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A Molecular Clasp in the Human Immunodeficiency Virus (HIV) Type 1 TM Protein Determines the Anti-HIV Activity of gp41 Derivatives: Implication for Viral Fusion

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We have previously reported that synthetic peptides representing the leucine zipper domain (DP107) and a second putative helical domain (DP178) of human immunodeficiency virus type 1 (HIV-1) gp41 exhibit potent anti-HIV activity. In this study we have used soluble recombinant forms of gp41 to provide evidence that the DP178 peptide and the DP178 region of gp41 associate with a distal site on the gp41 transmembrane protein whose interactive structure is influenced by the leucine zipper (DP107) motif. We also observed that a single coiled-coil-disrupting mutation in the leucine zipper domain transformed the recombinant gp41 protein from an inactive to an active inhibitor of HIV-1 fusion and infectivity, which may be related to that finding. We speculate that this transformation results from liberation of the potent DP178-related sequence from a molecular clasp with a leucine zipper, DP107, determinant. The results are discussed in the context of two distinct conformations for the gp41 molecule and possible involvement of these two domains in structural transitions associated with HIV-1-mediated fusion. The results are also interpreted to suggest that the anti-HIV activity of the various gp41 derivatives (peptides and recombinant proteins) may be due to their ability to form complexes with viral gp41 and interfere with its fusogenic processes.

The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein is synthesized as a gp160 precursor which is subsequently cleaved to the gp120 outer surface (SU) and gp41 transmembrane (TM) proteins. The gp120 protein serves to bind the virus to appropriate host cells through its high-affinity interaction with the CD4 molecule, while the gp41 molecule plays a major role in subsequent fusion of the virus and cell membranes. Although little is known about the three-dimensional structure of gp41, we became interested in two regions of the gp41 ectodomain which have been modeled as extended helices (11). One of these, on the N-terminal side of the molecule, was predicted to contain a leucine zipper-like motif (8). We reported that a synthetic peptide based on that sequence exhibited helical structures characteristic of coiled coils (29), which is consistent with that prediction. That peptide, DP107, was also found to effectively block virus-induced membrane fusion and inhibit HIV infection by cell-free virus (29). A single amino acid substitution in the synthetic peptide that disrupted the coiled-coil structure also compromised its anti-HIV activity. The importance of this leucine zipper motif in membrane fusion was indicated in several mutagenesis studies of gp41 (3, 6, 7, 9, 26). The second putative helical sequence (11) is located in the C-terminal end of the gp41 ectodomain. A peptide (DP178) synthesized to correspond to that sequence blocked HIV-1-induced membrane fusion at unusually low concentrations (<1 nM) (27, 30). The potent anti-HIV activity exhibited by DP178 and its specificity for HIV-1 rather than HIV-2 suggested a specific involvement of this region in virus fusion.

In this investigation we have extended our studies of the ectodomain of the HIV-1 transmembrane protein. The results are interpreted to suggest an interaction between the leucine zipper determinant and the putative C-terminal helical sequence in the gp41 transmembrane protein. We argue that the described interaction between these two regions of the HIV-1 transmembrane protein may be related to the mechanism of action of the potent anti-HIV-1 peptide (DP178) and soluble gp41 derivatives which were constructed for these studies. Finally, we discuss possible implications of these observations for the structure and function of gp41.

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MATERIALS AND METHODS

Construction of fusion proteins and mutants. Construction of the recombinant soluble gp41 fusion proteins and mutants was accomplished as follows. The DNA sequence corresponding to the extracellular domain of gp41 (amino acids 540 to 686 of HIV-1_{LAI}, numbered according to reference 20; the five N-terminal amino acids are M T L T V and the five C-terminal amino acids are N W L W Y) was cloned into the *Xmn*I site of the expression vector pMal-p2 (New England Biolabs) to obtain M41. The gp41 sequence was amplified from pgtat (18) by PCR with the upstream primer 5′-ATGACGCTGACGGTACAGG CC-3′ (primer A) and the downstream primer 5′-TGACTAAGCTTAATACCA CAGCCAATTTGTTAT-3′ (primer B). M41-P was constructed with the T7-Gen in vitro mutagenesis kit from United States Biochemicals by following the supplier's instructions. The mutagenic primer employed (5′-GGAGCTGCTTGGG GCCCCAGAC-3′) introduces an Ile-to-Pro mutation in M41 at position 578. The M41Δ107 construct was made with a deletion mutagenic primer, 5′-CCAAATCCCCAGGAGCTGCTCGAGCTGCACTATACCAGAC-3′ (primer C), by following the T7-Gen mutagenesis protocol. M41Δ178 was made by cloning the DNA fragment corresponding to gp41 amino acids 540 to 642 into the *Xmn*I site of pMal-p2. The DNA fragment encoding amino acids 540 to 642 was generated by PCR from the pgtat template by using primer A (see above) as the upstream primer and the downstream primer 5′-ATAGCTTCTAGATTA ATTGTTAATTTCTCTGTCCC-3′, which was designated primer D. The M41-P construct was used as a template with primers A and D in PCR to generate the DNA fragment used to make the M41-PΔ178 construct. All inserted sequences and mutated residues were checked by restriction enzyme analysis and confirmed by DNA sequencing.

ELISA. Either recombinant protein M41 or maltose-binding protein at 5 μg/ml in 0.1 M $NaHCO_3$ (pH 8.6) was used to coat 96-well Linbro ELISA plates (Flow Laboratories, Inc.), which were then incubated overnight. Each well was washed three times with distilled water and then blocked with 3% bovine serum

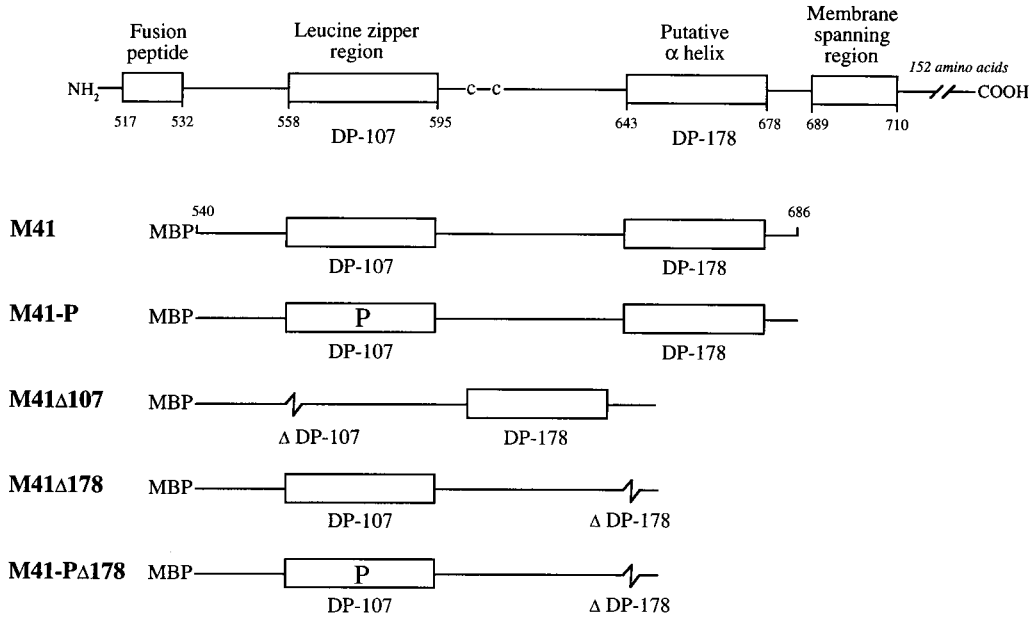

FIG. 1. Schematic representation of HIV-gp41 and maltose-binding protein (MBP)–gp41 fusion proteins. DP107 and DP178 are synthetic peptides based on the two putative helices of gp41. The letter P in the DP107 boxes denotes an Ile-to-Pro mutation at amino acid 578. Amino acid residues are numbered according to reference 20.

albumin (BSA) for 2 h. Antibodies (100 μl) were added in the presence of 0.5% BSA in TBST (40 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.05% Tween 20) and the plates were incubated at room temperature for 1 h. Fab-d was added at a concentration of 10 ng/ml with 0.5% BSA in TBST. The plates were washed three times with TBST after incubation at room temperature for 1 h. Horseradish peroxidase-conjugated goat anti-human Fab antiserum at a 2,000-fold dilution in TBST with 0.5% BSA was added to each well, and the plates were incubated at room temperature for 45 min. The plates were then washed four times with TBST. The peroxidase substrate *o*-phenylenediamine (0.4 mg/ml) and 0.03% H_2O_2 were added to develop the color. The reaction was stopped with an equal volume of 4.5 N H_2SO_4 after incubation at room temperature for 10 min. The optical density of the reaction mixture was then measured at 490 nm with a microplate reader (Molecular Devices). For the experiments demonstrating reconstitution of the Fab-d epitope (see Fig. 7), various concentrations of the peptide DP178 were added to the wells after blocking them with BSA. Peptide was incubated at room temperature for 1 h before the addition of antibody.

Protein purification and Western blotting (immunoblotting) analysis. The *Escherichia coli*-expressed fusion proteins were purified according to the protocol described in the manufacturer's (New England Biolabs) brochure of protein fusion and purification systems. Briefly, the bacterial extract in column buffer (150 mM NaCl, 10 mM Tris-HCl [pH 7.4], 1 mM EDTA) was applied to an amylose resin affinity column, and the column was washed with column buffer. The maltose-binding fusion proteins were then eluted from the column with buffer containing 10 mM maltose. The maltose was subsequently removed from the eluate by dialysis against phosphate-buffered saline (PBS), pH 7.4. Fusion proteins (10 ng) were analyzed by electrophoresis on sodium dodecyl sulfate (SDS)–8% polyacrylamide gels. Western blotting analysis was performed as described by Sambrook et al. (23). Either HIV-1-positive serum diluted 1,000-fold or human Fab (unpublished data) derived from repertoire cloning as described by Barbas and Lerner (1) was used to react with the fusion proteins. The second antibody was horseradish peroxidase-conjugated goat anti-human Fab. An enhanced chemiluminescence Western blotting detection system (Amersham) was used for detection of the bound antibody according to the manufacturer's instructions. Rainbow molecular weight markers (Amersham) were used to estimate the sizes of the fusion proteins.

Cell fusion assay. Cell fusion assays were performed as previously described (19). MOLT-4 cells (7×10^4) were incubated with CEM cells (10^4) chronically infected with HIV-1_{LAI} in 96-well half-area flat-bottomed plates (Costar) in 100 μl of culture medium. Peptide and fusion proteins at various concentrations in 10 μl of culture medium were incubated with the cell mixtures at 37°C for 24 h. Multinucleated syncytia were enumerated by microscopic examination of the entire contents of each well.

HIV-1 neutralization assay. Twenty microliters of a serially diluted HIV-1_{LAI} virus stock was incubated for 60 min at ambient temperature with 20 μl of various concentrations of purified recombinant fusion protein in RPMI 1640 containing 10% fetal bovine serum and antibiotics in a 96-well microtiter plate. Twenty microliters of CEM4 cells at a concentration of 6×10^5 cells per ml was added to each well, and cultures were incubated at 37°C in a humidified CO_2 incubator. The cells were cultured for 9 days, with fresh medium added every 2 to 3 days. On days 5, 7, and 9 postinfection, supernatant samples were harvested and assayed for reverse transcriptase activity, as described below, to monitor viral replication. The 50% tissue culture infective dose was calculated for each condition according to the formula of Reed and Muench (22). Reverse transcriptase activity was determined by modifications of the published methods of Goff et al. (12) and Willey et al. (32) as described in reference 5. Briefly, Triton X-100 was added to harvested culture supernatants to a final concentration of 1% (vol/vol). A 10-μl sample of each Triton X-100 lysate was mixed with 50 μl of a reaction cocktail containing 50 mM Tris-HCl [pH 7.8], 75 mM KCl, 2 mM dithiothreitol, 5 mM $MgCl_2$, 5 μg of poly(rA) per ml, 1.5 μg of oligo$(dT)_{12-18}$ per ml, 0.05% Nonidet P-40, and 10 μCi of [^{32}P]TTP per ml and incubated at 37°C for 90 min. Aliquots (40 to 50 μl) of reaction mixtures were spotted onto DE-81 paper (Whatman) in a minifold sample filtration manifold (Schleicher & Schuell) and washed several times with 2× SSC (0.3 M NaCl plus 0.03 M sodium citrate) followed by 2× SSC containing bromophenol blue to locate spots. Autoradiography was performed, and radioactivity was estimated with a Packard Matrix 9600 direct beta counter.

Flow cytometric analysis by FACS. Flow cytometric analysis was conducted by fluorescence-activated cell sorting (FACS) with CEM cells chronically infected with the HIV-1_{LAI} isolate. Briefly, 10^6 chronically infected CEM cells (CEM/HIV-1_{LAI}) were incubated for 30 min at 37°C with or without 10 μg of soluble CD4 (ST4) per ml. Primary antibodies (Fab-d at a final concentration of 5 μg/ml and HIV-1-positive or -negative serum at a 100-fold dilution) were then added to the samples, and the mixtures were incubated at 4°C for 30 min. After three washes with PBS–1% fetal calf serum, CEM/HIV-1_{LAI} cells were incubated with fluorescein isothiocyanate-conjugated anti-human $F(ab)_2$ (Cappel) in the dark at 4°C for 30 min. The cells were then washed three times with PBS-fetal calf serum, resuspended in PBS containing 1% formaldehyde, and subjected to flow cytometric analysis.

RESULTS

Construction and characterization of recombinant gp41 fusion proteins. To further investigate the roles of the leucine zipper domain and the putative C-terminal helical sequence of gp41, we expressed the gp41 ectodomain as a soluble maltose-binding fusion protein (M41) in *E. coli* (Fig. 1). The fusogenic peptide sequence at the N terminus of gp41 was omitted from this recombinant protein and its derivatives to improve solubility. The inclusion of the maltose-binding protein moiety facilitated purification of the fusion proteins under relatively mild, nondenaturing conditions. Because the M41 soluble re-

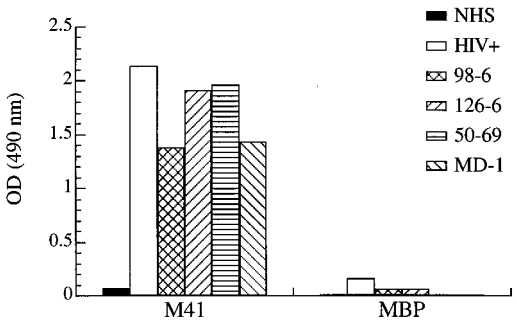

FIG. 2. Reactivity of M41 and maltose-binding protein (MBP) to conformational monoclonal antibodies. The reactivity of M41 to conformational antibodies was analyzed by ELISA as described in Materials and Methods. Human monoclonal antibodies 98-6, 126-6, and 50-69 (33) were diluted 100-fold in this assay, and monoclonal antibody MD-1, HIV-1-positive (HIV-1$^+$) serum from an HIV-1-positive individual, and normal human serum (NHS) were diluted 1,000-fold. OD, optical density.

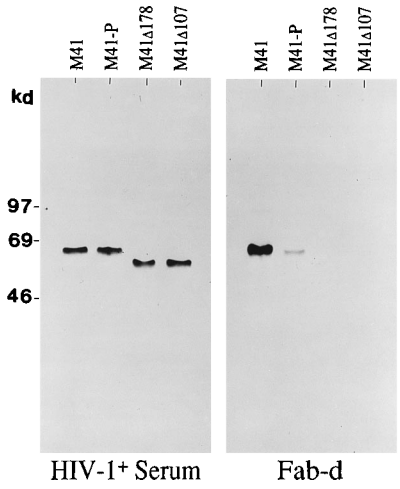

FIG. 3. Reactivity of Fab-d and HIV-1-positive (HIV-1$^+$) serum with wild-type M41 and mutant forms of the gp41 fusion protein. The fusion proteins were purified according to the protocol described in the manufacturer's (New England Biolabs) brochure of protein fusion and purification systems. Fusion proteins (10 ng) were analyzed by electrophoresis on SDS–8% polyacrylamide gels. It was found that a point mutation alters the conformation of M41.

combinant gp41 was not glycosylated, lacked several regions of the transmembrane protein (i.e., the fusion peptide as well as the membrane-spanning and cytoplasmic domains), and was expressed in the absence of gp120, we felt that it was unlikely to precisely reflect the structure of native gp41 on HIV-1 virions. Nevertheless, purified M41 folded in a manner that preserved certain discontinuous conformational epitopes, as evidenced by its reactivity with human monoclonal antibodies 98-6, 126-6, and 50-69 (Fig. 2), which had previously been shown to bind conformational epitopes on native gp41 expressed in eukaryotic cells (6). The 98-6 and 126-6 antibodies recognize conformational epitopes in the C-terminal half of the extracellular domain of gp41 (33), while the 50-69 antibody reacts with a conformation defined by the two cysteines of the gp41 ectodomain (33). The recombinant M41 protein was also reactive with the monoclonal antibody MD-1 (Fig. 2), which binds almost exclusively to oligomeric forms of gp41 on immunoblots (21). Thus, at least certain regions of native gp41 defined by these antibodies appear to be reproduced in the recombinant M41 fusion protein.

The M41 fusion protein also reacted with a human recombinant Fab designated Fab-d. The latter antibody fragment was constructed by using the system described by Barbas and Lerner (1) and was originally selected on the basis of HIV-1 gp160 binding (unpublished data). In Fig. 3, a Western blot illustrating the reactivity of the Fab-d with wild-type M41 and several mutant forms of the gp41 fusion protein is shown. The reactivity of Fab-d following SDS denaturation might suggest that its epitope is of a linear nature. Nevertheless, further experiments described below argue that its epitope is discontinuous. The positive Western blot result in Fig. 3 with wild-type M41 thus is probably related to the refolding of a fraction of the blotted gp41 molecules, which yielded the discontinuous epitope.

Fab-d also bound to HIV-1_{LAI}-infected CEM cells, as shown in Fig. 4. Interestingly, the binding of Fab-d to infected cells was significantly increased by treatment of the cells with soluble CD4 (ST4) under conditions which induce gp120 shedding (Fig. 4). Although the M41 fusion protein and native gp41 molecules differ significantly in many respects, the binding of Fab-d to infected cells suggests that the structure recognized by Fab-d on bacterially expressed M41 reflects a conformation of the native gp41 molecule.

Further mapping studies with Fab-d demonstrated binding to HIV-1 gp41 but not to gp120 or to a panel of overlapping peptides that span the entire ectodomain of gp41. Although the epitope recognized by Fab-d resides in the ectodomain of gp41 (Fig. 3), the inability of Fab-d to demonstrate reactivity with the panel of overlapping peptides suggested that the epitope might be conformational and/or discontinuous. Deletion of either the C-terminal or N-terminal helix motif (M41Δ107 and M41Δ178, respectively) of the M41 fusion protein eliminated Fab-d reactivity (Fig. 3), which is consistent with that notion. This suggests that both helical regions, separated by 60 amino acids in the primary sequence, are required to maintain the Fab-d epitope. We engineered a proline mutation into the leucine zipper sequence of M41 (creating the recombinant fusion protein M41-P) and found that this muta-

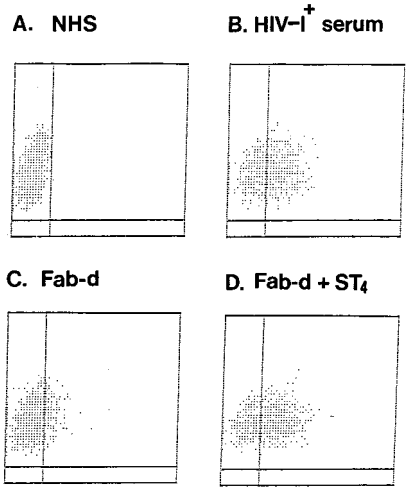

FIG. 4. Flow cytometric analysis (by FACS) demonstrates the binding of Fab-d to CEM cells chronically infected with HIV-1_{LAI}. The primary antibodies used for the FACS analysis are indicated in each panel. NHS, normal human serum (HIV-1 negative); ST4, soluble CD4. The *x* axis shows log fluorescence intensity, and the *y* axis shows the amount of forward scatter, which is a measurement of cell size. Fab-d did not bind to uninfected CEM cells (data not shown).

TABLE 1. Interaction of the two putative helices of gp41

Peptide	Concn inhibiting cell fusion (IC_{90})[a]	Fab-d binding (K_d)[b]	Concn inhibiting HIV infectivity (IC_{90})
DP107	1 μM	—[c]	1 μM
DP178	1 nM	—	80 nM
M41	>50 μM	3.5×10^{-9}	>16 μM
M41-P	80 nM	2.5×10^{-8}	70 nM
M41-PΔ178	>50 μM	—	>8 μM

[a] IC_{90}, 90% inhibitory concentration.
[b] The affinity constants for Fab-d binding were determined by using a protocol described by Friguet et al. (10).
[c] —, no detectable binding of Fab-d.

tion also severely reduced Fab-d reactivity (Fig. 3). This proline mutation has been found to disrupt the structure of the leucine zipper motif (26, 29) and to abolish gp41-mediated cell-cell fusion and HIV-1 infectivity (7, 26). It thus appears that a wild-type leucine zipper motif and presumably a corresponding coiled-coil oligomeric helix are necessary for epitope formation.

A mutation in the leucine zipper region changes the structure and function of soluble gp41. In the course of these studies, the wild-type M41 fusion protein was examined for anti-HIV-1 activity. As mentioned above, synthetic peptides corresponding to the leucine zipper (DP107) and the C-terminal putative helix (DP178) each exhibit potent anti-HIV activity. Despite inclusion of both of these regions, the recombinant M41 protein did not affect HIV-1-induced membrane fusion at concentrations as high as 50 μM (Table 1). In contrast, a single amino acid substitution, proline in place of isoleucine in the middle of the leucine zipper motif (I-578 to P), yielded a fusion protein (M41-P) which did exhibit antiviral activity (Table 1 and Fig. 5). As seen in Table 1, M41-P blocked syncytium formation by 90% at ~80 nM and neutralized $HIV\text{-}1_{LAI}$ infection by 90% at ~70 nM. We have reported earlier that the same substitution disrupted the helical structure of the DP107 peptide and rendered that peptide inactive in virological assays (26, 29). It therefore was unlikely that the biological activity noted in the M41-P fusion protein could be mediated by the leucine zipper domain. A more likely candidate was the DP178 region, and a variant of the M41-P protein lacking the DP178 region (M41PΔ178) was inactive (Table 1), which is consistent

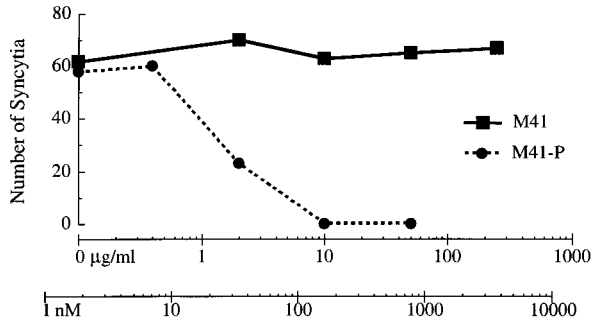

FIG. 5. Anti-HIV-1 activity of M41-P. Cell fusion assays were performed as previously described (19). CEM cells (7×10^4) were incubated in 96-well flat-bottomed half-area plates (Costar) in 100 μl of culture medium with CEM cells (10^4) chronically infected with HIV_{LAI}. Fusion proteins at various concentrations (indicated on the *x* axes) in 10 μl of culture medium were incubated with the cell mixtures at 37°C for 24 h. The numbers of multinucleated syncytia were estimated by microscopic examination. M41 and M41-P did not exhibit any cytotoxicity at the concentrations tested.

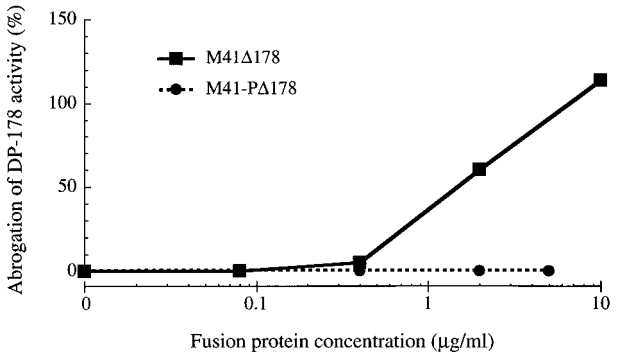

FIG. 6. Abrogation of DP178 anti-HIV activity by M41Δ178 but not by M41-PΔ178. Assays for HIV-1-induced cell-cell fusion were carried out in the presence of 10 ng of DP178 per ml and the indicated concentrations of either M41Δ178 or M41-PΔ178. The fusion assays were conducted as described in Materials and Methods. In the absence of added DP178 peptide, 88 syncytia were observed. The addition of 10 ng of DP178 per ml completely blocked syncytium formation. Percent abrogation of DP178 activity was calculated as follows: (number of syncytia in the presence of fusion protein and DP178/number of syncytia in the absence of peptide and fusion protein) × 100. Neither M41Δ178 nor M41-PΔ178 affected syncytium formation when tested alone.

with that possibility. Although other interpretations are possible, we feel that the lack of activity for wild-type M41 is related to structural constraints within the protein which act to sequester the DP178 sequence and prevent its access to its putative site of action. Stated another way, insertion of the proline mutation within the DP107 leucine zipper region of the M41-P fusion protein effectively liberates the C-terminal helical domain, enabling the interaction of the DP178 sequence with its target.

Abrogation of DP178 antiviral activity by a recombinant soluble gp41 fusion protein. The interpretation presented for the preceding result was reinforced by the following series of competition experiments in which the anti-HIV activity of the DP178 peptide was assessed in the presence of several soluble recombinant gp41 fusion proteins, as indicated in Fig. 6. We found that a truncated fusion protein lacking the DP178 sequence, M41Δ178, abrogated the potent antifusion activity of the DP178 peptide in a concentration-dependent manner (Fig. 6). The same truncated fusion protein containing the leucine zipper-disrupting proline mutation, M41-PΔ178, was not active in similar competition experiments (Fig. 6). These results suggest that the DP178 peptide associates with a second site on gp41 whose interactive structure is dependent on a wild-type leucine zipper sequence. The association of the DP178 peptide with this second site on gp41 could be an integral step in the mechanism of action of this potent anti-HIV peptide. These results also support the interpretation that an analogous intramolecular interaction may occur within the wild-type fusion protein M41, forming a molecular clasp which effectively sequesters the DP178 region and makes it unavailable for antiviral activity.

Reconstitution of a discontinuous epitope with a peptide and a protein fragment. A specific association between the two gp41 helical domains is also indicated by further studies using the human monoclonal antibody Fab-d discussed above. As shown in Fig. 3, both an intact leucine zipper DP107 region and the DP178 region are required for recognition of M41 by Fab-d. On the basis of those experiments, it was not surprising that Fab-d failed to bind either the DP178 peptide or the fusion protein M41Δ178, which lacked the DP178 sequence. However, we observed the striking result that the Fab-d epitope could be reconstituted by simply mixing the DP178

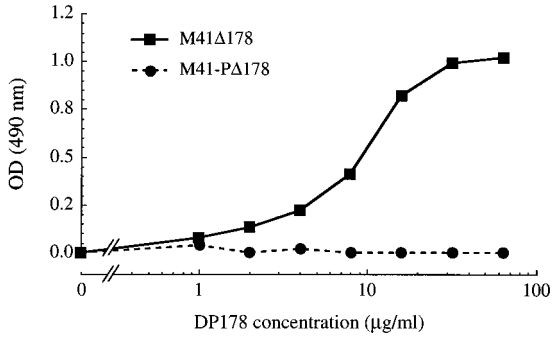

FIG. 7. Binding of DP178 to the leucine zipper of gp41 and reconstitution of a conformational epitope. Binding of DP178 to the leucine zipper motif of gp41 was analyzed by ELISA. The amino acid sequence of DP178 is YTSLIHSLIE ESQNQQEKNEQELLELDKWASLWNWF. Peptide binding assays were carried out and optical density (OD) was measured as described in Materials and Methods.

peptide and the M41Δ178 fusion protein (Fig. 7). Again, the proline mutation in the leucine zipper domain of the fusion protein M41-PΔ178 failed to reconstitute the epitope in similar mixing experiments. The latter result is consistent with the results in Fig. 3, which shows that the single proline mutation in the full-length fusion protein (M41-P) compromised recognition by the Fab-d antibody. These observations may also be related to those obtained with several other monoclonal antibodies whose epitopes are conformationally dependent and involve the DP178 region of gp41 (14, 33).

DISCUSSION

The experiments described above indicate that a peptide mimic of a C-terminal sequence in the gp41 ectodomain associates with a determinant of the gp41 leucine zipper domain. This peptide, DP178, has been shown to exhibit antiviral activity (27). The mechanism of action is unknown, but we propose, as a working hypothesis, that the DP178 peptide-recombinant protein interactions observed in this study might be involved. We also speculate that the two involved helical domains are stably associated with each other in recombinant gp41 proteins which contain a wild-type leucine zipper motif. Helix-breaking mutations in the leucine zipper domain impede the interaction and transform the recombinant gp41 fusion protein into an inhibitor of HIV-1 replication. We have interpreted the latter result to reflect the release of the potent DP178 sequence from an intramolecular clasp, thus making it available for interaction with the wild-type leucine zipper motif on infectious viruses.

In efforts to further test these ideas, we have attempted to isolate DP178-resistant mutants of HIV-1_{LAI} by serial passage in escalating doses of peptide. One such isolate has been identified. Sequence analysis suggests that a mutation in the leucine zipper domain is associated with resistance to the peptide (22a). In other studies, it was found that DP178 peptide alters the helical circular dichroism spectra of the DP107 peptide mimic of the leucine zipper motif (28). These results are consistent with the peptide-recombinant gp41 interactions noted here and support a mechanism of action for DP178 that involves association with a leucine zipper determinant.

It is of interest that a peptide which overlaps DP178 has also been reported to inhibit HIV-1 infection and fusion (15, 16). A related mechanism based on the binding of that peptide to the fusion peptide located at the N termini of gp41 about 30 residues removed from the leucine zipper motif was proposed. Even though the mechanisms may be related and though the inhibitory peptides overlap each other, the hypothetical targets of interaction appear to differ, since the recombinant gp41 analogs used in the present study do not contain the fusion peptide sequence described by Jiang et al. (15, 16).

The implications of the observations reported here for the tertiary or quaternary structure of gp41 are more speculative. No crystallographic information is currently available for the HIV-1 gp41 molecule, and structure models are in large part based on theoretical algorithms and comparison to other fusogenic viruses. The situation is also complicated since gp41, like the fusion proteins of other enveloped viruses, is thought to undergo conformational changes associated with its role in membrane fusion (24). Thus, the interactions evidenced in our studies may reflect structures found only during certain stages of the virus life cycle.

The most well-characterized viral fusion protein is the HA2 component of influenza virus (25, 31). In this case, details of the native conformation have been known for some time (25, 31). Insights into the transition of HA2 from a native to a fusogenic conformation have recently been reported by Carr and Kim (4) and Bullough et al. (2). On the basis of their studies, Carr and Kim proposed a model in which a nonhelical loop region of the native HA2 oligomer is rearranged into a helical coiled coil. The transition was proposed to extend the trimeric HA2 stem region and move the fusion peptide sequence from a buried site to an exposed site at the top of the elongated fibrous stem, making it available for insertion into the target membrane. In large measure, these predictions were borne out by the crystallographic studies of HA2 conducted by Bullough and colleagues (2), although the transition from a native to a fusogenic state is apparently even more complex than was anticipated, with structural rearrangements involving major refolding of much of the HA2 ectodomain.

As described by Carr and Kim (4) and others (11), the fusion proteins of other membrane viruses, including HIV, appear to contain features similar to those of the HA2 component of influenza virus. They are oligomeric and are usually synthesized as precursor molecules which upon proteolytic cleavage yield a conserved fusion peptide sequence at the N terminus. That site is often followed by a sequence predictive of a coiled-coil structure, which according to the flu model is a region critically involved in the structural transitions associated with the fusion process. For gp41, the analogous sequence is most likely the leucine zipper domain, and there is experimental evidence supportive of structural transitions at that site following receptor binding (13, 17, 24). On the basis of other considerations, we have also argued that the leucine zipper domain most likely exists as a coiled coil in the fusogenic state rather than in the native state (26). That interpretation is in good agreement with the Carr and Kim model for HA2 as well as with the structure of the TBHA2 molecule reported by Bullough et al. (2).

By further analogy to the Carr and Kim model of HA2, if the distal gp41 helical domain modeled with the DP178 peptide interacts with the leucine zipper domain proximal to the N termini, as suggested by the experiments described here, this would most likely occur in gp41 in the native state. Such an interaction might act as a clasp or one of the forces that holds gp41 in a native configuration. Following the extension of the fibrous stem during transformation to the fusogenic state, the two putative helical domains would become far removed from each other, according to that model. Several other possibilities, however, must be considered. One is that the two helical domains remain associated throughout the life cycle of the envelope. Another is that they associate only while they are in the

fusogenic state. The latter possibility might be more in line with the current HA2 model, which is based on the TBHA2 structure described by Bullough et al. (2). We hope to be able to distinguish between these possibilities with the use of conformation-specific antibodies, such as Fab-d, whose epitope appears to be dependent on an interaction between these two domains, as described in this communication. The results of the FACS analysis of chronically infected cells shown in Fig. 4 revealed high levels of Fab-d binding to resting cells. That result could be interpreted as being consistent with an association between the two helical domains in the resting, or native, configuration. That interpretation is complicated, however, since the level of Fab-d binding to chronically infected cells was found to intensify significantly upon treatment of cells with soluble CD4. Similar results were obtained with Fab-d binding to intact HIV-1 virions in the absence and presence of soluble CD4 (data not shown). If binding to CD4 induces a conformational switch from the native to the fusogenic configuration, then the results could indicate that the association of the two helical domains occurs in the fusogenic conformation. Clearly, more work will have to be done to address these issues. Some of the issues include the homogeneity of the envelope configuration (native or fusogenic) on infected cells or viruses and a better understanding of the requirements for transition of gp41 from the native to the fusogenic state. That is, is binding to soluble CD4 sufficient for transition, or does the triggering phenomenon require more complex interactions?

Given the difficulties associated with crystallization of HIV-1 envelope components, it is our hope that the protein fragment approaches described here might provide useful insights into the structure-function relationships of gp41. Several groups have addressed the functional role of various regions of gp41 in a number of studies using site-directed mutagenesis. These have included the functional assessment of mutations in both gp41 helical domains studied in the present work and others (3, 6, 7, 9). Of particular interest is the fact that mutations in the C-terminal helix motif tended to enhance fusion whereas mutations in the leucine zipper region more commonly decreased or abolished the fusion process (3). Considering the interactions described here, the mutagenesis results might reflect a direct involvement of the leucine zipper motif in fusion, while the putative C-terminal helix may serve to regulate the availability of the leucine zipper.

Finally, we are unaware of any precedent for the reconstitution of discontinuous antibody epitopes with synthetic peptides and protein fragments as described here for the Fab-d antibody. The specificity of the interactions described needs further definition, but taken together, the experiments presented argue that there is an association of the two gp41 helix motifs, which are separated by about 60 amino acids in the primary sequence of gp41, and that this interaction may form the basis for the mechanism of action of the DP178 peptide and the M41-P recombinant fusion protein. The results also raise the possibility that the interaction between these distal sites may be involved in the control of fusion mediated by the HIV-1 transmembrane protein.

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