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Sequential Interaction of CD4 and HIV-1 gp120 with a Reconstituted Membrane Patch of Ganglioside GM3: Implications for the Role of Glycolipids as Potential HIV-1 Fusion Cofactors

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The fusion of HIV-1 with CD4$^+$ cells involves, in addition to CD4, specific cell surface molecules acting as fusion cofactors. Recently, we reported that the V3 loop of HIV-1 gp120 binds to GM3, a ganglioside abundantly expressed on CD4$^+$ lymphocytes and macrophages. In the present study, we show that CD4 interacts with a reconstituted patch of GM3 by measuring the surface pressure with a Langmuir film balance. A biphasic increase in surface pressure is observed after the sequential addition of CD4 and gp120 under the GM3 monolayer, indicating the formation of the trimolecular complex GM3-CD4-gp120. Neutralization of gp120 with an anti-V3 antibody inhibits the secondary interaction with GM3, suggesting that the CD4-induced conformational change in gp120 allows the V3 loop to interact with GM3. In conclusion, this study supports the concept that glycolipids can function as HIV-1 fusion cofactors.

Key Words: **CD4; HIV-1; fusion; ganglioside; coreceptor; oligosaccharide.**

CD4 is a 55 kDa cell surface protein involved in the regulation of T-cell activation through major histocompatibility complex class II-restricted mechanisms (1). In addition to its immunological functions, CD4 serves as a specific receptor for HIV-1 on CD4$^+$ cells (1,2). The fusion of HIV-1 with the plasma membrane of CD4$^+$ cells requires additional human components (3,4) which act as coreceptors for the fusion process. Among the ten coreceptors potentially used by human and/or simian immunodeficiency viruses (5), the major HIV-1 coreceptors are the chemokine receptors CXCR4 and CCR5. These receptors belong to the family of seven-transmembrane segments receptors coupled to G proteins. CXCR4 serves as a fusion cofactor for T-lymphotropic strains of HIV-1 (6,7), whereas CCR5 mediates entry of macrophagetropic isolates (8,9). In both cases however, a common mechanism leads to the formation of a trimolecular complex between gp120, CD4 and either CXCR4 or CCR5. Following a primary interaction with CD4, a conformational change in gp120 renders the V3 domain of the viral glycoprotein available for secondary interactions with either CXCR4 or CCR5 (10-13). In absence of CD4, the V3 loop is not correctly exposed to allow direct binding of gp120 to chemokine receptors, although infection of some CD4-negative cells through CXCR4 and CCR5 have been reported in the case of HIV-2 (14) and SIV (5).

Recent studies from our laboratory suggested that the V3 loop of HIV-1 gp120 can bind to GM3, a glycolipid abundantly expressed by human CD4$^+$ lymphocytes (15,16). Moreover, CD4$^+$ non-human cells are rendered competent to CD4-dependent HIV-1 fusion by transfer of human erythrocyte glycolipids (17). Taken together, these data support the concept that some glycolipids may function as alternative fusion cofactors (5). To investigate this possibility, we have studied the interaction of CD4 and gp120 with reconstituted patches of glycolipids. Monomolecular films of glycolipids at the air-water interface were used as model for membrane patches (18). The insertion of

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Abbreviations used: HIV-1, type 1 human immunodeficiency virus; GalCer, galactosylceramide; LacCer, lactosylceramide; PBMC, peripheral blood mononuclear cells.

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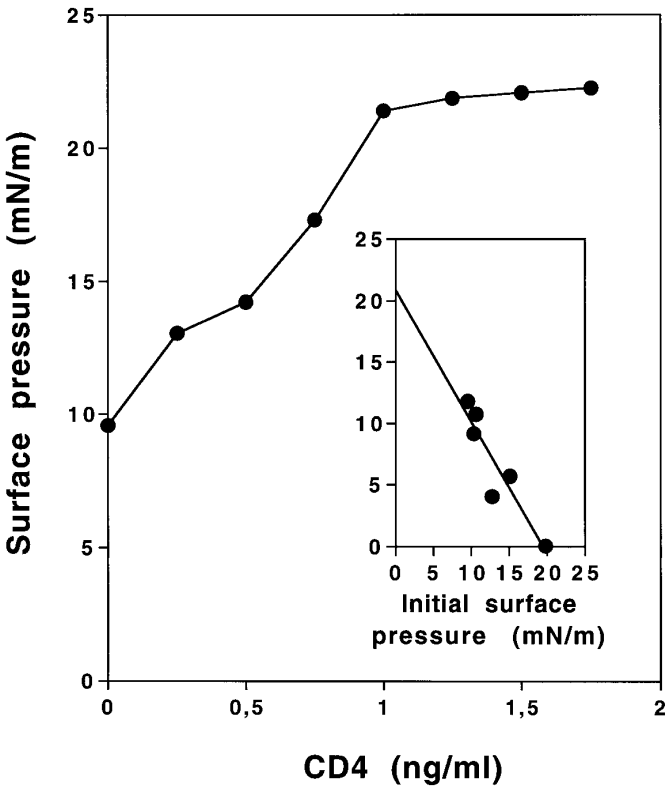

FIG. 1. Variations in surface pressure of a monolayer of human GM3 after injection of soluble recombinant CD4. (Inset) Maximal surface pressure increase reached after injection of soluble recombinant CD4 (1 ng/ml) under a monomolecular film of human GM3 at various initial surface pressures.

CD4 and gp120 into the glycolipid films was detected by measuring the variations of the interfacial pressure with a Langmuir film balance (19,20).

MATERIALS AND METHODS

Chemicals. GalCer, LacCer, GM1, GM2, GM3 from human erythrocytes, and GD3 were purchased from Sigma (Les Ulis, France). Bovine milk GM3 was from Matreya, Inc. (Pleasant Gap, PA). When indicated, GM3 was purified from peripheral blood mononuclear cells (PBMC) from healthy donors. 3′Sialyllactose purified from human milk was from Oxford GlycoSystems (Abinbgdon, UK). The monoclonal antibody 110-H, which recognizes the epitope GPGRAFVTI in the V3 loop of HIV-1 gp120 was obtained through the Agence Nationale de Recherches sur le SIDA. Recombinant soluble CD4 was generously provided by Dr. David Klatzmann (Paris, France) and by the Medical Research Council (UK). The multimeric V3 loop peptide SPC3 (15) was obtained from Eurethics (Paris, France). Solvents and reagents were of the highest purity available.

HIV-1 surface envelope glycoprotein. A stably transfected Chinese hamster ovary (CHO) cell line expressing HIV-1 gp120 (IIIB isolate, BH10 clone) was kindly provided by Celltech through the Medical Research Council. The gp120 secreted by this cell line was purified by lectin affinity chromatography (21) with slight modifications (16).

Surface pressure measurements. The surface pressure was measured with a Langmuir film balance (A & D Instruments, Oxford, UK). Data were saved every 30 sec using the Collect software (Labtronics Inc., Guelph, Ontario, Canada). After being dissolved in a mixture of hexane: chloroform: ethanol 11: 5: 4 (v: v:v) (22), lipids were spread inside a Teflon tank. In all experiments, the subphases were pure water obtained by filtration through a milli-Q water purification system (Millipore, Saint-Quentin, France). When the barrier of the film balance was run across a subphase of pure water no change in surface tension was observed. As a further precaution against the introduction of surface active contaminant with the pure water, the surface layer of water was removed with a Pasteur pipette connected to a water-driven aspirator. This compression cleaning cycle was repeated twice before films of lipids were spread. Each run was performed with a fresh film and subphase. To measure the interaction of proteins with monolayers, the lipids were spread inside the Teflon tank and various concentrations of ligand were added into the subphase. The increase in surface pressure was then measured as a function of ligand concentration. When indicated, the variations of surface pressure were measured as function of time.

RESULTS

Interaction of CD4 with a monolayer of GM3 purified from human erythrocytes. The interaction of recombinant soluble CD4 with GM3 was followed by measurement of the surface pressure increase in a constant area set up. As shown in Figure 1, the addition of CD4 under a human GM3 monolayer of 9.7 mN/m resulted in a maximal pressure increase of 11.8 mN/m. The maximal effect was obtained with 1 ng/ml of CD4. To investigate the specificity of the penetration process, the in-

TABLE 1

Interaction of CD4 with Glycolipid Monolayers

Glycolipid	Structure	ΔΠ (mN/m)
GM3 (human)	NeuAcα2-3Galβ1-4Glcβ1-1Cer	11.8
GM3 (bovine)	NeuAcα2-3Galβ1-4Glcβ1-1Cer	2.5
GM2	GalNAcβ1-4[NeuAcα2-3]4Galβ1-4Glcβ1-1Cer	0.8
GM1	Galβ1-3GalNAcβ1-4[NeuAcα2-3]4Galβ1-4Glcβ1-1Cer	0.6
GD3	NeuAcα2-8NeuAcα2-3Galβ1-4Glcβ1-1Cer	0.3
GalCer	Galβ1-1Cer	1.5
LacCer	Galβ1-4Glcβ1-1Cer	3.6

Note. Monolayers of the indicated glycolipid were formed at an initial surface pressure of 10-12 mN/m. Soluble CD4 (1 ng/ml) was added in the aqueous phase and the maximal surface pressure variation (ΔΠ) obtained after reaching equilibrium is indicated.

crease in surface pressure ($\Delta\Pi$) caused by the addition of CD4 under the human GM3 monolayer was measured at various initial surface pressures. As shown in Figure 1 (inset), the compressibility of the monolayer was gradually decreased as the initial pressure of the monolayer increased. The influence of the initial surface pressure on the compressibility of the monolayer demonstrates the high specificity of the interaction, as previously established for several other lipids and ligands (19,20). Similar results were obtained with three different batches of recombinant CD4 from two distinct origins, and with GM3 purified from human PBMC. The specificity of the GM3/CD4 interaction was demonstrated by the total lack of activity of several gangliosides, including GM3 from bovine milk (Table 1). Interestingly, among the glycolipids tested, only LacCer showed a significant interaction with CD4, although with less efficiency than GM3 ($\Delta\Pi$ of 3.6 mN/m). Since LacCer is the nonsialylated precursor of GM3, these data suggest that the sialic acid of GM3 is an important determinant for CD4 association. The involvement of the oligosaccharide moiety of GM3 in CD4 binding was further demonstrated by the inhibitory effect of 3′sialyllactose on the reaction (data not shown).

Insertion of HIV-1 gp120 into a GM3/CD4 monolayer. In a recent study, we showed that gp120 binds to GM3 through an interaction with the V3 loop (16). Thus, experiments were conducted to assess whether a trimolecular complex could be formed between GM3, CD4 and gp120. The addition of recombinant soluble CD4 under a monolayer of human GM3 resulted in a progressive increase in the surface pressure which followed a logarithmic curve fit (Figure 2A). The addition of gp120 induced an upsetting of the equilibrium until reaching a new plateau value corresponding to a net increase of 3.9 mN/m. In absence of CD4, the effect of gp120 at the same concentration (1.85 nM) on a monolayer of GM3 at the same initial surface pressure was negligible (<0.5 mN/m). Moreover, the surface pressure increase induced by gp120 on the GM3/CD4 monolayer was totally abrogated when the viral glycoprotein was preincubated with 110-H, a monoclonal antibody directed to the V3 loop (Figure 2B). However, the monolayer was fully functional since a marked surface pressure increase was obtained with the V3 loop peptide SPC3. Finally, when bovine instead of human GM3 was used, recombinant soluble CD4 induced only a very weak increase in surface pressure, while gp120 had no effect (data not shown). Interestingly, the V3 loop peptide SPC3 could not discriminate between the bovine and human GM3, confirming the poor specificity of this peptide compared with the native V3 loop (16).

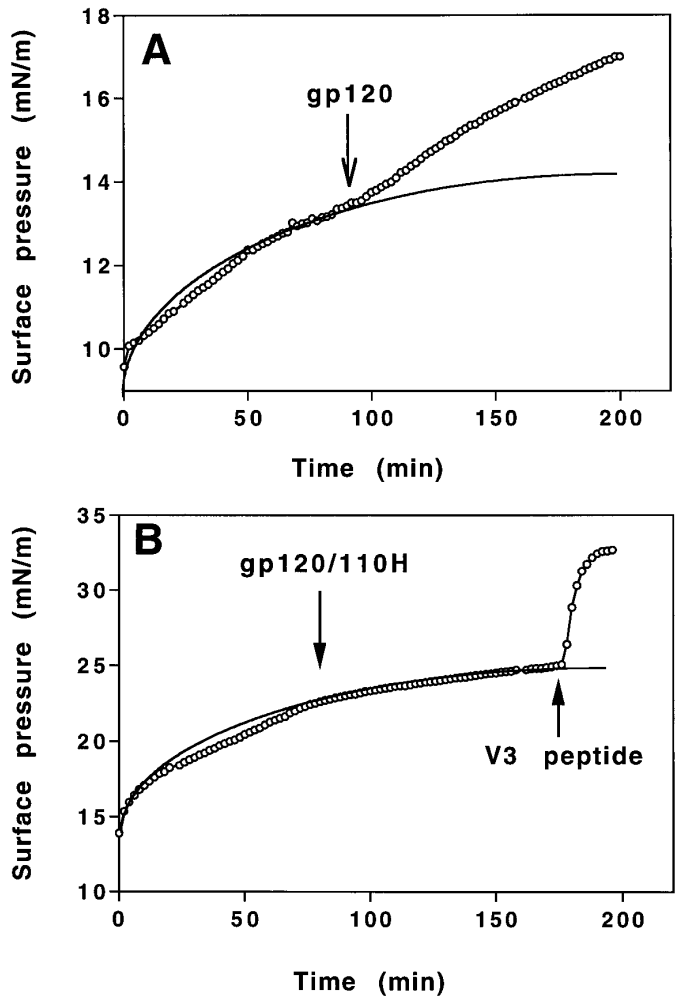

FIG. 2. CD4-induced interaction of gp120 with GM3. (A) At time 0, recombinant soluble CD4 (0.5 ng/ml) was added under a monolayer of human GM3. The increase in surface pressure induced by CD4 followed a logarithmic curve fit as indicated. The addition of gp120 (1.85 nM) induced a further increase in surface pressure. (B) In this experiment, gp120 was preincubated with the anti-V3 mAb 110H for 30 min at 37°C before its addition underneath the GM3 monolayer. In this case, no further increase in surface pressure was noted. However, the monolayer was fully functional since a marked surface pressure increased was obtained with the V3 loop peptide SPC3 (300 nM).

DISCUSSION

Lipid-protein interactions can be studied by measuring the surface pressure increase induced by the protein added in the aqueous phase under a monomolecular film of lipid (19). According to this model, the insertion of the protein in the lipid monolayer results in a compression of the monomolecular film which can be measured with a Langmuir balance (19,20). Since the compressibility of the monolayer decreases as its initial surface pressure increases, the specificity of the interaction can be demonstrated by performing the experiment at various surface pressures (Figure 1, inset). Using this approach, we could demonstrate that recombinant soluble CD4 interacts specifically with GM3, a

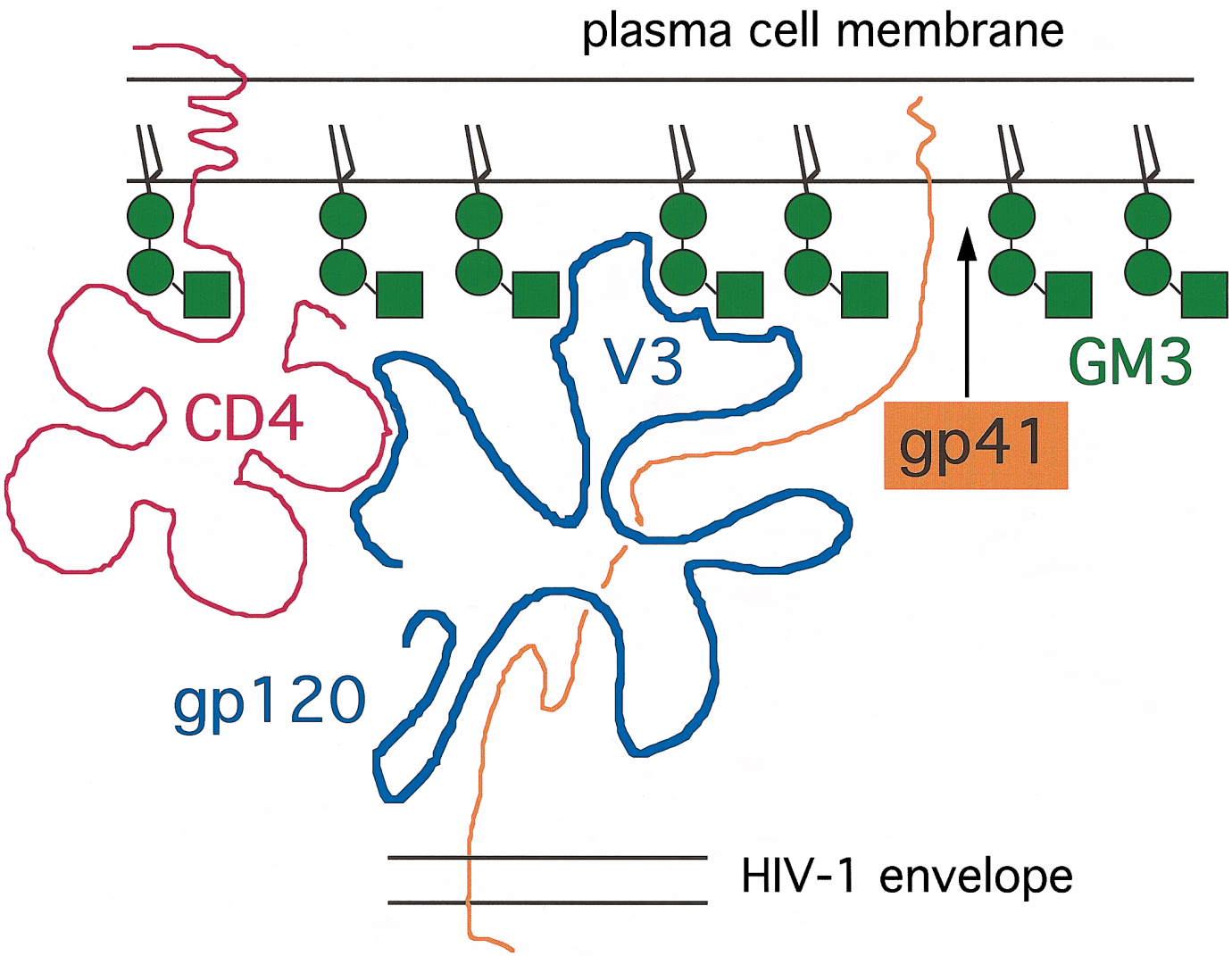

FIG. 3. Interaction of CD4 and gp120 with a patch of GM3. The binding of gp120 to CD4 triggers a conformational change within the V3 loop. The high concentration of GM3 around CD4 is consistent with the establishment of secondary interactions between the V3 loop and GM3. The binding of the V3 loop to the plasma membrane of the target cell allows the fusion peptide of gp41 to penetrate the cellular membrane.

ganglioside expressed in various cell types and particularly abundant in the plasma membrane of human lymphocytes where it represents up to 70% of the total ganglioside. Recently, Sorice et al. reported that GM3 and CD4 are co-localized in the same detergent-insoluble microdomain of the lymphocyte plasma membrane, and that the ganglioside was co-immunopurified by anti-CD4 antibodies (23). These data, which supported the possibility of a GM3-CD4 interaction, can now be explained by our study which presents for the first time an experimental evidence that CD4 binds to GM3. The lack of interaction of CD4 with other gangliosides including mono- and di-sialylated species (Table 1) demonstrates that the association does not rely on weak electrostatic interactions with sialic acid residues. Interestingly, GM3 purified from bovine milk showed a very poor interaction with human CD4. Human and bovine GM3 have the same oligosaccharide motif, but differ in their ceramide moiety and especially in its fatty acid composition (24). The nature of the fatty acid influences the orientation of the sugar part of glycolipids and, consequently, their binding properties (25,26). Thus, it is likely that the lack of activity of bovine GM3 is due to an inadequate conformation of its oligosaccharide moiety.

One of the major outcome of this study is that gp120 increases the surface pressure of a human GM3 monolayer that has been previously incubated with CD4. This pressure increase demonstrates the insertion of gp120 into the monomolecular film of GM3. Although

gp120 can interact with a GM3 monolayer in absence of CD4 (16), this is not the case under the experimental conditions of Figure 2A. Indeed, when the experiment was performed without CD4, addition of gp120 at the same concentration (1.85 nM) did not result in compression of the monomolecular film of GM3. Thus, these data suggest that gp120 binds first to CD4, and subsequently interacts with GM3 through a domain distinct from the CD4-binding site. The inhibition of the surface pressure increase by an anti-V3 mAb (Figure 2B) strongly suggests that this domain is actually the V3 loop.

According to recent data suggest that the main function of HIV-1 coreceptors may be to provide a cellular binding site for the V3 loop (27,28). This step would be necessary to ensure the correct orientation of the fusion peptide, in the N-terminal part of gp41, towards the plasma membrane of target cells (27). In absence of a V3 loop-binding site, gp41 remains associated with gp120 in the viral spike, and the fusion process cannot start. In the plasma membrane of $CD4^+$ lymphocytes and macrophages, it is clear that the major fusion cofactors are CXCR4 and CCR5 (5). Most striking is the observation that heat- and protease-resistant membrane components from human red blood cells can stimulate CD4-dependent HIV-1 fusion (3,4). In absence of any potential HIV-1 coreceptor in these cells (the erythrocyte chemokine receptor, i.e. the Duffy antigen, does not promote HIV-1 fusion (29)) one can hypothesize that erythrocyte glycolipids are responsible for the observed enhancement of fusion. This hypothesis was recently confirmed by Puri et al. (17) who observed that non-human $CD4^+$ cells become competent for CD4-dependent HIV-1 fusion following transfer of human red blood cells glycolipids. We believe that GM3 is the most likely candidate for a glycolipid fusion cofactor, based on the following data: i) GM3 is associated with CD4 in the plasma membrane (23); ii) like the previously characterized HIV-1 coreceptors, it is recognized by the V3 loop of HIV-1 gp120; iii) its organization in patches at the cell surface (30) is consistent with the formation of a multimolecular complex between CD4 and gp120 (Figure 3); iv) its bovine counterpart is not recognized by CD4. Noteworthy, glycolipids such as GalCer, which recognize the V3 loop of gp120 (31) but not CD4, do not promote the CD4-dependent fusion of HIV-1 (17). We are aware that our experiments, together with those of other laboratories (17) demonstrate that human erythrocyte glycolipids and most likely GM3, may function as surrogate fusion cofactors *only* in cells expressing CD4 but lacking the HIV-1 coreceptor activity (Figure 3). However, several lines of evidence suggest a role for glycolipids in the fusion between HIV-1 and $CD4^+$ lymphocytes and/or macrophages: i) HIV-1 infection of these cells is blocked by SPC3 (32), a multimeric V3 loop peptide which binds to GM3, but not to CD4, CXCR4 or CCR5 (15); ii) the N-acetylation of the eight GPGRAF motifs of SPC3 results in a total loss of antiviral activity, which correlates with decreased affinity for GM3 (data not shown); iii) HIV-1 infection of $CD4^+$ cells is affected by inhibitors of glycolipid biosynthesis (17,33), possibly through masking of CD4 (34). The association of CD4 with a patch of GM3, freely moving in the outer leaflet of the plasma membrane, may facilitate the migration of the CD4-gp120 complex towards CXCR4 or CCR5. Moreover, the binding of the V3 loop of gp120 to GM3 may stabilize the complex until a functional interaction between gp120 and its corresponding coreceptor (CXCR4 or CCR5) can take place. Further studies are warranted to determine whether HIV-1 coreceptors may also interact with GM3 patches on the plasma membrane of $CD4^+$ cells.

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