Covalent Trimers of the Internal N-terminal Trimeric Coiled-coil of gp41 and Antibodies Directed against Them Are Potent Inhibitors of HIV Envelope-mediated Cell Fusion*

We have engineered two soluble, covalently linked, trimeric polypeptides, N35CCG-N13 and N34CCG comprising only the internal trimeric coiled-coil of the ectodomain of HIV-1 gp41. Both trimers inhibit human immunodeficiency virus, type 1 (HIV-1) envelope (Env)-mediated cell fusion at nanomolar concentrations by targeting the exposed C-terminal region of the gp41 ectodomain in the prehairpin intermediate state. The IC₅₀ values for N35CCG-N13 and N34CCG are ~15 and ~95 nM, respectively, in a quantitative vaccinia virus-based reporter gene assay for HIV-1 Env-mediated cell fusion using Env from the T cell line tropic strain LAV. Polyclonal antibodies were raised against N35CCG-N13 and a tightly binding fraction of anti-N35CCG-N13 inhibits T cell and macrophage tropic HIV-1 Env-mediated cell fusion with respective IC₅₀ values of ~0.5 and ~1.5 μg/ml at 37 °C. The tightly binding anti-N35CCG-N13 antibody fraction targets the exposed internal trimeric coiled-coil in the prehairpin intermediate state of gp41 in a manner analogous to peptides derived from the C region of the gp41 ectodomain. The potency of the tightly binding anti-N35CCG-N13 antibody fraction in the fusion assay is comparable with that of the broadly neutralizing monoclonal antibody 2G12. These results indicate that N35CCG-N13 is a potential anti-HIV therapeutic agent and represents a suitable immunogen for the generation of neutralizing monoclonal antibodies targeted to the internal trimeric coiled-coil of gp41. The data on the tightly binding anti-N35CCG-N13 antibody fraction demonstrate that the internal trimeric coiled-coil of gp41 in the prehairpin intermediate state is accessible to antibodies and that access is not restricted by either antibody size or the presence of a kinetic barrier.

The first step in HIV infection involves virus-cell or cell-cell fusion mediated by the viral envelope glycoproteins (Env) gp41, transmembrane subunit of HIV envelope) and gp120 (surface envelope glycoprotein of HIV) (1). Both proteins therefore present highly attractive targets for the development of antiviral agents as well as broadly neutralizing antibodies. HIV Env-mediated cell fusion involves a complex series of events. gp120 first binds to CD4 and a chemokine receptor; this triggers conformational changes in the gp120-gp41 complex that lead to the insertion of the gp41 fusion peptide into the target membrane and ultimately to cell fusion (2, 3).

The structure of the ectodomain of gp41 (e-gp41) in its fusogenic/post-fusogenic state has been solved by both NMR (4) and crystallography (5–8) and consists of a trimer of hairpins comprising an internal parallel trimeric coiled-coil of N-terminal helices (residues 542–592 of HIV-1 Env (4)) surrounded by antiparallel C-terminal helices (residues 625–663 of HIV-1 Env (4)) (left-hand side of Fig. 1). The formation of the fusogenic/post-fusogenic state of e-gp41 provides the driving force for the apposition of the virus and cell membranes, thereby promoting membrane fusion (2). Prior to the formation of the fusogenic trimer of hairpins, e-gp41 exists in a “prehairpin” intermediate state (2, 9–12), so-called because the C region of e-gp41 (corresponding to the C-helices in the fusogenic/post-fusogenic state) is not yet associated with the internal trimeric coiled-coil of N-helices (Fig. 1, middle). In the prehairpin intermediate state both the internal trimeric coiled-coil (9, 10) and the C region of e-gp41 are accessible to inhibitors (13–15).

There are three classes of fusion inhibitors that target the prehairpin intermediate state of e-gp41 (right-hand side of Fig. 1). Class 1 inhibitors (shown in blue at the top right of Fig. 1) are directed against the internal trimeric coiled-coil of N-helices. Examples of class 1 inhibitors include peptides derived from the C-helices such as C34 (residues 628–661 of HIV-1 Env(5) and T20 (also referred to as DP175, residues 638–673 of HIV-1 Env which extends 10 residues beyond the C-terminal end of the C-helix), both of which have IC₅₀ values in the low nanomolar range (16–18). T20 is currently in the final stages of high pressure liquid chromatography; PBS, phosphate-buffered saline; GnHCl, guanidine hydrochloride; Ni-NTA, nickel-nitrilotriacetic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; M tropic, macrophage tropic; T tropic, T cell line tropic.

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‡ To whom correspondence may be addressed: Laboratory of Chemical Physics and Bioorganic Chemistry, NIDDK, National Institutes of Health, Bethesda, Maryland 20892

§ To whom correspondence may be addressed: Laboratory of Chemical Physics, Bldg. 5, Rm. B1–30I, NIDDK, National Institutes of Health, Bethesda, Maryland 20892

The abbreviations used are: HIV, human immunodeficiency virus; Env, viral envelope glycoproteins; e-gp41, ectodomain of gp41; HPLC,

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phase 3 clinical trials (19, 20). The class 2 inhibitors (shown in red at the bottom right of Fig. 1) are directed against the exposed C region of e-gp41. Examples include engineered proteins such as NCCG-gp41 (13) and IQN17 (21), both of which expose the internal trimeric coiled-coil in a stable manner, and the protein 5-helix, which exposes only a single helix of the internal trimeric coiled-coil (14). NCCG-gp41, IQN17, and 5-helix have IC50 values in the 15–25 nM range.

Finally, the class 3 inhibitors (shown in yellow, middle right of Fig. 1), exemplified by the construct N36 Mut(e,g), which comprises a mutated version of the trimeric coiled-coil that can no longer interact with the C region of e-gp41, act by forming heterotrimers of the N-terminal coiled-coil (22).

The chimeric protein NCCG-gp41 (Fig. 2) comprises an exposed trimeric coiled-coil of N-helices (residues 546–580) with L576C, Q577C, and A578G mutations, N34 spans residues 542–592 and 623–663, respectively, of HIV-1 Env (4). There are three classes of inhibitors (right-hand side of Fig. 1) that target the prehairpin intermediate and prevent its collapse into the trimer of hairpins, thereby rendering it fusion incompetent. Class 1 shown in blue (e.g. C34 (17) and the anti-N35CCG-N13 antibodies described in the present paper) target the exposed trimeric coiled-coil of N-helices; class 2 shown in red (e.g. NCCG-gp41 (13) and N34CCG and N35CCG-N13 described in the present paper) target the C region; and class 3 shown in yellow (e.g. N38Mut(e,g) (22)) interact with the prehairpin intermediate state to form heterotrimers.

EXPERIMENTAL PROCEDURES

Peptides—The C34 peptide (residues 628–661 of HIV-1 Env), purchased from Commonwealth Biotechnologies (Richmond, VA), was synthesized on a solid phase support, purified by reverse-phase high pressure liquid chromatography (HPLC), and verified for purity by mass spectrometry and amino acid analysis. C34 bears an acetyl group at the N terminus and an amide group at the C terminus.

Generation of N34CCG and N35CCG-N13 Constructs—The synthesis and cloning of the chimeric protein NCCG-gp41 (Fig. 2) has been described previously (13). NCCG-gp41 comprises the engineered construct N35CCG-N34-(L6)-C28, where N35CCG spans residues 546–580 of HIV-1 Env with L576C, Q577C, and A578G mutations, N34 spans residues 542–592 and 623–663, respectively, of HIV-1 Env (4). There are three classes of inhibitors (right-hand side of Fig. 1) that target the prehairpin intermediate and prevent its collapse into the trimer of hairpins, thereby rendering it fusion incompetent.
The N34-CCG-gp41 core has been solved crystallographically (7). The model of N34-CCG-gp41 was constructed by grafting N35 onto the N terminus of the crystal structure to generate a contiguous 69-residue helix comprising N35 and N34. The three intermolecular disulfide bridges formed by the two cysteines introduced at positions 576 and 577 are shown in gold, and the three subunits of the trimer are shown in red, blue, and green. The models of N35-CCG-N13 and N34-CCG are directly derived from N34-CCG-gp41. B, sequences of N35-CCG-N13 and N34-CCG (Note that the expressed constructs of N35-CCG-N13 and N34-CCG contain an additional 20-residue N-terminal region with a His6 tag; see “Experimental Procedures.”) The residue numbering is that of HIV-1 Env; the engineered Cys-Cys-Gly at positions 576–578 that have replaced the wild type Leu-Gln-Ala sequence are shown in red; N13 (residues 546–659 of HIV-1 Env) is indicated in purple; the letters α–γ indicate the positions in a helical wheel presentation.

Purification and Protein Folding—The cells were grown at 37 °C in Luria-Bertani medium to an optical density of 0.7, induced with 2 mM isopropyl-β-D-thiogalactoside for 4 h, and harvested by centrifugation. The supernatant was subjected to Ni-NTA-agarose affinity column (bed volume, 10 ml) chromatography at room temperature. The column was washed in buffer A, and bound protein was eluted in the same buffer containing 0.2 M imidazole. The protein was concentrated on a Centriprep YM-3 device (Millipore Corporation, Bedford, MA) and applied at room temperature at a flow rate of 3 ml/min to a Superdex-75 column (HiLoad, 2.6 × 60 cm; Amersham Biosciences) equilibrated in 50 mM Tris-HCl, pH 8, 4 mM GdnHCl, 5 mM EDTA, and 5 mM dithiothreitol. The peak fractions were then subjected to reverse-phase HPLC on POROS 20 R2 resin (Applied Biosystems, Foster City, CA) using a linear gradient of 0–60% acetonitrile/0.05% trifluoroacetic acid. The peak fractions were pooled and stored at −80 °C.

~3.2 mg of C34 peptide (residues 628–661 of HIV-1 Env) was dissolved in 35% acetonitrile, 0.05% trifluoroacetic acid, H2O containing ~2.2 mg of either N34-CCG or N35-CCG-N13 protein at a concentration of 0.3 mg/ml. The polypeptide mixture (N34-CCG + C34 or N35-CCG-N13 + C34), kept in a Slide-A-Lyzer cassette (3.5 molecular weight cutoff; Pierce), was folded by dialysis against 2 liters of 50 mM sodium formate buffer, pH 3.0, for 1.5 h at room temperature. The intermolecular disulfide bonds were then allowed to form by oxidation using the following dialysis scheme: 20 mM sodium phosphate, pH 6.25, for 2 h; 50 mM sodium formate, pH 3.0, for 3 h; 20 mM sodium phosphate, pH 4.25, for 15 h; and finally 50 mM sodium formate, pH 3.0, for 24 h. The N34-CCG/C34 and N35-CCG-N13/C34 complexes were concentrated to ~1 mg and analyzed by SDS-PAGE under nonreducing conditions to verify that N34-CCG and N35-CCG-N13 were predominantly disulfide-linked trimers. The N34-CCG/C34 and N35-CCG-N13/C34 complexes were subsequently denatured in 7.5 M GdnHCl, applied on a Superdex-75 column (2.6 × 60 cm; Amersham Biosciences) and fractionated under denaturing conditions in 4 M GdnHCl, 50 mM sodium formate, pH 4. The peak fractions corresponding to the trimeric, disulfide-linked, N34-CCG or N35-CCG-N13 proteins, stripped of C34, were pooled, dialyzed (3.5 MWCO; Pierce) against excess 50 mM sodium formate buffer, pH 3.0, concentrated (YM-3, Millipore), and stored at 4 °C.

Concentrations of all samples were determined spectrophotometrically; the calculated absorbances at 280 nm (1 cm path length) for a concentration of 1 mg/ml of N34-CCG-gp41, N34-CCG, N35-CCG-N15, and C34 are 2.026, 0.987, 0.786, and 2.90, respectively. The corresponding molecular masses as monomers are 11863, 6011, 7546, and 4286 Da, respectively.
**HIV Fusion Inhibitors and Fusion Inhibitory Antibodies**

**Circular Dichroism**—CD spectra of N34CCG (10 μM) and N35CCG-N13 (8 μM) were recorded in 20 mM sodium formate buffer, pH 3.0, at 25 °C on a JASCO J-720 spectropolarimeter using a 0.05-cm path length cell. Quantitative evaluation of secondary structure from the CD spectrum was carried out using the program CDNN (www.bioinformatik.biochemtech.uni-halle.de/cd_spect/index.html) (26).

**Production of Antibodies and Purification of IgG**—Antibodies to the intermolecular disulfide-linked 6H-N34CCG and 6H-N35CCG-N13 trimers were raised in rabbits using the accelerated protocol services provided by Covance (Covance Research Products, Denver, PA) (27). 125 μg of N34CCG and 200 μg of N35CCG-N13 was used for each initial and subsequent boost injections. Enzyme-linked immunosorbent assays to estimate antibody titers and IgG affinity purifications on immobilized protein A were performed according to established methods (28). An average value of 1.7 absorbance units at 280 nm for 1 mg/ml was used to calculate the concentration of purified antibodies.

**Western Blotting**—Proteins, nonreduced and reduced (in the presence of β-mercaptoethanol), were subjected to SDS-PAGE on precast 10–20% linear gradient Tris-Tricine gels (Invitrogen). The gels were soaked for 10 min and then transferred onto a nitrocellulose membrane (Schleicher & Schuell) in 25 mM Tris buffer, pH 8.0, 190 mM glycine, and 20% methanol using a mini-electrophoretic transfer apparatus (BioRad). The blots were then processed using a WesternBreeze kit, a chromogenic immunogenic system for the detection of rabbit primary antibodies (Invitrogen).

**Purification of Anti-N35CCG-N13-specific Antibodies**—N35CCG-N13 was bound to Ni-NTA-agarose via its N terminus His6 tag as follows. 1.2–1.3 mg of N35CCG-N13 in 15 ml of PBS, pH 6.5, was slowly dispensed into a beaker containing Ni-NTA-agarose (7.5 ml of packed volume) suspended and kept stirred in 15 ml of PBS, pH 6.5, at room temperature. The agarose was packed in a XK-26 column (Amersham Biosciences) and equilibrated in PBS, pH 6.5. 10 ml of protein A affinity chromatography-purified IgG (1.8 mg/ml) derived from rabbit antiserum was used to calculate the concentration of purified antibodies.

**RESULTS AND DISCUSSION**

**Rationale for the Design of N34CCG and N35CCG-N13**—We have previously shown that the chimeric protein NCCG-gp41 (Fig. 2), which comprises an exposed, stable disulfide-linked, trimeric coiled-coil of N-helices grafted in helical phase onto the minimal thermostable core of e-gp41, inhibits HIV-1 Env-mediated cell fusion with an IC50 value of 15–20 nM. The NCCG-gp41 construct is as follows: N35CCG-N34-L6-C28 (see “Experimental Procedures”). The exposed, disulfide-linked, internal trimeric coiled-coil of e-gp41 is formed by N35CCG and the minimal thermostable core of e-gp41 is formed by N34-L6-C28.

From the perspective of a potential therapeutic, the NCCG-gp41 trimmer suffers from being rather large (~35 kDa). In terms of an immunogen, antibodies raised against NCCG-gp41 will be targeted against two distinct epitopes: namely the exposed N-helical trimERIC coiled-coil and the outer surface of the minimal e-gp41 core consisting of the C-helices. Thus, polyclonal antibodies raised against NCCG-gp41 cannot be used to assess whether the internal trimeric coiled-coil represents a suitable target for neutralizing antibodies. We therefore sought to design smaller, covalently linked, soluble constructs consisting solely of the internal trimERIC coiled-coil of e-gp41. To this end we engineered the two constructs N34CCG and N35CCG-N13 (Fig. 2) by introducing stop codons at position Ile13 of the N35 portion and position Ile13* of the subsequent N34 portion, respectively, into the re-engineered 6H-NCCG-gp41 construct having a His6 tag at the N terminus (see “Experimental Procedures”).

**Preparation and Characterization of Disulfide-linked Trimers of the N94CCG and N35CCG-N13 Analogues of the Internal Trimeric Coiled-coil of gp41**—The chimeric protein NCCG-gp41 folds spontaneously into a trimer, which becomes disulfide-linked upon air oxidation. In contrast, the same procedure applied to both N34CCG and N35CCG-N13 yields trimers only to an extent of ~10%. More than 95% yield of stabilized trimmer of N34CCG and N35CCG-N13, however, can readily be obtained in a three-step procedure: N34CCG and N35CCG-N13 are first folded in the presence of C34 peptide (which comprises the C-helix region of the trimer of hairpins in the fusogenic/post-fusogenic state of e-gp41). This yields a trimer of the form (N34CCG/C34)3 (equivalent to the ectodomain core of fusogenic/post-fusogenic gp41) and (N35CCG-N13/C34)3, as evidenced by the elution profile of the complex at pH 3.0 on a Superdex-75

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3 R. Willey and M. A. Martin, personal communication.
column (Fig. 3A, profile shown by the red dashed line for (N34CCG/C34)3). This elution pattern is nearly the same as that for N34CCG-gp41, which only forms trimers (Fig. 3A, profile shown by the black line). Intermolecular disulfide bond formation between the three chains of N34CCG or N35CCG-N13 in the (N34CCG/C34)3 or (N35CCG-N13/C34)3 complexes is then achieved by air oxidation, upon shifting the pH of the solution to 6.25. Finally, the N34CCG and N35CCG-N13 disulfide-linked trimers are stripped from the C34 peptide by denaturation in 7.5 M GnHCl followed by size exclusion column chromatography (in 4 M GnHCl) and reverse-phase HPLC (see “Experimental Procedures”).

Analysis of the purified folded trimeric form of N34CCG on a Superdex-75 column is shown by the red trace in Fig. 3A. SDS-polyacrylamide gel analysis of N34CCG and N35CCG-N13 subsequent to folding by dialysis against 50 mM sodium formate buffer, pH 3, is shown in Fig. 3B. Both N34CCG and N35CCG-N13 are completely trimeric under nonreducing conditions (Fig. 3B, lanes 3 and 5, bands labeled T2 and T3, respectively). Treatment of the samples with a reducing agent prior to electrophoresis clearly results in both N34CCG and N35CCG migrating to the position of a monomer (Fig. 3B, lanes 4 and 6, labeled M2 and M3, respectively).

**Characterization of disulfide-linked trimeric N34CCG and N35CCG-N13 by CD spectroscopy.** CD spectra of the trimeric forms of N34CCG (10 μM) and N35CCG-N13 (8 μM) were recorded in 20 mM sodium formate buffer, pH 3, at ambient temperature.

**CD Spectra of Disulfide-linked Trimeric N34CCG and N35CCG-N13—**CD spectra of disulfide-linked trimeric N34CCG and N35CCG-N13 are shown in Fig. 4. Both spectra display a double minimum at 208 and 222 nm, indicative of the presence of α-helix. Quantitative analysis of the spectra using the neural network program CDNN (26) yields an overall helical content of 43.0 ± 1.5% for N34CCG and 51.4 ± 0.9% for N35CCG-N13. These values, however, also reflect the presence of the 20-residue His6 tag at the N terminus that is expected to be random coil. Thus, the number of helical residues/subunit present in trimeric N34CCG and N35CCG-N13 is 23.7 ± 0.8 and 35.5 ± 0.6, respectively. The difference in the number of helical residues between N34CCG and N35CCG-N13 reflects the 14-residue extension of the trimeric coiled-coil in N35CCG-N13. Assuming that the His6 tag is random coil, these values yield percentage helicities within the e-gp41-derived regions of the N34CCG (34 residues) and N35CCG-N13 (48 residues) constructs of 69.7 ± 2.4% for N34CCG and 74.0 ± 1.3% for N35CCG-N13.

The CD data are therefore consistent with the models of N34CCG and N35CCG-N13 displayed in Fig. 2A. The models, however, in Fig. 2A are depicted as fully helical. In reality, it is clear from the CD data that the N-terminal 9–10 residues of the e-gp41 derived regions of both constructs are likely to be frayed, and in the case of N35CCG-N13, possibly the last 1–2 C-terminal residues as well.

N34CCG and N35CCG-N13 Are Potent Inhibitors of HIV Env-mediated Cell Fusion—N34CCG and N35CCG-N13 were tested in a quantitative vaccinia virus-based reporter gene assay to determine their effect on HIV-1 Env-mediated cell fusion (using Env from the Tropic HIV-1 strain LAV). The results are shown in Fig. 5. The IC50 values for N34CCG and N35CCG-N13 are 96 ± 7 and 15.5 ± 1.3 nM, respectively. Also shown for comparison in Fig. 5 is the inhibition curve for NCCG-gp41, which has an IC50 value of 19.3 ± 1.4 nM, consistent with previous data using the same fusion assay (13). (For reference, the IC50 for the C34 peptide, a potent class 1 inhibitor, under the same assay conditions is 2.3 ± 0.5 nM.) Thus, one can conclude that N35CCG-N13 is equipotent with NCCG-gp41, and the presence of the additional N13 segment coupled with the intermolecular disulfide bridge is sufficient to stabilize the appropriate region of the trimeric coiled-coil of N-helices in N35CCG-N13. The 5–6-fold lower inhibitory activity of N34CCG relative to both N35CCG-N13 and NCCG-gp41 is presumably due to its slightly lower helical content, as a consequence of fraying at the N terminus.

**Polyclonal Antibodies Specific to the Internal Trimeric Coiled-coil of gp41—**Polyclonal antibodies to N34CCG and N35CCG-N13 were raised in rabbits according to a standard protocol (27). The estimated 50% titers (i.e. the dilution at
which 50% of antigen is bound using a concentration of 1 μg/ml of antigen) for antibodies directed against N34CCG and N35CCG-N13 were 2.8 × 10^4 and 1.0 × 10^5, respectively, after 3–4 months. Sera from rabbits immunized with either N34CCG or N35CCG-N13 reacted strongly with disulfide-linked NCCGgp41, N34CCG, N35CCG-N13 trimers on Western blots (data not shown), indicating that anti-N34CCG and anti-N35CCG-N13 antibodies recognize the trimeric coiled-coil of N-helices. Immunoblots using purified anti-N35CCG-N13 antibodies are discussed below and displayed in Fig. 7B. In contrast, the native N36 peptide (residues 546–581 of HIV-1 Env) reacts very poorly with the polyclonal antibodies (data not shown). N36, in the absence of C34, aggregates and does not form a unique species in solution (21, 22). This provides further evidence that the anti-N34CCG and anti-N35CCG-N13 antibodies are targeted mainly to the folded trimeric coiled-coil of N-helices.

Because the 50% titer for the anti-N35CCG-N13 serum was 3–4-fold higher than that for the anti-N34CCG serum, we chose to focus further purification on the anti-N35CCG-N13 antibodies. The scheme employed is shown in Fig. 6 (see “Experimental Procedures” for details). Total IgG was first purified by protein A affinity chromatography (28). Fractions of the total IgG specific to the internal trimeric coiled-coil were then obtained by affinity chromatography using a N35CCG-N13 immobilized NiNTA-agarose column. After elution of the unbound fraction (which comprises 90–95% of the total protein A purified IgG) and extensive washing with PBS, the IgG fraction bound to the immobilized N35CCG-N13 was eluted at low pH (pH 3.0). At pH 3.0, elution of N35CCG-N13 is also expected to occur because of protonation of the histidine residues in the His6 tag, which therefore no longer bind nickel ions. Upon raising the pH of the peak fractions to 8.5, co-precipitation of a pool of tight binding anti-N35CCG-N13-specific IgG and N35CCG-N13 takes place, because of the poor solubility of N35CCG-N13 at high pH. The distribution of IgG in the co-precipitate (pellet) and soluble (supernatant) fractions is approximately equal. The protein in the pellet fraction (harvested by centrifugation) was solubilized and denatured in 6 M GnHCl (33), passed through a column containing an excess of fresh NiNTA-agarose to completely remove any co-precipitated N35CCG-N13, and refolded. IgG in the supernatant fraction was purified in exactly the same manner as the pellet fraction. The resulting purified tight (from the pellet) and weak (from the supernatant) anti-N35CCG-N13-specific antibodies contain no residual N35CCG-N13 as judged by SDS-PAGE (Fig. 7A) and mass spectrometry and react specifically with trimeric NCCGgp41, N34CCG, and N35CCG-N13 proteins observed by Western immunoblotting analysis (Fig. 7B). The absence of contaminating N35CCG-N13 in the purified tight binding anti-N35CCG-N13-specific IgG fraction was further confirmed by immunoblot analysis of the tight binding anti-N35CCG-N13-specific IgG probed against itself (Fig. 7C).

Effect of Purified Anti-N35CCG-N13-specific Antibodies on HIV-1 Env-mediated Cell Fusion—At 37 °C, serum from rabbits immunized against N35CCG-N13 has no effect on HIV-1 Env mediated cell fusion in a vaccinia-virus based reporter gene assay. The purified tight binding anti-N35CCG-N13-specific IgG, however, inhibits fusion completely at concentrations as low as 10 μg/ml; the weak binding anti-N35CCG-N13-specific IgG inhibits fusion by ~90% at ~100 μg/ml. As a control, both protein A-purified preimmune IgG and preimmune IgG purified in exactly the same manner as that used for the anti-
N35CCG-N13-specific antibodies (i.e. including a GnHCl denatured and refolded IgG) react with purified denatured/refolded tight binding anti-N35CCG-N13-specific IgG; green solid squares, weak binding anti-N35CCG-N13-specific IgG; blue solid diamonds, 2G12 monoclonal antibody directed against gp120; black open circles, preimmune IgG. The solid lines represent best fits to the data using the simple activity relationship: percentage of fusion = 100(1 + [I]/IC50), where [I] is the inhibitor concentration. The vertical bars indicate standard deviations for the experimental data points. The calculated IC50 values for strong binding anti-N35CCG-N13, weak binding anti-N35CCG-N13, and 2G12 are 0.42 ± 0.04 μg/ml, 21.2 ± 1.9 μg/ml, and 1.1 ± 0.2 μg/ml, respectively. The broadly neutralizing monoclonal antibody 2G12 is directed against an Asn-linked glycosylation site on gp120 (36, 37).

2G12, a broadly neutralizing monoclonal antibody directed against gp120 (23, 24), using Env from a Tropic strain of HIV-1 (LAV) is shown in Fig. 8. The apparent IC50 values for the tight and weak binding anti-N35CCG-N13-specific antibodies are −0.4 and −20 μg/ml, respectively, compared with −1.5 μg/ml for 2G12. Cell fusion assays using an M Tropic strain of HIV-1 were also carried out for the tight binding anti-N35CCG-N13-specific antibodies and 2G12; inhibition of M Tropic Env-mediated cell fusion by both antibodies is highly effective, although the IC50 values are increased about 4-fold relative to those obtained with Tropic Env (Table I). These results therefore allow one to conclude that the tight binding anti-N35CCG-N13-specific antibodies are highly potent inhibitors of HIV-1 fusion and are comparable in potency to the monoclonal antibody 2G12, which has just entered phase I clinical trials (25).

It is worth noting that antibodies raised against the native N36 peptide, which aggregates and does not form a helical trimeric, dimeric, and monomeric N35CCG-N13 protein, respectively. Densitometric scanning (National Institutes of Health Image version 1.6.1; rsh.info.nih.gov/nih-image) of the two N35CCG-N13 lanes (lanes 4 and 8) indicates that the monomer M3 band represents ~3.3% of the total (T3+D3+M3) intensity, which corresponds to a protein quantity of ~65 ng. Western blot analysis of the N35CCG-N13 and N35CCG-N13 proteins reacted with purified denatured/refolded tight binding anti-N35CCG-N13-specific IgG (i.e. antibody shown in lanes 3 and 7 of panel A) at a 1000-fold dilution from a 3.7 mg/ml stock solution. T1, T2, and T3 denote the trimeric forms of N35CCG, N34CCG, and N35CCG-N13 proteins, respectively. C, a Western blot of overloaded purified tight binding anti-N35CCG-N13-specific IgG (3 μg) probed against itself does not reveal the presence of any N35CCG-N13 left-hand lane. Control bands (T3) containing 50 and 10 ng of trimeric N35CCG-N13 protein are also shown in the middle and right lanes, respectively. One can therefore conclude that the upper limit for any possible contaminating N35CCG-N13 in the purified tight binding anti-N35CCG-N13-specific antibody preparation is less than 0.5%. The samples were processed for SDS-PAGE as described in the Fig. 3 legend.

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HIV Viral Fusion Inhibitors

**TABLE 1**

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**REFERENCES**