concentrations of sCD4 that result in enhancement represent a large molar excess over gp120 present in the culture. If those concentrations are increased substantially, many primary isolates can be neutralized. These observations suggest that only when a sufficient number of spikes per virion are fully occupied will sCD4 effectively inhibit virus entry (24). The concentration required to achieve that state is likely to be extremely high for two reasons. First, sCD4 must compete with surface-bound CD4 receptors, which are presented in bulk on the surface of a target cell. Because sCD4-gp120 ligation involves slow binding kinetics and substantial changes in entropy, gp120 avidity effects strongly favor membrane-associated receptors (25). Second, sCD4-gp120 ligation promotes a high affinity interaction between gp120 and CCR5 (26, 27). Enhancement of viral entry is not solely a consequence of sCD4-gp120 ligation. It is well established that many monoclonal antibodies (mAbs), including neutralizing mAbs also enhance viral entry at suboptimal concentrations (19, 20). Moreover, polyclonal antibodies derived from HIV-infected individuals can also enhance viral replication (19). To develop more potent entry inhibitors and more effective vaccine immunogens it is important to better define the biochemical events surrounding this phenomenon and to develop strategies that eliminate it.

We hypothesized that if sCD4 was modified such that it could more efficiently compete with membrane-associated CD4, block interactions between activated spikes and CCR5, and occupy multiple gp120s within a spike, then it would no longer enhance viral replication at suboptimal concentrations. In this regard, we adopted a strategy that utilizes the 18-amino acid secretory tailpiece (otp) of IgA (30–32), otp is encoded at the extreme carboxyl terminus of IgA and bears significant sequence homology to the μ tailpiece (μtp), which is encoded at the carboxyl terminus of IgM. Unlike μtp, which, in conjunction with the j chain, promotes the formation of pentamers, otp does not promote oligomerization of IgA beyond a dimer. However, when fused to the carboxyl terminus of an IgG1 heavy chain, otp promotes the formation of a large recombinant immunoglobulin that is predicted to consist of 12 IgG1 heavy chains (30).

The two N-terminal domains of CD4, termed D1 and D2 (33), encode the gp120 binding epitope and, when expressed in the absence of the remaining domains of CD4, retain the capacity to bind gp120 (34). We fused the coding sequences of D1 and D2 to that of IgGtp, creating a recombinant protein we term D1D2-IgGtp. Such a recombinant protein should theoretically exhibit a mass close to 800 kDa and present 12 gp120 binding sites. We predicted that the presentation of 12 closely spaced gp120 binding sites should improve the capacity of sCD4 to compete with membrane CD4 for virion-associated gp120. We further hypothesized that the extremely large size of this protein should preclude any interaction between activated virion spikes and CCR5 on the target cell membrane. In this report, we demonstrate that this protein, termed D1D2-IgGtp, in contrast to sCD4, does not enhance the entry of primary isolates into CD4+ T cells at suboptimal concentrations.

2 P. Kwong, personal communication.

EXPERIMENTAL PROCEDURES

Virus Entry

Virus entry into primary lymphocytes was measured using a quantitative real-time PCR assay based upon the generation of early long terminal repeat transcripts, adapted from a method previously described (35). Briefly, freshly isolated peripheral blood mononuclear cells (PBMCs) were activated (OKT3 (1 μg/ml)/interleukin-2 (25 units/ml)) for 3 days and then depleted of CD8+ T cells by magnetic bead selection (Dynal, Lake Success, NY). 3 × 10^6 cells were incubated in a volume of 100 μl with the addition of titrated viral stocks (Advanced Biotechnologies, Columbia, MD) for 2 h at 37 °C. Where specified, monomeric sCD4 and D1D2-IgGtp (see below) were preincubated with virus stocks for 10 min at 37 °C prior to cell inoculation. Cells were washed with phosphate-buffered saline, pelleted through a 100% fetal bovine serum (FBS) cushion (heat-inactivated), and then resuspended in Dulbecco’s modified Eagle’s medium/FBS (heat-inactivated) and incubated an additional 4 h. Cells were washed and then lysed in a buffer containing an anionic detergent (Gentra, Minneapolis, MN) and RNase A. DNA was precipitated from lysates in isopropl alcohol and resuspended in distilled H_2O. Quantitative real time PCR was carried out using the following primers and probe: R5/forward primer, 5’-gtaactgggaccaactgttc-3’; R5/reverse primer, 5’-aacacagaggggaacctact-3’; R5 probe, 5’-agactcaataaggctgctggagttc-3’. Copy numbers were standardized against genomic DNA obtained from an ACH-2 cell line carrying a single integrated HIV-1 genome in each diploid cell (36).

Expression and Purification of D1D2-IgGtp

The two N-terminal domains of CD4, termed D1 and D2, encode the gp120 binding epitope and, when expressed in the absence of the remaining domains of CD4, retain the capacity to bind gp120 (34). We fused the coding sequences of D1D2 (34) to that of IgGtp (30), creating a recombinant protein that we term D1D2-IgGtp. The D1D2-IgGtp is predicted to be a hexamer of a dimer (12 binding sites). The D1D2-IgGtp expression vector was designed after a CD86-IgGtp constructed by Sweet and colleagues (30) using standard recombinant DNA methodologies (37). This vector contains a cytomegalovirus promoter for high level expression of D1D2-IgGtp as well as a gene cassette containing dihydrofolate reductase for amplification in dihydrofolate-reductase-deficient Chinese hamster ovary cells (American Type Culture Collection catalogue no. CRL9096). Purified plasmids were transfected into dihydrofolate reductase-deficient Chinese hamster ovary cells by a modified calcium phosphate transfection procedure (Invitrogen). Positive transfectedants were initially selected by growth in α-aminol essential medium without nucleosides supplemented with dialyzed fetal calf serum (Invitrogen). To increase expression, positive transfectedants were pooled and cultured in the presence of increasing concentrations of methotrexate (Sigma) as previously described (34). Cell clones expressing high levels of D1D2-IgGtp were identified by Western blot with a rabbit polyclonal antiserum raised against sCD4. Clones were subsequently cultured in hollow-fiber cartridges (Flow Systems, Erkert, MD) using Dulbecco’s modified Eagle’s medium plus 4% heat-inactivated FBS without methotrexate. Proteins were harvested daily from the extracapillary space, yielding greater than 5 mg/harvest. D1D2-IgGtp protein was purified in two steps. Initially, supernatants from the extracapillary space of the hollow fiber cartridge were passed over a Hi-Trap protein A column (Amershams Biosciences). Bound protein was eluted in 0.1 M sodium citrate, pH 3.0, and rapidly neutralized with 2 M Tris-HCl, pH 8.0. Peak fractions were subsequently pooled, concentrated, and passed over either a Superdex Hi-load 26/60 or a Superdex 200 10/30 gel filtration column (Amersham Biosciences) in phosphate-buffered saline, and the peak fraction was collected. With the exception of analytical ultracentrifugation and dynamic light scattering experiments, this was the fraction employed in all biological assays. Silver staining of SDS-PAGE gels indicated that the purity of protein obtained in this manner was >98%. Protein preparations were determined to be endotoxin-free using the Chromogenic Limulus Amebocyte Lysate method (BioWhittaker, Walkersville, MD).

Optical Biosensor Analysis

General Procedures—All binding assays were performed using a BIA3000 optical biosensor (Biacore, Inc., Uppsala, Sweden). Ligands were immobilized onto the surface of a CM5 sensor chip using the standard amine coupling procedure described by Biacore, Inc. Briefly, the carboxyl groups on the sensor surface were activated by injecting 35 μl of 0.2 M N-ethyl-N-(3-dimethylaminopropyl) carbodiimide, 0.05 M N-hydroxy succinimide. The ligand, suspended in 10 mM acetate buffer, pH 4.0–5.5 (depending on the ligand used) was passed over the activated
FIG. 1. Gel filtration of purified D1D2-Igatp and D1D2-Igatp-gp120 complexes. D1D2-Igatp was purified from Chinese hamster ovary culture supernatants and passed over a Superdex-200 gel filtration column at a flow rate of 0.5 ml/min (a). Absorbance was measured at 280 nm, and 0.5-ml fractions were collected. Molecular weight standards were also run under the same conditions to generate a standard curve (left inset). The void volume of this column was determined to be 7.45 ml. Peak fractions were collected and electrophoresed through a denaturing SDS-polyacrylamide gel and analyzed by Western blot with an anti-CD4 polyclonal antiserum (right inset). sCD4 was used as a positive control in the Western blot. D1D2-Igatp-gp120 complexes were passed over a Superose-6 gel filtration column at a flow rate of 0.3 ml/min (b). D1D2-Igatp and gp120 alone were also passed over the same column for comparison. Both gp120 and D1D2-Igatp were demonstrated to reside in the high molecular weight fraction (9.2 ml) by Western blot analysis with either anti-CD4 or anti-gp120 antiserum (b, left inset).
surface until the desired surface density was reached. Unreacted carboxyl groups were capped by injecting 35 µl of 1 x ethanolamine (pH 8.0). Bovine serum albumin was immobilized on the surface of one flow cell as a reference surface to control for nonspecific binding of analyte. The running buffer used was 10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.01% surfactant P-20, 0.5% soluble carboxymethyl dextran (Fluka BioChemika, Inc.). All binding experiments were performed in duplicate and at 25 °C.

**Interaction Analysis between sCD4 or D1D2-Igtp and HIV-1 gp120**—sCD4 or HIV-1 gp120 was directly immobilized onto the surface of CM5 sensor chips as described above to surface densities of ~250 response units (RU) for sCD4 and 500 RU for gp120. This was followed by injection of increasing concentrations of gp120 or sCD4, respectively. The surface was regeneration after each cycle by injecting 25 µl of 5 mM NaOH, 1 x NaCl, followed by a second injection of 25 µl of 4.5 % MgCl2. Association and dissociation rate constants were calculated using the BiaEvaluation 3.1 software (Biacore, Inc., Uppsala, Sweden).

**Determination of D1D2-Igtp/gp120 Binding Ratios**—To determine the ratio of gp120 monomers bound per D1D2-Igtp construct, D1D2-Igtp in running buffer was passed over a sensor surface to which protein A had been previously immobilized (surface density ~1500 RU). The final surface density of D1D2-Igtp was ~250 RU, and it was reloaded to this density at the beginning of each cycle. After loading of D1D2-Igtp, the surface was allowed to stabilize for 5 min, at which time serial solutions of gp120 in running buffer were passed over the surface for a total of 10 min. The surface was completely regenerated using three sequential 25-µl injections of 10 mM HCl. Stiochiometries were calculated from the experimentally derived amount of D1D2-Igtp and gp120 bound per cycle (in RU) using the conversion factor 1 RU = 1 pg of protein bound/mm2 of flow cell surface area and the molecular masses of the proteins (D1D2-Igtp = 800,000 Da, gp120 = 120,000 Da).

**Virus Coculture**

Virus coculture was carried out as previously described (35). Briefly, PBMCs from HIV-1-infected donors were isolated by Ficoll-Hypaque and enriched for CD4+ T lymphocytes by negative selection with a mixture of antibody-conjugated magnetic beads (StemCell Technologies, Vancouver, Canada). Cells were cultured in RPMI, 10% FBS (heat-inactivated) plus OKT3 (1 µg/ml) and interleukin-2 (50 units/ml). In addition, 3-day activated CD8+ T cell-depleted PBMCs from infected donors were added at a ratio of ~2:1 as necessary. Cultures treated with monoclonal sCD4 or D1D2-Igtp were fed with media containing these proteins such that the original concentration was maintained. Virus replication was assessed by harvesting culture supernatants at regular intervals and measuring p24 antigen using an HIV-1 p24 Antigen Capture Kinetic ELISA (Couffer, Miami, FL).

**Acute Infection**

Freshly isolated donor PBMCs were propagated in RPMI supplemented with 10% FBS and stimulated with OKT3 (1 µg/ml) and interleukin-2 (50 units/ml). Prior to infection, cells were screened by PCR for CCR5 wild-type homozygosity. Three days after stimulation, CD8+ T cell-depleted PBMCs from infected donors were added. Supernatants were collected every other day, and virus replication was assessed by measuring the level of the initial reverse transcription products in the R and U5 regions of the HIV-1 long terminal repeat. The target cells we utilized in this assay were 3-day activated, CD8+ T cell-depleted PBMCs. After optimization, the linear range of this assay typically fell between 25 and 300,000 copies of reverse transcribed product (data not shown). We employed two viruses, JR-FL and BAL, both of which utilize the CCR5 coreceptor and were derived after minimal passage of primary isolates. To establish the conditions under which sCD4 would enhance viral entry, we briefly preincubated viral inoculi with various concentrations of monoclonal sCD4 and then carried out the entry assay. Under these conditions, sCD4 at a concentration of either 6.25 or 12.5 nM repeatedly increased viral entry by 1.5–3-fold for both JR-FL and BAL (Fig. 2). At high concentrations (2400 nM), sCD4 reduced viral entry to levels close to background (Fig. 2, a and c).

**RESULTS**

**D1D2-Igtp Is Expressed as a Highly Oligomerized Protein**—We first asked whether D1D2-Igtp was expressed in a highly oligomerized form. To this end, D1D2-Igtp was purified from culture supernatants by protein-A affinity chromatography and analyzed by standard size exclusion chromatography. When passed over an analytical Superdex-200 gel filtration column, a major peak appeared in that fraction corresponding to a molecular mass greater than 650 kDa (Fig. 1). A minor fraction, comprising less than 5% of total protein, eluted in the 50–100-kDa range. Because the major fraction appeared close to the void volume of the column, we were unable to accurately estimate its molecular weight from these data. These fractions were then reduced and electrophoresed under denaturing conditions. Western blot analysis with a polyclonal antiserum specific for CD4 indicated that D1D2-Igtp resided primarily in the peak fraction (Fig. 1a, right inset). We then asked whether D1D2-Igtp recognized gp120. D1D2-Igtp was incubated for 1 h with gp120 and then passed over a Superose-6 gel filtration column. The peak fraction was observed at 9.2 ml (Fig. 1b). In contrast, when D1D2-Igtp and gp120 were passed separately over the same column, peak fractions were observed at 10.9 and 15.2 ml, respectively. To verify that complexes of D1D2-Igtp and gp120 were present in the 9.2-ml fraction, we carried out a Western blot analysis under denaturing conditions with an anti-CD4 antisemir and anti-gp120 antisemir (Fig. 1b, left inset). Both proteins were determined to elute in this fraction. We conclude from these results that D1D2-Igtp is expressed as a highly oligomerized CD4-IgG1 fusion protein. Furthermore, D1D2-Igtp recognizes HIV-1 gp120.

**Comparison of D1D2-Igtp and Monomeric sCD4 in a Quantitative HIV Entry Assay**—To determine the efficiency with which D1D2-Igtp inhibited HIV entry, we established a real time PCR-based quantitative viral entry assay. Virion entry was detected by measuring the level of the initial reverse transcription products in the R and U5 regions of the HIV-1 long terminal repeat. The target cells we utilized in this assay were 3-day activated, CD8+ T cell-depleted PBMCs. After optimization, the linear range of this assay typically fell between 25 and 300,000 copies of reverse transcribed product (data not shown). We employed two viruses, JR-FL and BAL, both of which utilize the CCR5 coreceptor and were derived after minimal passage of primary isolates. To establish the conditions under which sCD4 would enhance viral entry, we briefly preincubated viral inoculi with various concentrations of monoclonal sCD4 and then carried out the entry assay. Under these conditions, sCD4 at a concentration of either 6.25 or 12.5 nM repeatedly increased viral entry by 1.5–3-fold for both JR-FL and BAL (Fig. 2). At high concentrations (2400 nM), sCD4 reduced viral entry to levels close to background (Fig. 2, a and c).

**Analytical Ultracentrifugation and Dynamic Light Scattering**

Sedimentation velocity experiments were conducted with the Beckman Optima XL-IA analytical ultracentrifuge using interference optics, with 400 g of protein (1 µg/µl) dissolved in phosphate-buffered saline at a rotor speed of 30,000 rpm and a rotor temperature of 20 °C. Data were analyzed by direct boundary modeling with a continuous distribution of Lamm equation solutions (38) and algebraic noise decomposition (39). The distribution of Lamm equation solutions c(s) was calculated with maximum entropy regularization with s = 0.68. For deconvolution of the diffusion, the best fit average fractional ratio of 1.5 was used (40), resulting in root mean square deviations of the direct boundary fit of ~0.004 fringes in all cases. Sedimentation equilibrium experiments were performed with the absorbance optics at a wavelength of 280 nm and a rotor temperature of 4 °C. Equilibrium was attained at rotor speeds of 3000, 5000, and 7500 rpm with best fit distributions with a single species model for the determination of the weight-average molar mass (41). Using tabulated values of the partial specific volume of amino acids (42) and 0.62 ± 0.02 ml/g for the average partial specific volume of the carbohydrate component (43), and with an average glycosylation of 5 and 15 kDa at the two glycosylation sites per chain, we estimate a molar mass of 140 kDa and a partial specific volume of 0.699 ml/g for a monomeric unit (two chains). Dynamic light scattering experiments were conducted using a Protein Solutions DynaPro instrument with a NanoPro-99 instrument (Protein Solutions, Charlottesville, VA). 20 µl of sample was inserted in the cuvette with the temperature control set to 20 °C. The light scattering signal was collected at 90 °C at a wavelength of 808.3 nm. Data acquisition and initial analysis was performed with the instrument software, and data were exported for analysis with the maximum entropy method (43) in the software SEDFIT (38).
PCR-based viral entry assay. PBMCs were inoculated with HIV-1 JR-FL (a and b) or Bal (c and d) alone or in the presence of either sCD4 or D1D2-Igtp. The number of virions entering cells within 6 h post-infection was determined using a real time PCR-based viral entry assay in which early long terminal repeat reverse transcripts were enumerated. A standard curve was generated from genomic DNA obtained from an ACH-2 cell line carrying a single integrated HIV-1 genome (not shown). a, a direct comparison of sCD4 versus D1D2-Igtp inhibition of JR-FL entry. b, a further titration of D1D2-Igtp inhibition of JR-FL entry. c and d, the effect of sCD4 and D1D2-Igtp, respectively, on the entry of Bal. Correlation coefficients corresponding to the standard curves for each of these experiments ranged from 0.993 to 0.998 with slopes falling between 0.993 and 0.998. All experiments were carried out in triplicate. These results are representative of at least three independent experiments using different donor PBMCs.

In contrast, at 6.25 or 12.5 nM, D1D2-Igtp reduced JR-FL and Bal entry to levels close to background (Fig. 2, a and b, respectively). Thus, at the concentrations in which sCD4 provides optimal enhancement of viral entry, D1D2-Igtp strongly inhibits viral entry. Because each D1D2-Igtp molecule presents multiple gp120 binding sites, we considered the possibility that it might enhance entry at even lower concentrations. We titered D1D2-Igtp down to 50 pM; however, we failed to observe enhanced viral entry (Fig. 2, b and d). Therefore, we conclude that, unlike sCD4, D1D2-Igtp does not enhance viral entry at low concentrations.

**D1D2-Igtp versus Monomeric sCD4 Inhibition of Primary Viral Isolates from Patient PBMCs**—We compared the capacity of monomeric sCD4 and D1D2-Igtp to inhibit the replication of HIV-1 in cultures of PBMCs derived from HIV-1-infected patients. CD4+ T cells were isolated from patients and placed into culture along with activated PBMCs from uninfected donors. To these cultures we added concentrations of sCD4 that enhanced entry of Bal and JR-FL in our viral entry assay. In two of the three cocultures, this resulted in enhanced replication (Fig. 3, a–c), while in the third coculture sCD4 inhibited viral replication to a limited degree (Fig. 3d). The same donor CD4+ T cells were treated in parallel with D1D2-Igtp. At the concentrations of sCD4 that enhanced viral replication in two of three donor cultures, D1D2-Igtp strongly inhibited viral replication in all three donor cells (Fig. 3). We conclude that unlike monomeric sCD4, which enhances viral replication at low concentrations, D1D2-Igtp actively inhibits the replication of HIV-1 in cocultures derived from infected patient PBMCs.

**D1D2-Igtp Inhibits mAb-mediated Enhancement of HIV-1 Replication**—Similar to sCD4, a number of mAbs specific for gp120 have been shown to enhance replication of HIV-1 at suboptimal concentrations (19, 20). One of these mAbs, termed 17b, recognizes an epitope on gp120 that overlaps the CCR5 binding surface (44). This epitope is exposed subsequent to envelope-CD4 ligation (26, 45). Consequently, 17b reacts more efficiently with gp120 in the presence of sCD4. We asked whether D1D2-Igtp could prevent 17b-mediated enhancement of viral replication. PBMCs were acutely infected with two primary isolates derived from patients within the first 6 months following seroconversion. Parallel cultures were treated with 17b, sCD4, 17b plus sCD4, D1D2-Igtp, or 17b plus D1D2-Igtp, and the extent of viral replication was determined by measurement of p24 antigen in culture supernatants. For both primary isolates, 17b alone enhanced viral replication relative to control cultures (Fig. 4). The combination of 17b plus sCD4 also resulted in enhanced replication relative to control cultures. sCD4 alone enhanced replication of primary isolate 202 to a modest degree (Fig. 4a). We were surprised to observe that the combination of sCD4 and 17b appeared to enhance replication in an additive manner (Fig. 4a), suggesting that higher concentrations of one or both of these ligands would be required to observe synergistic inhibition of viral entry. sCD4 demonstrated no enhancing or inhibitory effect on primary isolate 202 (Fig. 4b). In contrast, D1D2-Igtp dramatically inhibited replication of both primary isolates. Of note, D1D2-Igtp fully suppressed 17b-mediated enhancement of both primary isolates.
Therefore, we regard the 10:1 ratio as a minimum number of gp120s bound per D1D2-Ig. Nevertheless, this analysis demonstrates that D1D2-Ig can bind many gp120s simultaneously.

**Stoichiometry of gp120-D1D2Ig-tp Binding**—To better understand why D1D2Ig-tp fails to enhance viral replication, we characterized two biochemical properties of this recombinant protein. Initially, we asked how many gp120s could be loaded onto a single D1D2-Igtp. In addition, we examined the kinetics of these interactions. D1D2-Igtp, once assembled into an oligomer, should theoretically present 12 independent gp120 binding sites. However, it is unclear whether steric constraints would limit the number of gp120s that actually bind at any given point in time. To address this issue, we established a biosensor assay that would measure the ratio of gp120 to D1D2-Igtp under conditions in which the number of gp120s bound to D1D2-Igtp approached equilibrium. Protein G was covalently coupled to a biosensor chip, which was subsequently loaded with fixed concentrations of D1D2-Igtp. To this surface we then added increasing concentrations of gp120. Once the level of gp120 approaches equilibrium, the number of gp120s bound per D1D2-Igtp can be determined by employing a standard calculation that relates Biacore RU to the mass of protein bound (see “Experimental Procedures”). Using a sensor chip loaded with 270 pg of D1D2-Igtp, we observed that concentrations of gp120 above 1800 mU approximated equilibrium (Fig. 5a). From these curves, we derived the number of gp120s recognized by a single D1D2-Igtp (Fig. 5b). Under the conditions we employed, D1D2-Igtp bound 10 gp120s simultaneously. Practical limitations, including injection volumes, protein concentration, and a very slow apparent off-rate of gp120 from D1D2-Igtp, allowed us to establish conditions at which we approached but did not actually achieve equilibrium. Therefore, we regard the 10:1 ratio as a minimum number of gp120s bound per D1D2-Igtp. Additionally, because gp120s can vary up to 30 kDa in size, this ratio may change when different envelopes are employed. Nevertheless, this analysis demonstrates that D1D2-Igtp can bind many gp120s simultaneously.

**Kinetics of gp120-D1D2Ig-tp Binding**—We next asked whether differences in the binding kinetics of D1D2-Igtp versus monomeric sCD4 might help explain the difference in activity of these two inhibitors at low concentrations. Either sCD4 or D1D2-Igtp was coupled to a biosensor chip, and the binding properties of four different envelope proteins were compared. The four gp120s we employed were 92MW959, an R5-specific clade C envelope; TH14–12, an R5-specific clade B envelope; 92Ug21–9, an X4-specific clade A envelope (46); and NL4-3, an X4-specific clade B envelope. With the exception of NL4-3, each of these envelopes was cloned after minimal passage in vitro. We noted a dramatic difference in the manner in which all of these envelopes dissociated from D1D2-Igtp relative to monomeric sCD4. Fig. 6 (a–d) displays the dissociation curves of each of the envelopes from either D1D2-Igtp or sCD4. The rate of dissociation is reflected in the slope of the curve such that the more negative the slope, the faster the rate of dissociation, while a slope of zero reflects constitutive binding. It is clear from each of the dissociation curves that all of the envelopes dissociate more slowly from D1D2-Igtp than from sCD4. Of note, each of these curves of D1D2-Igtp dissociating from gp120 approaches a slope close to zero. These observations are most easily explained by assuming that once an envelope dissociates from one chain of D1D2-Igtp, it immediately rebinds to the same molecule. Under conditions where this type of rebinding is likely to occur, we are unable to calculate an accurate dissociation constant ($k_d$). Nevertheless, by comparing the sCD4 and D1D2Ig-tp dissociation curves, we conclude that gp120 dissociates from D1D2Ig-tp at a much slower rate than it dissociates from monomeric sCD4.

We then carried out the converse assay in which we coupled gp120 to the biosensor surface and observed the kinetics of soluble D1D2-Igtp binding to that surface. As is evident from the virtually flat curve in the dissociation phase (Fig. 6c), the rate at which D1D2Ig-tp dissociated from surface-bound gp120 was extremely slow. Of note, the binding kinetics of D1D2-Igtp for monomeric gp120 employed in these assays are likely to be different from those for gp120 presented on the surface of an infectious virion. However, the virion as a target theoretically presents 216 gp120s distributed as trimers among 72 spikes (47). To the extent that D1D2-Igtp may bind more than one virion-associated gp120 simultaneously, avidity effects will result in an extremely slow rate of dissociation from the virion in a manner similar to the dissociation of D1D2-Igtp from surface-bound gp120 (Fig. 6c).
Size and Molar Mass Distribution of D1D2-Igtp.—We initially postulated that if D1D2-Igtp were sufficiently large, it would prevent the enhancement of viral entry that is associated with suboptimal concentrations of monomeric sCD4. Additionally, establishing the size of D1D2-Igtp would further help us to determine whether it is sufficiently large to span multiple spikes on the surface of a virion. D1D2-Igtp was initially fractionated by gel filtration, and the peak fraction and trailing fraction were collected (data not shown). Because of the well known difficulty of precisely measuring the molar mass of large glycoproteins by gel filtration, we characterized the size of D1D2-Igtp in more detail by analytical ultracentrifugation and dynamic light scattering. We first assessed the homogeneity of the peak protein fraction by sedimentation velocity, which showed a broad sedimentation coefficient distribution indicating a heterogeneous size distribution. The large majority of protein in the peak fraction exhibited a sedimentation coefficient between 14 and 25 S (Fig. 7, solid line). Consistent with this observed heterogeneity, the average molar mass measured by sedimentation equilibrium was dependent on rotor speed, ranging from 5.8 to 8.8 monomer units (Fig. 7, top inset). To simplify the analysis of the size distribution, we also studied the trailing fraction which exhibited less heterogeneity (Fig. 7, dashed line). By comparing the shape of both curves and with consideration of the range of molar mass values, we estimated the range of sedimentation coefficients for each oligomer (Fig. 7, arrows). From the estimated pairs of sedimentation and mass, we calculated the hydrodynamic radius of the dimers of pentamers up to dimers of octamers, which clearly represent the majority of the molecules, to be 11.9–13.5 nm (Fig. 7). This range of hydrodynamic radii was not very sensitive to the assignment of oligomers to s values. This was in excellent agreement with a direct measurement of the hydrodynamic radius by dynamic light scattering, which resulted in a peak at 12.5 nm for the trailing fraction and significant scattering from the larger oligomers contained in the peak fraction (Fig. 7, bottom inset). Although the hydrodynamic radius by itself does not contain information about the precise shape of the molecules, for fundamental reasons at least in one dimension the molecules will measure at least twice the hydrodynamic radius. Therefore, we can conclude that D1D2-Igtp preparation consists of molecules that are at least 24 nm in length. Given that a spike protrudes 10 nm from the surface of a virion (48), we consider that, once engaged by D1D2-Igtp, spikes are impeded from interacting with the target cell membrane. Furthermore, on the surface of an intact and well assembled virion, the distances from the center and edge of one virion spike to an adjacent spike are 22 and 8 nm, respectively (47–50). Thus, our data indicate that a D1D2-Igtp may span multiple spikes on the virion membrane.

DISCUSSION

In the present study, we have demonstrated that by increasing both the size and the valence of sCD4 one can generate a protein that does not enhance virus replication at suboptimal concentrations. This has potentially important implications for therapeutic and vaccine strategies. Unlike coreceptor epitopes on gp120, the CD4 receptor binding site is highly conserved, making it an attractive target for both antiviral therapy and antibody-based vaccines. We consider the enhancing activity of sCD4 on HIV entry to be one of the critical unintended effects of this failed antiviral agent that must be overcome in order to develop maximally effective CD4 binding site-based therapies. Similarly, vaccines targeting gp120 are likely to be more effective if they avoid the elicitation of enhancing antibodies.

The CD4 receptor is thought to extend about 7 nm from the membrane of a lymphocyte, while the extracellular loops of CCR5 lie closer to the cell surface (50). One of the proposed functions of membrane-bound CD4 is to bring the virion into close proximity to CCR5 and thus to the cell membrane so that the fusion process can proceed. Fusion is dependent in part on CD4-induced conformational changes in gp120 (26–29, 45). We reasoned that by generating a molecule of sufficient size, the attachment of that molecule to the surface of a virion would prevent the virion from gaining close proximity to fusion components on the cell surface. In this instance, any conformational changes in gp120 induced by such an agent are unlikely to promote fusion. To this end, we constructed an extremely large immunoglobulin derivative termed D1D2-Igtp composed of, on average, 12 IgG1 heavy chains, each fused to the
two amino-terminal domains of CD4. Viral spikes are estimated to rise 10 nm from the surface of a virion (47, 48). Our data from dynamic light-scattering experiments and sedimentation velocity centrifugation indicate that the hydrodynamic radius of D1D2-Igtp is ~12 nm (diameter = 24 nm). Thus, we believe that once D1D2-Igtp engages a spike, the bulk of D1D2-Igtp will prevent that spike from gaining close proximity to the target cell membrane.

Several multivalent CD4 fusion proteins have been shown to neutralize primary isolates more efficiently than monomeric sCD4 (8–10), but little data on the binding kinetics and potential avidity effects associated with these recombinant proteins are available. In this report, we have investigated the binding kinetics of D1D2-Igtp-gp120 in order to better understand how this protein is able to inhibit entry of primary isolates at relatively low concentrations. When we compared the dissociation of gp120 in solution from either surface-bound sCD4 or surface-bound D1D2-Igtp, we found that gp120 dissociated much more slowly from D1D2-Igtp than from sCD4. Conversely, when we measured the dissociation of soluble D1D2-Igtp from surface-bound gp120, we observed an extremely slow rate of dissociation, suggesting a highly avid interaction between multiple gp120s and a single D1D2-Igtp.

It is important to note that the binding kinetics of D1D2-Igtp for soluble gp120 versus virion-associated gp120 are probably different. Nevertheless, the general trend is likely to hold (i.e. D1D2-Igtp dissociates from virion spikes more slowly than does monomeric sCD4). The critical importance of increasing the avidity of CD4-envelope interactions is underscored by our observation that at the same concentrations of sCD4 that enhance viral replication D1D2-Igtp inhibited viral replication by greater than 90% (Figs. 2 and 3). Depending upon the viral isolate employed, we required concentrations of sCD4 between 300- and 1000-fold greater than D1D2-Igtp to achieve the same level of inhibition (Figs. 2 and 3 and data not shown).

In light of the inefficient inhibitory activity of monomeric sCD4 in neutralizing HIV, these observations underscore the important role that avidity is likely to play in the interaction between virions and clusters of CD4 receptors on target cell membranes in the course of viral attachment and entry. A single D1D2-Igtp may bind more than one of the three gp120s included in a spike. We determined that one D1D2-Igtp is either sufficiently flexible or otherwise folded to accommodate at least 10 gp120s, supporting the possibility that two or even three of the envelopes on a spike could be occupied by different chains of a single D1D2-Igtp. Additionally, because spikes on an intact virion are arranged ~22 nm apart (center to center), a single D1D2-Igtp, with an estimated
diameter of 24 nm, may span multiple spikes (51). Binding of one D1D2-IgGtp to multiple envelopes on a virion, whether within or across spikes, should significantly slow the rate at which it dissociates from that virion. To the extent that spikes are occupied and kept sufficiently distant from the cell membrane, they cannot participate in the fusion process. Thus, we conclude that the size and capacity for multivalent ligation confer upon D1D2-IgGtp two properties that distinguish it from monomeric sCD4; it does not enhance viral replication at sub-optimal concentrations, and it efficiently inhibits replication of primary isolates.

Similar to sCD4, mAbs specific for gp120 can enhance the replication of many primary isolates (20, 31). Additionally, polyclonal sera from infected patients or individuals vaccinated with envelope-based immunogens also enhance HIV-1 replication (31). As with sCD4, this effect is seen as the concentration of the serum or mAb is titered out to very high dilutions. It has been noted elsewhere that this property of gp120-specific antibodies may negatively impact on the effectiveness of anti-envelope humoral responses, both in the context of HIV disease and vaccination (20). We asked whether D1D2-IgGtp would interfere with antibody-mediated enhancement of viral replication. We employed mAb 17b, an antibody that has previously been shown to enhance viral replication. Of note, 17b recognizes gp120 more efficiently in the presence of monomeric sCD4 (26, 45). D1D2-IgGtp eliminated the enhancing effects of 17b. This may have occurred because the size of D1D2-IgGtp limits the access of 17b to the virion or, as has been discussed above, it may have prevented 17b enhancement by keeping virion spikes at a distance from the target cell membrane. In this respect, D1D2-IgGtp illustrates two highly desirable attributes of a potential neutralizing antibody; it dissociates slowly from gp120, and it is large and therefore likely to keep virions separated from the cell membrane.

In summary, we have addressed one of the unintended properties of sCD4 that prevented it from being developed as an effective antiviral agent. Since sCD4 binds to one of the few structures on gp120 that is almost invariably conserved in replication-competent viruses, the CD4 binding epitope on gp120 remains a highly attractive target for both therapeutic strategies and vaccines. We have identified one strategy through which the intrinsic capacity of sCD4 to enhance viral replication can be removed. This information should aid in the design of effective inhibitors of viral entry.

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