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Trimeric Membrane-anchored gp41 Inhibits HIV Membrane Fusion*

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The HIV-1 envelope glycoprotein is composed of a receptor binding subunit, gp120 that is non-covalently linked to the membrane-anchored fusion protein, gp41. Triggered by cellular receptor binding, the trimeric envelope complex mediates the fusion of viral and cellular membranes through the rearrangement of the fusion protein subunit into a six-helical bundle core structure. Here we describe the biophysical and functional properties of a membrane-anchored fragment of gp41 (gp41ctm) that includes the complete C-terminal heptad repeat region 2, the connecting part, and the transmembrane region. We show that the transmembrane domain of the envelope glycoprotein is sufficient for trimerization *in vitro*, contributing most of the α-helical content of gp41ctm. Trimeric gp41ctm is protease-resistant and recognizes neutralizing antibodies 2F5 and 4E10. However, gp41ctm and gp41ctm proteoliposomes elicit no clear neutralizing immune responses in preliminary mouse studies. We further show that gp41ctm and surprisingly also gp41ctm proteoliposomes have potent anti-viral activity. Our data suggest that liposome-anchored gp41ctm exerts its inhibitory action outside of the initial fusion contact site, and its implications for the fusion reaction are discussed.

The human immunodeficiency virus, type 1 (HIV-1)[1] envelope (env) glycoprotein gp160 is synthesized as a precursor that is post-translationally cleaved into the receptor binding subunit gp120 and the membrane-anchored fusion protein gp41, which associate into non-covalently linked trimers in the viral membrane. Trimeric env interacts sequentially with cellular receptors such as CD4 and chemokine co-receptors CXCR4 or CCR5 that trigger a cascade of conformational changes in gp120 and the gp41 fusion subunit (1–3). This leads to the exposure of the N-terminal gp41 fusion peptide sequence and its interaction with the target membrane, thus creating a prehairpin structure that may bridge both viral and cellular membranes (4, 5). Subsequent refolding of both gp41 heptad repeat regions, HR1 (N-terminal) and HR2 (C-terminal), into a six-helical bundle structure is thought to pull viral and cellular membranes into close proximity, thus leading to membrane fusion (4, 6–8). The gp41 prehairpin structure is the target for inhibitory peptides such as T-20 that prevent gp41 refolding into the six-helical bundle structure (9–18). Such a mechanism is further supported by escape mutations that map to the N-terminal triple-stranded coiled-coil region (19, 20). Additional evidence suggests that co-receptor usage influences sensitivity to T-20 inhibition (21–23). The transition of gp41 into the six-helical bundle most probably drives membrane fusion (24, 25), although other studies implicate the six-helical bundle directly in the fusion reaction (26, 27).

The C-terminal membrane proximal part of gp41 is involved in the fusion reaction (28) and contains epitopes for neutralizing antibodies 2F5, 4E10, and Z13 (29–33). The efficiency of mAb 2F5 in the prevention of infection and the reduction of viral load has been demonstrated in a number of studies (34–36), and structural studies reveal a rather extended conformation of its epitope (37, 38). Although the epitopes recognized by mAbs 2F5 and 4E10 have been mapped to linear sequences (29, 32), the peptides or constraint peptides thereof failed to induce a mAb 2F5-like immune response (39–42), indicating additional components of env in mAb 2F5-mediated neutralization. It has been suggested that mAb 2F5 binds to native gp160 and that CD4 receptor binding reduces mAb 2F5 interaction (43). In contrast, other studies indicate that mAb 2F5 targets the gp41 fusion intermediate prehairpin structure (44, 45). The latter scenario is also supported by mutations that destabilize the six-helical bundle formation and enhance mAb 2F5-neutralization activity (46). Due to the broadly neutralizing activity of mAb 2F5, it is of considerable interest to understand the antigenic properties and eventually stabilize them in a model antigen (37–39, 42) that are then capable of inducing a 2F5-like immune response.

Here we report the structural and functional characterization of a gp41 construct comprising the env transmembrane domain and the extracellular C-terminal region including the peptide regions that have been shown to exert fusion inhibition (C34, T-20). We show that the env transmembrane domain constitutes the trimerization domain. Trimeric gp41ctm is protease-resistant and is recognized by mAbs 2F5 and 4E10. gp41ctm exerts potent anti-viral activity in solution and surprisingly when incorporated into liposomes. However, initial immunization studies in mice indicate low immunogenicity of gp41ctm in solution, whereas gp41ctm incorporated into liposomes generates IgG and IgA responses, which have no clear neutralization capacity.

EXPERIMENTAL PROCEDURES

Expression Constructs—HIV-1 (strain HXB2R) gp41 cDNA (nucleic acid position 1885–2118; gp41 residues 118–195) was amplified by

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[1] The abbreviations used are: HIV, human immunodeficiency virus; gp, glycoprotein; env, envelope; HR, heptad repeat region; TM, transmembrane region; mAb, monoclonal antibody; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; β-OG, β-octylglucopyranoside; PBS, phosphate-buffered saline; EGS, ethylene glycol bis(-succinimidylsuccinate); ELISA, enzyme-linked immunosorbent assay.

standard PCR and cloned into the expression vector pRSET (Invitrogen). DNA sequencing revealed the C-terminal addition of 18 amino acids (APRALEIILFNFKKEIYI) encoded by the vector sequence (gp41ctm). HIV-1 gp41 cDNA (nucleotides 1605–1743; gp41 residues 24–70) (gp41-N-FLAG; N-terminal helix) were amplified by PCR with the addition of a C-terminal FLAG tag and cloned into a pRSET expression vector. The sequence was confirmed by DNA sequencing.

Protein Purification—gp41ctm and gp41-N-FLAG were expressed in *Escherichia coli* host strain BL21(DE3) pUBS. The expression of the proteins was induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 3 h at 37 °C. gp41ctm-expressing cells were lysed by sonication in buffer A (50 mM Tris, pH 8, 100 mM NaCl, 1% CHAPS). The cleared supernatant was loaded onto a Q-Sepharose column in buffer A, and gp41ctm was eluted by applying a 0.1–1 M NaCl gradient. gp41ctm-containing fractions were pooled and dialyzed against H_2O, subsequently adjusted to buffer A, and subjected to one more Q-Sepharose purification step. Final purification included separation on a Superdex 200 column (Amersham Biosciences) in buffer B (20 mM Hepes, pH 8, 0.1 M NaCl, 1% β-octylglucopyranoside (OG)). Lysates from cells expressing gp41-N-FLAG were loaded onto a S-Sepharose column in 50 mM Tris, pH 8, 0.1 M NaCl and eluted by applying a 0.1–1 M NaCl gradient. Fractions containing gp41-N-FLAG were identified by SDS-PAGE und used for pull-down experiments.

CD Analysis—The CD spectrum of gp41ctm (0.16 mg/ml) in 10 mM phosphate, pH 7.2, 50 mM NaCl was recorded at 20 °C using a 1-mm cell on a Jasco J-810 spectropolarimeter equipped with a thermoelectric controller. The thermodynamic stability was measured at 222 nm by monitoring the CD signal in the temperature range of 4–98 °C. The measured signal was then converted into molar ellipticity.

Chemical Cross-linking—gp41ctm (PBS, 1% β-OG) and gp41ctm incorporated into liposomes (PBS) were incubated with ethylene glycol bis(-succinimidylsuccinate) (EGS, Pierce) for 20 min at room temperature as indicated. The reaction was quenched by the addition of 50 mM Tris, pH 8.0, for 5 min. Samples were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions, and the bands were visualized by Coomassie Brilliant Blue staining.

Liposome Preparation and Flotation Assay—gp41ctm proteoliposomes (25% L-α-phosphatidylcholine, 50% L-α-phosphatidylserine, 25% cholesterol) were prepared as described previously (47) with minor modifications. Lipid films were resuspended with gp41ctm in buffer A, and the detergent was removed by dialysis with a liposomat (Dianorm). The proteoliposomes were adjusted to 50% sucrose (in PBS), overlaid with 40, 30, 10, and 0% sucrose (in PBS), and centrifuged in a SW 41 rotor at 40,000 rpm for 12 h.

After incubation (15 min at room temperature) of gp41ctm proteoliposomes with either mAb 2F5 or 4E10, similar sucrose gradient centrifugation analyses were applied to separate free mAb and gp41ctm/mAb proteoliposomes. Samples of fractions containing gp41ctm/mAb proteoliposomes were separated on 12% SDS-PAGE and stained with Coomassie Brilliant Blue.

For the immunization studies, the liposomes were produced by the cross-flow injection technique using a multiple injection mode (48, 49). The lipid mixture was dissolved in 95% ethanol at 50 °C, and the temperature of the protein/detergent solution was equilibrated at 50 °C at a β-OG concentration of 1%. For vesicle formation, the protein/detergent solution was pumped from container A to container B, and while pumping the micellar protein solution through the cross-flow injection module, the ethanol/lipid solution was injected followed by immediate dilution with PBS in vessel B. After dilution, the β-OG concentration was reduced to 0.1% resulting in a stable liposome solution. The successful incorporation of gp41ctm was confirmed by Western blot and fluorescence-activated cell sorter analysis using mAb 2F5.

Pull-down Assay—gp41-N-FLAG and gp41ctm were incubated for 30 min at room temperature in buffer B, diluted 1:2 in Tris-buffered saline, 1% Triton-100 and loaded onto a FLAG-agarose column, washed extensively with Tris-buffered saline, 1% Triton-100, and eluted with 0.1 M glycine, pH 3.5. Proteins were analyzed by SDS-PAGE and visualized by Coomassie Brilliant Blue staining.

HIV Neutralization Assay—TZM-b1 cells, a HeLa cell line derivative expressing CD4, CXCR4, and CCR5, and firefly luciferase upon infection with HIV were seeded at a density of 3×10^3 cells/96-well plate (50). The next day cells were pretreated with T-20, gp41ctm, and liposomes with and without incorporated gp41ctm at different concentrations for 30 min at room temperature. 1000 infectious units/well (as determined on TZM-b1 cells) of $HIV_{NL\text{-}4.3}$ were used to challenge the cells, and 2 days later the cells were lysed and the activity of firefly luciferase activity was determined (Steady-Glo luciferase system, Promega). Because of the induction of firefly luciferase upon infection, the reduction of the relative light units detectable correlates with the inhibition of infection by T-20, gp41ctm, and liposomes with and without incorporated gp41ctm, respectively. The viability of the cells was not affected by the addition of gp41ctm or gp41ctm liposomes. The same assay and HIV strain were also used to measure the neutralization capacity of the sera obtained by gp41ctm immunization of mice. The sera were diluted 1:10, 1:25, 1:50, and 1:100.

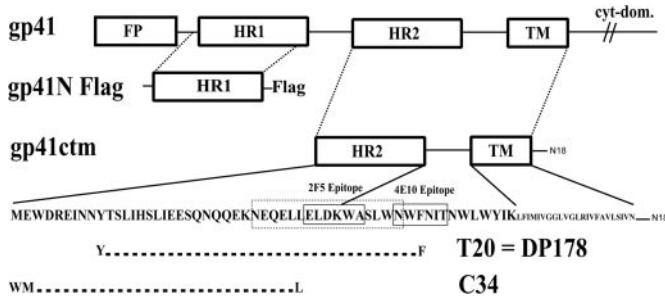

FIG. 1. **Schematic drawing of gp41 and gp41ctm.** The N-terminal helical region is designated as HR1, and the C-terminal helical region is designated as HR2. gp41-N-FLAG is indicated. The sequences of inhibitory peptides C34 and T-20 and of 2F5 and 4E10 antibody epitopes are marked. For the mAb 2F5, the core epitope (*solid line*) and the extended epitope (*dashed line*) are shown. *FP*, fusion peptide.

Immunization—BALB/c inbred female mice 6–8 weeks of age were immunized intraperitoneally (intraperitoneal). Six mice per group were primed with gp41ctm or with gp41ctm proteoliposomes. The second immunization was performed 3 weeks later using the same preparations. Control groups were immunized with an empty liposome preparation utilizing the same immunization procedures. Two weeks following the boost, mice were bled from *Sinus orbitalis* and sera were stored at −20 °C before further analyses.

Enzyme-linked Immunosorbent Assay (ELISA)—An ELISA protocol was performed as described earlier (31) utilizing gp41ctm protein (2.5 μg/ml carbonate buffer, pH 9.6) as coating antigen. Serial dilutions of sera in PBS/Tween 20 containing 1% skim milk were added to the coated plates, and the mixtures were incubated for 1.5 h at room temperature. Bound antibodies were detected with goat anti-mouse IgG1 and IgG2a (γ-chain-specific) conjugated with horseradish peroxidase (Zymed Laboratories Inc.). Following additional washing steps, plates were stained with 3,3′,5.5′-tetramethylbenzidine as substrate. The reaction was stopped with 1.25 M H_2SO_4, and the plates were measured (wavelength 450 nm). The cutoff value is defined as the mean value of absorption of serum samples of mice immunized with empty liposome preparations plus two standard deviations.

Syncytium Inhibition Assay—The inhibition of HIV-1 replication by serum samples was assessed in a standard syncytium inhibition assay. Experiments were performed with sera from mice immunized with gp41ctm or gp41ctm-containing liposomes. Sera from mice, which had received liposomes only, were run in parallel as negative controls. AA-2 cells were used as the indicator cell line with syncytium formation as readout. 10 serial 2-fold dilutions of serum samples in cell culture medium (RPMI 1640 medium, 10% fetal calf serum, 4 mM L-Gln, 5 μg/ml polybrene) were preincubated with 10^2-10^3 $TCID_{50}$ of tissue culture line adapted, HIV-1 RF, for 1 h at 37 °C before the addition of the AA-2 cells. Cells were incubated for 5 days before the assessment of syncytium formation. Experiments were performed with four replicates per dilution step. The presence of at least one syncytium per well was considered as an indication for HIV-1 infection. The 50% inhibiting titer was calculated according to the method of Reed and Muench (51). Unspecific inhibition by control sera was used as cutoff. All of the assays included a virus titration of the inoculum to confirm the infectious titer.

RESULTS

Oligomerization and Protease Sensitivity of gp41ctm—Initial attempts to express gp41 residues 118–195 comprising the C-terminal helical region, the connection to the transmembrane region, and the gp41 transmembrane region (Fig. 1) failed because of bacterial cytotoxicity. However, we were able to select a clone that has an extended 3′-open reading frame derived from the plasmid sequence that includes 18 residues and allows bacterial expression. gp41ctm can be purified to homogeneity and is monodisperse in solutions containing 1%

detergent (β-OG or CHAPS). It forms soluble aggregates in PBS or H_2O (data not shown) but elutes from a gel filtration column at ~14 ml in PBS containing 1% CHAPS or 1 β-OG (data not shown), indicating a distinct oligomeric state. The elution volume may also indicate an extended conformation of gp41ctm, because a 50-kDa marker protein (antibody Fc fragment) elutes at ~15 ml and a 220-kDa protein (catalase) elutes at 12 ml under the same conditions (data not shown). Chemical cross-linking of gp41ctm in detergent (Fig. 2*A*) and of gp41ctm incorporated into liposomes (Fig. 2*B*) with increasing concentrations of EGS cross-linking reagent gives rise to a second band migrating at ~30 kDa and a third band migrating at ~45 kDa in both experiments (Fig. 2). This is consistent with the trimeric nature of the envelope glycoprotein (52–56) and indicates that the transmembrane region is a potential minimal trimerization domain of HIV-1 env gp160.

To test the presence of structural features, we subjected gp41ctm alone or incorporated into liposomes to protease digestion. This shows cleavage of gp41ctm at high protease concentrations (1:100 w/w) resulting in two smaller tryptic bands migrating at ~9 and ~4 kDa as well as two slightly smaller chymotryptic fragments migrating at ~8 and ~3 kDa (Fig. 2*C*). In contrast, gp41ctm incorporated into liposomes was not cleaved under the same conditions, thus indicating increased resistance (Fig. 2*D*).

CD Analysis of gp41ctm—Secondary structure analysis of gp41ctm by circular dichroism revealed α-helical spectra with an approximate calculated helical content of ~30%. This is independent of the buffer conditions, because the same spectra were obtained in buffers containing either H_2O or PBS or PBS and 1% β-OG (Fig. 3*A*). The thermal stability of gp41ctm is indistinguishable in PBS and in PBS containing 1% β-OG and shows somewhat greater stability in H_2O. The unfolding transition of gp41ctm occurs at ~48 °C in PBS buffers and at ~52 °C in H_2O (Fig. 3*B*).

gp41ctm Binding to the gp41 N-terminal Helix—gp41ctm contains the C-terminal helical region (HR2), which interacts with the N-terminal triple-stranded coiled-coil region (HR1) in the gp41 core structure (Fig. 1) (4, 6). To show that the C-terminal helical region of gp41ctm is available for binding to gp41 HR1, we performed a pull-down assay with FLAG-tagged gp41-N-FLAG. As expected gp41-N-FLAG bound to the column and could be eluted (*lane 1*), whereas gp41ctm did not bind (*lane 2*). When we preincubated gp41-N-FLAG and gp41ctm prior to column binding, the complex composed of both fragments was eluted, indicating that HR2 from gp41ctm interacts with HR1 of gp41-N-FLAG (Fig. 4*A*, *lane 3*).

gp41ctm Interaction with mAbs 2F5 and 4E10—gp41ctm contains two membrane proximal epitope sequences (Fig. 1) that are recognized by broadly neutralizing human antibodies, mAb 2F5 and 4E10. Therefore we analyzed whether these mAbs recognize trimeric gp41ctm in a membrane environment. gp41ctm was incorporated into liposomes and incubated with mAbs, and free mAbs were separated by sucrose density gradient centrifugation. This revealed that both mAbs 2F5 and 4E10 bound to gp41ctm and were found in the upper fraction of the gradient (Fig. 4*B*) similar to the floatation of gp41ctm liposomes alone (Fig. 4*B*), whereas antibodies incubated with empty liposomes remain in the loading zone of the gradient (data not shown). This indicates that gp41ctm can be incorporated into the liposomes with the extracellular region being accessible for mAb 2F5 and 4E10 interaction.

Immunogenicity of gp41ctm—Here we have shown that mAbs 2F5 and 4E10 interact with membrane-anchored gp41ctm. Although interaction of these antibodies can be mapped to linear gp41 sequences (Fig. 1), the nature of the antigen, which is capable of inducing such an immune response, remains elusive so far. Thus we tested the immunogenicity of trimeric gp41 in solution and when incorporated into

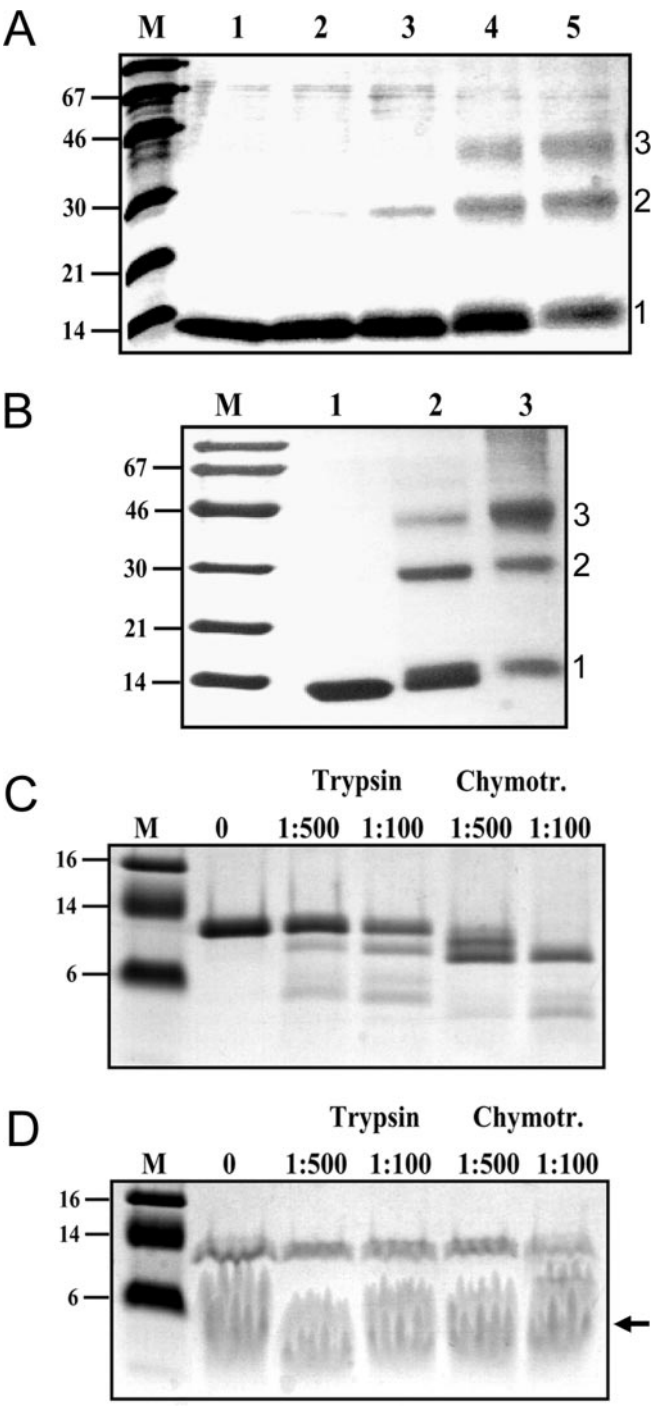

FIG. 2. **Oligomerization and stability of gp41ctm in solution and incorporated into liposomes.** *A*, soluble gp41ctm (PBS, 1% β-OG) was incubated with increasing concentration of EGS (*lane 1*, no EGS; *lane 2*, 0.1 mM; *lane 3*, 0.25 mM; *lane 3*, 0.5 mM; *lane 4*, 1 mM; *lane 5*, 5 mM). *B*, sucrose gradient-purified gp41ctm liposomes were incubated with EGS (*lane 1*, no EGS; *lane 2*, 1 mM; *lane 3*, 5 mM). The samples were separated on a reducing SDS-PAGE followed by Coomassie Brilliant Blue staining. gp41ctm monomers and putative dimers and trimers are indicated by *numbers*. *C* and *D*, protease digestion of gp41ctm (*C*) and of gp41ctm incorporated into liposomes (*D*). Trypsin and chymotrypsin were used at the concentrations (w/w) indicated. Samples were analyzed by SDS-PAGE followed by Coomassie Brilliant Blue staining. Note that gp41ctm incorporated into liposomes is resistant to trypsin and chymotrypsin digestion. The *fuzzy broad band* (*arrow*) in *D* migrating at ~6 kDa corresponds to lipids. *M*, molecular weight standards.

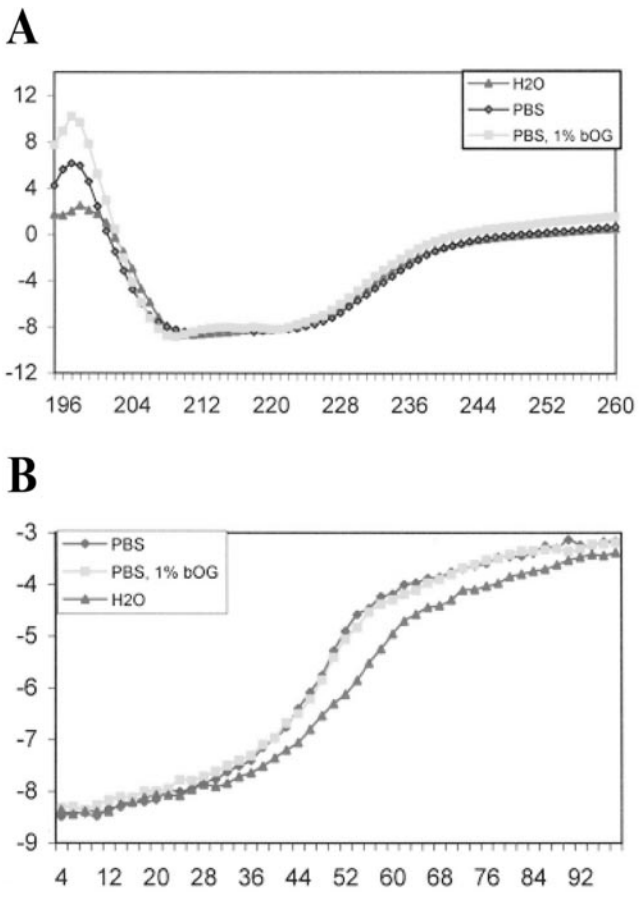

FIG. 3. **Circular dichroism spectra of gp41ctm.** *A*, the two minima at 208 and 222 nm are characteristic for α-helical content. Spectra were measured in PBS, PBS + 1% β-OG, and H_2O. *B*, thermal denaturation curves of gp41ctm recorded at 222 nm in the same buffers as used in *A*. The measured ellipticity was converted to molar ellipticity.

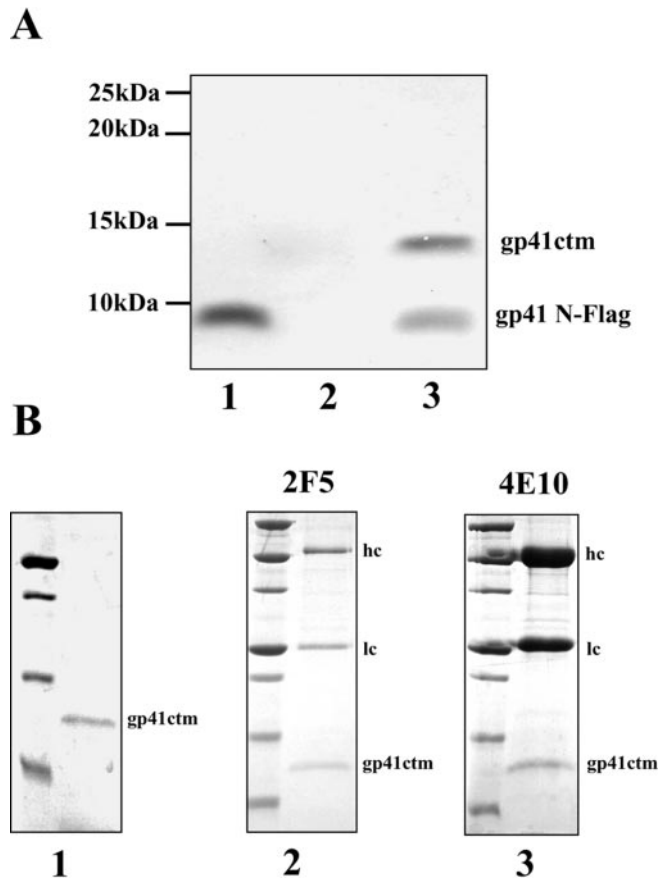

FIG. 4. **gp41ctm binds gp41-N-FLAG and mAbs 2F5 and 4E10.** *A*, gp41ctm pull down of gp41-N-FLAG. gp41-N-FLAG, gp41ctm, and potential complexes formed by gp41ctm and gp41-N-FLAG were passed over a anti-FLAG column and washed, and bound protein was eluted by low pH treatment. This shows elution of gp41-N-FLAG (*lane 1*), no binding of gp41ctm (*lane 2*), and elution of a complex formed by gp41ctm and gp41-N-FLAG (*lane 3*). Samples were separated on SDS-PAGE, and bands were visualized with Coomassie Brilliant Blue. *B*, gp41ctm containing proteoliposomes were purified over a sucrose gradient (*panel 1*, gp41ctm) and then incubated with either mAb 2F5 or 4E10 and subjected to a further round of sucrose gradient purification to remove unbound mAbs. The upper fraction of the gradient contains both gp41ctm and either mAb 2F5 (*panel 2*) or 4E10 (*panel 3*). *lc*, antibody light chain; *hc*, antibody heavy chain. Samples were analyzed using SDS-PAGE followed by Coomassie Brilliant Blue staining. Only the sample from the top of the gradient is shown in *panels 1–3*.

liposomes. Mice were immunized intraperitoneally (intraperitoneal) with gp41ctm liposomes or with gp41ctm alone or as a control with empty liposomes. After two rounds of immunization, the mice were bled and the reactivity of the sera against gp41ctm was tested using a standard ELISA protocol (Fig. 5). All of the mice immunized with gp41ctm liposomes showed a IgG1 and IgG2a-specific immune response against gp41ctm up to a reciprocal titer of 2560. In contrast, only 3 of 5 mice immunized with soluble gp41ctm showed a weak response with reciprocal titers between 250 and 640 (Fig. 5). Although low reciprocal titers for IgA (~150) could be detected in 2 of 5 mice immunized with gp41ctm liposomes, no IgA response could be determined in the gp41ctm group (Fig. 5). This indicates that gp41ctm has poor antigenicity by itself. However, when incorporated into liposomes, gp41ctm showed antigenicity after only two rounds of immunization.

The sera were then tested for their neutralizing activity using two different assays, a syncytium inhibition assay and a single round infection assay as described above. In both cases, sera from the control group (liposomes only) showed considerable unspecific inhibition of virus replication, which in addition varied among the different mice. Sera derived from mice immunized with either gp41ctm or gp41ctm incorporated into liposomes showed some variation in comparison to the negative control; however, no clear significant neutralization activity could be observed with either assay (data not shown), indicating that gp41ctm might not expose the 2F5 or 4E10 epitopes properly.

Antiviral Activity of gp41ctm—gp41ctm contains peptide sequences that had been previously shown to exert potent antiviral activity such as C34 and T-20 (Fig. 1). These C-terminal peptides are thought to interact with the N-terminal coiled-coil region of gp41, thus blocking the refolding of gp41 into the six-helical bundle structure (2, 3, 5, 18). Therefore it was of interest to analyze whether trimeric gp41ctm in solution or when incorporated into liposomes had a similar inhibitory effect. Using a sensitive single round infection assay that measures infection based on the stimulation of luciferase activity (50), we found that soluble gp41ctm (in PBS buffer) has a strong inhibitory effect with an IC_{50} of 0.4 μg/ml (34 nM), which is comparable with an IC_{50} of 0.2 $\pm$ 0.02 μg/ml (45 $\pm$ 4.5 nM) calculated for T-20 using this assay (50). Surprisingly, gp41ctm incorporated into liposomes also had a strong inhibitory effect on HIV-1 entry with an IC_{50} of 0.15 μg/ml (12 nM). Empty liposomes tested as a control showed no inhibitory activity (Fig. 6). In contrast, a high concentration of empty liposomes enhanced the efficiency of the HIV infection in this assay, but the mechanism of this effect is unclear. Together, these data indicate that soluble gp41ctm or membrane-anchored gp41ctm has a potent anti-viral activity, which is similar to therapeutic fusion inhibitors such as T-20 (18).

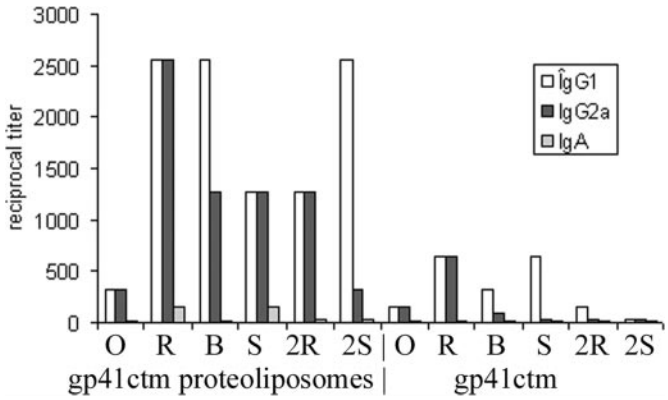

FIG. 5. **Immunogenicity of gp41ctm.** Serial dilution of sera from mice immunized with gp41ctm alone or gp41ctm-liposomes was incubated with gp41ctm coated onto ELISA plates. Bound antibodies were detected with anti-mouse IgG1, IgG2a, and IgA antibodies followed by incubation with horseradish peroxides-conjugated secondary antibodies. The emitted signal was measured at 450 nm after the addition of substrate 3,3′,5,5′-tetramethylbenzidine. Sera from individual mice are *numbered*. *gp41ctm*, mice immunized with gp41ctm; *gp41ctm-lip*, mice immunized with gp41ctm proteoliposomes. The *y* axis shows the reciprocal titer of the antibodies.

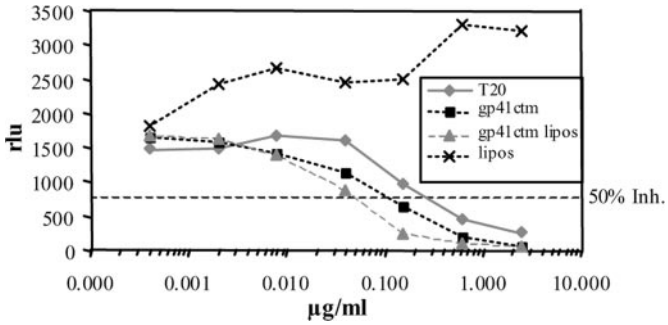

FIG. 6. **gp41ctm inhibits HIV-1 entry.** gp41ctm alone, gp41ctm containing liposomes, the HIV fusion inhibitor T-20, and empty liposomes as negative control were added in the concentrations indicated to TZM-b1 cells, and the cells were challenged with $HIV_{NL-4.3}$. Two days after infection cells were lysed and the activity of firefly luciferase was measured as relative light units (*rlu*) (*y* axis), which correlates with the inhibition of infection. The *x* axis indicates the concentration of the compounds added in a logarithmic scale. Each experiment was run in triplicate and repeated with three different gp41ctm liposome preparations.

DISCUSSION

The HIV and simian immunodeficiency virus envelope glycoproteins form trimers on infectious virions (54, 55), consistent with a trimeric structure of the gp41 core domain observed *in vitro* (4, 6–8, 52, 53, 56). Depending on the HIV or SIV strain, env ectodomains lacking the transmembrane region form either monomers or oligomers that tend to aggregate (57–59). Only in some cases are native trimeric structures formed by the ectodomain alone (60, 61). Therefore a number of strategies have been employed to stabilize trimerization of env (58, 62–65). Here we show that the transmembrane region (TM) of gp41 forms defined trimers in solution or in a lipid bilayer environment. The α-helical content, as measured by circular dichroism, is consistent with a predicted helical TM similar to that of the TM from influenza virus hemagglutinin (66). There is, if at all, little contribution from the extracellular region, consistent with structural studies on 2F5 epitope containing peptides (38) and the random coil conformation of the isolated HR2 region (52, 56). Our *in vitro* data clearly implicate the TM of gp41 in trimerization of env, which is further supported by the expression of mixed HIV-1 and HIV-2 oligomers with identical env TMs (67). Thermodynamic stability measurements reveal that gp41ctm or its TM is stable, underlining its role in trimerization; however, the stability of the trimeric TM is also low enough to exert its proposed role during membrane fusion (68, 69).

gp41ctm contains the conserved membrane proximal region, which harbors linear epitopes of broadly neutralizing antibodies 2F5, 4E10, and Z13 (29, 31, 32). In addition, the same region has been directly implicated in the fusion process (28, 70). As env is the only HIV target protein to elicit neutralizing antibodies, considerable interest is directed to understanding the conformational requirements for the induction of neutralizing antibodies (33, 71). We showed that gp41ctm interacts with neutralizing antibodies 2F5 and 4E10 when anchored to liposomes, which is in agreement with a recent suggestion that a lipid environment enhances the binding of both mAbs (38). Our preliminary immunization studies in mice revealed poor immunogenicity of soluble gp41ctm and considerable immunogenicity of proteoliposomes containing gp41ctm. However, all of the sera failed to exert significant neutralizing activity. This suggests that gp41ctm might not be in a proper conformation to act as an immunogen that could induce 2F5 and/or 4E10-like neutralization activity. The induction of such a neutralizing activity is complicated by the fact that it is not yet clear at which stage during infection these epitopes are exposed and targeted for neutralization, which may be native env or the gp41 prehairpin fusion intermediate structure (43–46, 72). Peptide binding and structural studies indicate a certain epitope length for high affinity binding (30, 39, 73) and only little conformational requirements for 2F5 antibody interaction (37–39). Conversely, an increase in the α-helical content of T-20 improved 2F5 immunoreactivity (74). Likewise, a peptide containing the 4E10 epitope was mostly α-helical in membrane-mimetic environments (75). In addition, because of the long heavy chain CDR3 region of mAb 2F5, it is questionable whether mouse models are actually helpful in establishing induction of neutralization activity by any env antigen (33). Therefore further structural information is needed to better understand the three-dimensional requirements of these epitopes for neutralization and such information may be ultimately used for gp41-based vaccination purposes.

gp41ctm contains C-terminal gp41 regions that have been previously shown to inhibit membrane fusion, such as T-20 (DP178) (10) and C34 (14). It is commonly thought that a gp41 prehairpin intermediate conformation is the target for peptide inhibition. Peptide binding to the prehairpin structure thus abrogates the formation of the six-helical bundle that leads to membrane fusion (2, 3, 12, 24, 25). This dominant negative effect is supported by several independent observations (14, 15, 19, 20) and is exerted by peptides as well as by protein inhibitors (76–78).

gp41ctm forms soluble aggregates under physiological buffer conditions (PBS), and these aggregates exert inhibitory activity, which is comparable to that of T-20. The pull down of gp41ctm by gp41-N-FLAG, confirms that the C-terminal HR2 region of gp41ctm is accessible for the interaction with the N-terminal HR1 region, the proposed target for its inhibitory function (12, 15). Interestingly, gp41ctm incorporated into liposomes revealed the same inhibitory action. This poses the question of how accessible the gp41 prehairpin structure is, because the gp41ctm proteoliposomes were ~50–100 nm in diameter (as measured by electron microscopy). CD4-env gp120/gp41 binding, which is sufficient and necessary for inducing the gp41 prehairpin target structure (12, 79), may form a 25–30-nm-long bridge between the viral and cellular membranes. Therefore the size of the gp41ctm proteoliposomes probably excludes the possibility that gp41ctm HR2 can bind in an anti-parallel way to the exposed HR1 region

in such a setting. A potentially similar inhibitory action has been described for an artificially plasma membrane-anchored version of T-20, which reduces the cell susceptibility to infection (80, 81).

Membrane fusion reactions catalyzed by viral fusion proteins require the concerted action of multiple fusion protein trimers, and their cooperative refolding might result in the synchronized release of conformational energy driving the fusion reaction (82). Consistent with such a proposal, it was recently shown that, in case of hemagglutinin-mediated fusion, hemagglutinin molecules outside of the direct hemagglutinin/receptor membrane contact site play a crucial role in the fusion reaction. Notably, hemagglutinin fusion was inhibited by proteases or antibodies coupled to beads with similar sizes as the gp41ctm proteoliposomes, although the beads were too big to enter the direct contact zone (83). The same authors hypothesized that fusion activation of the so-called "bystanders" helped to induce stresses on membranes, which translates into membrane curvature required for fusion (84). Our data on the fusion inhibition of gp41ctm proteoliposomes are thus consistent with this hypothesis and indicate that gp41ctm proteoliposomes might exert its inhibitory potential also on bystander env molecules, thus blocking membrane fusion. The inhibitory fusion potential of such membrane-anchored molecules suggests also that this formula may be useful for developing a topical microbicide that prevents HIV transmission (85). Interestingly, a proof of principle for such an approach was recently reported for a modified version of the chemokine RANTES (regulated on activation normal T cell expressed and secreted) (86).

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