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Role of glycosphingolipid microdomains in CD4-dependent HIV-1 fusion

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The fusion of HIV-1 with the plasma membrane of CD4$^+$ cells is triggered by the interaction of HIV-1 surface envelope glycoprotein gp120 with the CD4 receptor, and requires coreceptors (CCR5 and CXCR4). Recent advances in the study of HIV-1 entry into CD4$^+$ cells suggest that glycosphingolipids (GSL) may also participate in the fusion process. GSL are organized in functional microdomains which are associated with specific membrane proteins such as CD4. GSL-enriched microdomains were purified from human lymphocytes and reconstituted as a monomolecular film at the air–water interface of a Langmuir film balance. Surface pressure measurements allowed to characterize the sequential interaction of GSL with CD4 and with gp120. Using this approach, we identified globotriaosylceramide (Gb3) and ganglioside GM3 as the main lymphocyte GSL recognized by gp120. In both cases, the interaction was saturable and dramatically increased by CD4. We propose that GSL microdomains behave as moving platforms allowing the recruitment of HIV-1 coreceptors after the initial interaction between the viral particle and CD4. According to this model, the GSL microdomain may : i) stabilize the attachment of the virus with the cell surface through multiple low affinity interactions between the V3 domain of gp120 and the carbohydrate moiety of GSL, and ii) convey the virus to an appropriate coreceptor by moving freely in the outer leaflet of the plasma membrane. This model can be extrapolated to all envelope viruses (e.g. influenza virus) that use cell surface GSL of the host cells as receptors or coreceptors.

Keywords: **glycolipids, HIV-1, air–water interface monolayer, fusion, membrane, CD4**

Introduction

HIV-1 is an enveloped virus which fuses with the plasma membrane to deliver its genomic RNA into the host cells. The entry of HIV-1 into cells requires the sequential interaction of the viral surface envelope glycoprotein gp120 with the CD4 receptor and a coreceptor (or a fusion cofactor) on the cell surface [1]. The coreceptors identified so far for human and simian immunodeficiency viruses include chemokine receptors (mainly CXCR4, CCR5, CCR3, CCR2b) and a series of orphan receptors including virus-encoded receptors all belonging to the family of seven-transmembrane domains receptors [2]. Following a primary interaction with CD4, a conformational change in gp120 renders cryptic regions of the viral glycoprotein (including the V3 domain) available for secondary interactions with either CXCR4 or CCR5. As seven-transmembrane domains receptors are almost flush with the cell membrane, binding of gp120 to the coreceptor is necessary to move the viral spike close to the target membrane. Finally, gp120-coreceptor interactions trigger additional conformational changes in the HIV-1 envelope glycoprotein trimer that lead to exposure of the fusion peptide at the N-terminus of the transmembrane glycoprotein gp41 [2]. Overall, these molecular rearrangements brings the transmembrane anchor (in the viral membrane) and the hydrophobic fusion peptide at the same end of a long rod-shaped molecule [3].

The involvement of cellular glycosphingolipids (GSL) in the attachment and fusion of enveloped viruses has been recognized for a long time [4]. GSL are ubiquitous membrane components located almost exclusively at the outer leaflet of the plasma membrane. All GSL share a common hydrophobic backbone dipped in the membrane, i.e. ceramide (Cer) which consists of a fatty acid chain linked to the sphingosine base [5]. In contrast, the hydrophilic oligosaccharide residues of GSL protrude into the extracellular space. GSL are classified into three main series, i.e. ganglio-, globo- and lacto-series, according to their carbohydrate structure which may include

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one of two hundred different oligosaccharides. In the outer leaflet of the plasma membrane, glycolipids organize into moving platforms, or rafts, onto which specific proteins attach within the bilayer [6]. This lateral organization probably results from preferential packing of sphingolipids and cholesterol, based on physico-chemical properties of their hydrophobic and hydrophilic parts [7]. Consequently, sphingolipids-cholesterol rafts are insoluble in the detergent Triton X-100 at 4°C and those detergent-insoluble membranes (DIMs) can be purified by centrifugation on a sucrose-density gradient [5]. In this report, we propose a model for HIV-1 fusion that takes into account the interaction of the virus with GSL microdomains.

HIV-1-induced perturbations of GSL metabolism

With the aim to determine the involvement of GSL microdomains in HIV-1 infection, we first studied the biosynthetic metabolic pathways of GSL in normal and HIV-1-infected peripheral blood mononuclear cells (PBMC). In these experiments, the GSL composition of human PBMC isolated from normal or infected individuals was analyzed after metabolic labeling of the cells with [^{14}C]galactose [8]. After lipid extraction, the neutral and acidic GSL were partitioned according to the Folch procedure and resolved by HPTLC (Fig. 1). The neutral GSL of the Folch lower phase of normal PBMC were composed mainly of four types of lipids : ceramide monohexoside (GlcCer), ceramide dihexoside (LacCer), ceramide trihexoside (Gb3) and tetraosylceramide (Gb4). Compared with PBMC from seronegative donors, the GSL metabolism in PBMC from HIV-1-infected individuals was characterized by an increased synthesis of two GSL, i.e. Gb3 (+28.6%) and GM3 (+96.5%). These data demonstrate that HIV-1 infection up-regulates the biosynthesis of Gb3 and GM3, suggesting the involvement of these GSL in HIV-1 replication.

Purification and characterization of GSL-enriched microdomains from human PBMC

Plasma membrane microdomains were isolated from 2×10^9 PBMC from seronegative donors by centrifugation of Triton X-100 insoluble material on a sucrose density gradient [9]. The microdomains were recovered as molecular complexes from the buoyant fractions (12.8–14.0% sucrose) in agreement with previously characterized DIMs [9]. The glycolipid analysis of DIMs from PBMC demonstrated the presence of GM3 and GD3 as the main ganglioside species. Neutral GSL were essentially ceramide monohexoside (GlcCer), ceramide dihexoside (LacCer), ceramide trihexoside (Gb3), and ceramide tetrahexoside (Gb4). Thus, most of the GSL synthesized by human PBMC (Fig. 1) are recovered in membrane microdomains which can be purified on the basis of their lack of solubility in Triton X-100 at 4°C.

Interaction of CD4 with GSL-enriched microdomains

In the plasma membrane of human T-lymphocytes, CD4 and GM3 are co-localized in the same detergent-insoluble microdomain [10,11]. These data raise the interesting possibility that CD4 may bind to some GSL and that this interaction could stabilize the association of CD4 with the microdomain. In order to investigate the potential interaction between CD4 and GSL, the lipids were spread at the air–water interface of a Langmuir film balance and the interaction of soluble CD4 added underneath the monomolecular film was analyzed by surface pressure measurements [12]. As shown in Figure 2, the addition of CD4 under a GM3 monolayer resulted in a maximal pressure increase of 11.8 mN/m. To

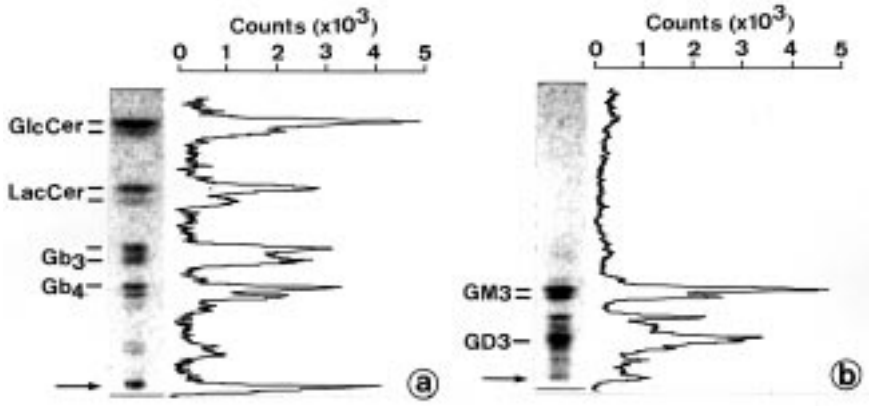

Figure 1. Patterns of [^{14}C]galactose-labeled neutral GSL and gangliosides from normal human PBMC. The cells were metabolically labeled with [^{14}C]galactose for 16 hr. GSL were then extracted, purified, separated by HPTLC and analyzed with a phosphoimager. The pattern of neutral GSL (**a**) and gangliosides (**b**) is shown with the corresponding quantitative scanning of the HPTLC plate. The position of standard GSL ran in parallel and stained with orcinol is indicated in the margin. The material left at the depot point (arrow) corresponds to minor nonlipid components, presumably proteins, that are carried over during Folch extraction.

investigate the specificity of the penetration process, the increase in surface pressure ($\Delta\Pi$) caused by the addition of CD4 under the GM3 monolayer was measured at various initial surface pressures. As shown in the 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 [13].

To rule out the possibility that the surface pressure increase could be due to an impurity in the recombinant preparation, CD4 was depleted with anti-CD4 antibodies and protein-A Sepharose. The CD4-depleted supernatant did no longer react with the GM3 monolayer. In contrast, the activity of CD4 was not altered by immunoprecipitation with control anti-HLA antibodies. Moreover, when CD4 was preincubated with 3′sialyllactose (the oligosaccharide corresponding to the sugar moiety of GM3), there was no increase in the surface pressure of the GM3 monolayer. The specificity of 3′sialyllactose as an inhibitor of GM3/CD4 association was demonstrated by the incapacity of the oligosaccharide to affect the interaction of cholera toxin with a reconstituted patch of its ganglioside receptor GM1. Taken together, these data suggest that CD4 binds to GM3 through a specific interaction with the carbohydrate domain of the GSL. Similar results were obtained with a monolayer of Gb3 [14]. Overall, GM3 and Gb3 were the only GSL from PBMC microdomains that interacted with CD4 in the monolayer assay. These data provide a molecular basis for explaining the recovery of CD4 with plasma membrane microdomains purified from human lymphocytes.

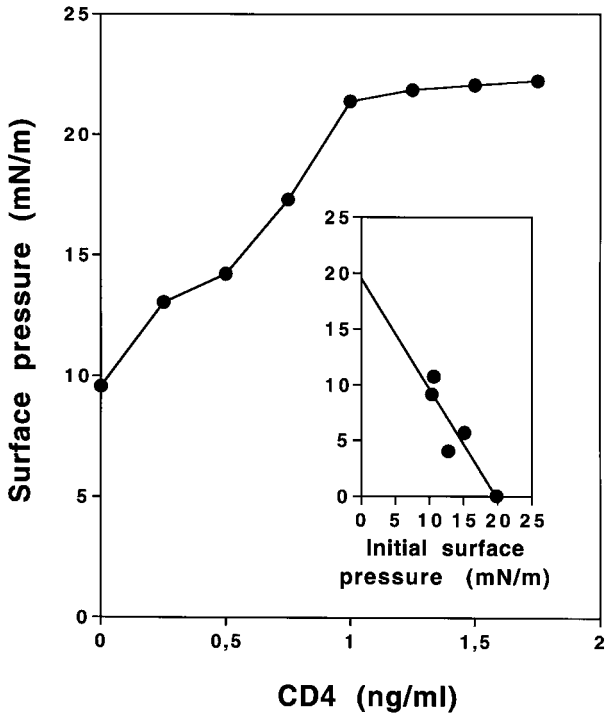

Figure 2. Variations in surface pressure of a monolayer of human GM3 after injection of soluble recombinant CD4. Inset: Maximal surface pressure increase reached after injection of soluble recombinant CD4 (1 ng/ml) under a monomolecular film of human GM3 at various initial surface pressures.

Interaction of gp120 with GSL purified from DIMs

Previous data from our laboratory demonstrated that the HIV-1 surface envelope glycoprotein gp120 binds to various GSL through an interaction between the V3 domain of the viral glycoprotein and the carbohydrate moiety of the GSL [15,16]. Thus, we analyzed the interaction of gp120 with the GSL extracted from PBMC microdomains. Following purification on sucrose density gradient, the DIMs were dissolved in chloroform:methanol (1:1, vol:vol), dried under a chemical hood, and eventually resuspended in hexane:chloroform:ethanol (11:5:4, vol:vol:vol). The lipids were spread at the air–water interface of a microtensiometer trough (μTrough SX, Kibron Inc.) and the variations of surface pressure ($\Delta\Pi$, expressed in mN/m) induced by gp120 added in the aqueous subphase were measured as a function of time. The insertion of gp120 in the lipid monolayer resulted in a compression of the monomolecular film which could be measured with the sensor probe of the microtensiometer. A typical experiment is shown in Figure 3. Recombinant gp120 (IIIB isolate) was added at a concentration of 8 nM underneath the monolayer of GSL extracted from PBMC microdomains. The maximal surface pressure increase (8.07 mN/m in this assay) was reached after 240 minutes of incubation. The interaction of gp120 from various HIV-1 isolates with GSL purified from PBMC microdomains is indicated in Table 1. All the tested viral glycoproteins could recognize these GSL, although with differences in the binding capacity. By using monolayers of pure GSL, we could determine that the most potent GSL in the microdomain fraction were GM3 and Gb3 [14]. Therefore, we concluded that GSL-enriched microdomains on the cell surface of human PBMC may bind HIV-1 through gp120 interactions with Gb3 and/or GM3.

Reconstitution of a functional HIV-1 fusion complex

As discussed above, GSL-enriched microdomains on the surface of $CD4^+$ cells are likely to participate in the HIV-1 fusion process because i) they are associated with the CD4 receptor [10,11], and ii) they are recognized by the V3 domain of HIV-1 gp120 [16]. Using the monolayer approach, we have been able to visualize the sequential interaction of a reconstituted microdomain of GSL with CD4 and gp120 [12,14]. A typical experiment is shown in Figure 4. The ordered addition of CD4 and gp120 at suboptimal concentrations induced a biphasic increase of the surface pressure of a Gb3 monolayer. The first response corresponded to the insertion of CD4 in the Gb3 patch, and the second one to

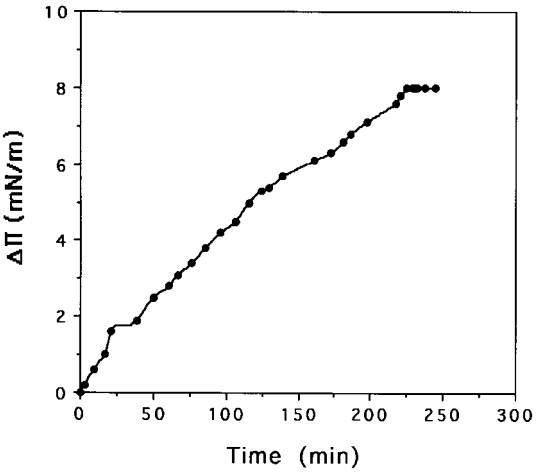

Figure 3. Interaction of HIV-1 gp120 with GSL extracted from PBMC microdomains. A monolayer of GSL extracted from PBMC microdomains (DIMs) was prepared at the air–water interface and recombinant gp120 (IIIB isolate) was then added in the aqueous phase at a concentration of 8 nM. The kinetics of the surface pressure increase induced by the viral glycoprotein are shown.

Table 1. Interaction of gp120 from various HIV-1 isolates with GSL extracted from PBMC microdomains.

HIV-1 isolate	$\Delta \prod max$ *(mN/m)*
IIIB (laboratory X4 isolate)	8.23
SEN (primary R5X4 isolate)	5.56
89.6 (primary R5X4 isolate)	7.48
NDK (laboratory X4 isolate)	4.07

HIV-1 gp120 from the indicated isolates were purified by lectin-affinity chromatography [16]. HIV-1 isolates are functionally classified with respect to their ability to use a given coreceptor : viruses using CXCR4 but not CCR5 are referred to as X4 isolates, whereas isolates using CCR5 but not CXCR4 are called R5. Dual-tropic viruses able to use either CXCR4 or CCR5 are referred to as R5X4. The results are expressed as the mean surface increase (n = 2, S.D. < 10%) induced by the indicated gp120 added in the aqueous subphase of a monomolecular film of GSL purified from PBMC microdomains. A typical experiment is shown in Figure 3.

the CD4-induced penetration of gp120, through its V3 domain, in the glycolipid monolayer. Similar data were obtained with GM3 [12]. In this case, the gp120-induced increase of surface pressure was specifically abrogated with anti-V3 antibodies, confirming that the V3 domain of gp120 interacts with the GSL. Finally, it is important to note that in the presence of CD4, gp120 from HIV-1(IIIB) (a T-cell line adapted X4 isolate) interacted preferentially with Gb3, whereas gp120 from HIV-1(89.6) (a dual-tropic, primary R5X4 isolate) interacted exclusively with GM3. Therefore, HIV-1 isolates might select GSL of their choice in addition to the chemokine receptors in the early steps of viral fusion [14].

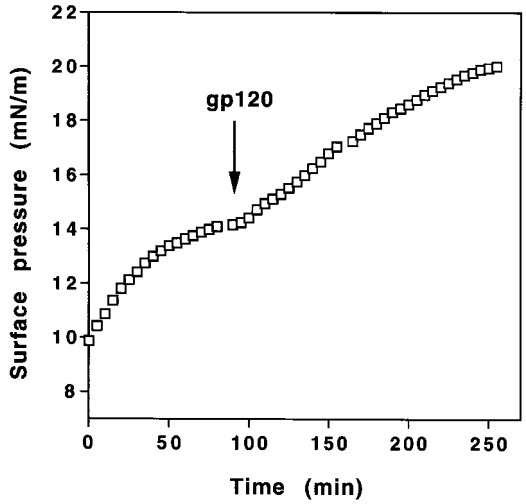

Figure 4. CD4-induced interaction of HIV-1 gp120 with a reconstituted microdomain of Gb3. At time 0, recombinant CD4 (0.5 ng/ml) was added under a monolayer of human Gb3 prepared at an initial surface pressure of 10 mN/m. The first increase in surface pressure ($\Delta\Pi$) is due to the interaction of CD4 with the monolayer of Gb3. After reaching a plateau value, HIV-1 gp120 was added at a concentration of 1.85 nM. Secondary gp120-Gb3 interactions are evidenced by a second phase of surface pressure increase.

Role of GSL-enriched microdomains in HIV-1 fusion

Several lines of evidence support the concept that HIV-1 fusion occurs in GSL-enriched microdomains of the plasma membrane of $CD4^+$ cells. i) The CD4 receptor interacts with GM3 and Gb3, and is accordingly localized in GSL-enriched microdomains [10–12,14]. Indeed, GM3 and Gb3 are highly expressed in human macrophages but are also present in $CD4^+$ lymphocytes and in T-cell lines [8,15]. ii) Inhibitors of glycolipid biosynthesis affects HIV-1 infection through cell surface masking of CD4 [17]. iii) Restoration of CD4/CXCR4-dependent HIV-1 fusion after GSL depletion is obtained by complementation with Gb3 purified from human erythrocytes [18–20]. iv) HIV-1 infection increases GM3 and Gb3 levels in naturally-infected human PBMC [8]. v) HIV-1 gp120 binds to several GSL, including GM3 and Gb3 [14,16]. vi) V3-derived synthetic peptides inhibit HIV-1 infection through interaction with cellular GSL [15]. vii) Synthetic analogs of GSL bind to the V3 domain of HIV-1 gp120 and block HIV-1 fusion [21,22].

From a molecular point of view, GSL able to interact with both CD4 and gp120 (i.e. GM3 or Gb3) may induce the formation of a trimolecular complex CD4-GSL-gp120 [14]. The role of the GSL in this multimolecular organization could be to facilitate the migration of the CD4-gp120 complex to an appropriate coreceptor. Indeed, CD4 and the CXCR4 co-receptor are not physically associated in absence of gp120 [23]. The situation is more complex for CCR5 since this coreceptor may directly interact with CD4 even in absence of HIV-1 [24]. Given the privileged association of CD4 with

GSL-enriched microdomains, this would suggest the existence of specific microdomains bringing together CD4 and CCR5 in the same restricted area of the plasma membrane. We are currently tempting to characterize such microdomains. In the model we propose for HIV-1 fusion, we consider the GSL patch as a raft dragging the CD4 receptor and taking aboard the viral particle (Fig. 5). The binding of the virion to the raft is stabilized by secondary interactions between the polar heads of glycolipid molecules and the V3 loop of gp120. The raft may then float on the cell surface until finding an adequate coreceptor which can displace the glycolipid-V3 loop interactions to its own profit, resulting in the initiation of the fusion process. This coreceptor may be either associated or not with a GSL microdomain. In absence of any available coreceptor, the GSL may eventually allow the conformational change of gp41, as may be the case for human erythrocyte glycolipids transferred into murine cells expressing human CD4 [19]. In this model, the stabilization of the virus onto the GSL moving platform results from multiple low affinity interactions between the V3 domain of gp120 and the carbohydrate moiety of GM3 and/or Gb3. This would allow the HIV-1 particle to 'browse over the cell surface' [4] until finding a second receptor exhibiting a high affinity for the V3 loop (i.e. CCR5 or CXCR4, according to the tropism of the virus and the nature of the GSL microdomain chosen by the virus). A very convincing study published by R. Blumenthal and co-workers [19] has demonstrated the requirement of Gb3 in CD4/CXCR4-dependent HIV-1 entry. These data fit with our biochemical results and support the HIV-1 fusion model described here.

GSL-enriched microdomains and virus-cell fusion: a unitary model

The binding of viral envelope glycoproteins to GSL may involve a complex network of molecular interactions between the glycoprotein and several domains of the GSL, including both the polar carbohydrate head and the ceramide moiety. This would lead to a two-step binding process with i) the carbohydrate part of the GSL (primary interaction), and ii) the hydrophobic core of the GSL (secondary interaction). As previously discussed by Haywood [4], the secondary binding step would strenghten viral adhesion and prepare the fusion event. The underlying idea is that GSL binding sites for enveloped viruses might represent low affinity receptors whose function would be to maintain the virus attached to the cell surface until it could find a favorable site for fusion. We believe that it is the ability of GSL microdomains to move freely in the outer leaflet of the plasma membrane that allows the virus to reach a convenient fusion site before its possible desorbtion from the cell surface. Multiple low affinity interactions between the viral spikes and several GSL molecules of the moving platform are required for this stabilization process. The reproducible observation that a minimal amount of GSL molecules is required to mediate viral attachment to GSL-containing liposome membranes [25,26] or reconstituted GSL monolayers [27] is consistent with this view. In this context, it should be recalled that initial studies of influenza virus binding led to the suggestion that viruses could move freely ('browse') over the cell surface [28]. A particularly interesting comparison of this process with the rolling of lymphocytes on the surface of endothelial cells has been proposed by Haywood [4].

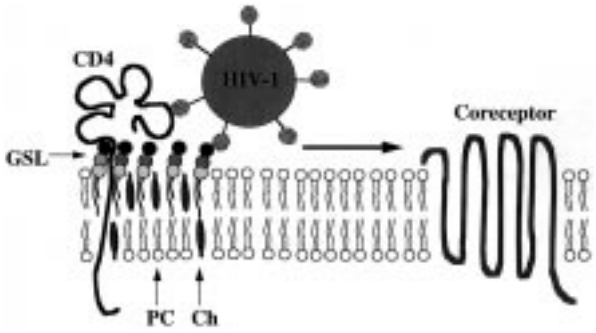

Figure 5. Plasma membrane glycosphingolipid microdomains as preferential sites of formation of the HIV-1 fusion complex. In the plasma membrane of $CD4^+$ cells, CD4 is present in microdomains enriched in GSL (GM3/Gb3) and cholesterol. Once bound to CD4, the viral particle is conveyed to an appropriate coreceptor by the GSL raft which moves freely in the outer leaflet of the plasma membrane. Ch, cholesterol; GSL, glycosphingolipid; PC, phosphatidylcholine.

Conclusion

GSL have been recognized for a long time as receptors for various enveloped viruses. However, the impact of their self-organization in membrane microdomains on their receptor function has received scant appraisal. Our data allowed us to propose a model for viral adhesion in which GSL platforms stabilize and convey the viral particles towards a high affinity receptor able to trigger the fusion process. The interaction of enveloped viruses with GSL microdomains may be inhibited by synthetic analogs of GSL [22] or by synthetic peptides derived from the GSL-binding domain of viral envelope glycoproteins [15]. The design of such inhibitors may reinforce the current arsenal of antiviral drugs.

Acknowledgements

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Note added at proof:

During the submission of this manuscript, Robert Blumenthal and coworkers published a very elegant study confirming that GSL may play an active role in HIV-1 fusion (*P. Hug, H-M.J. Lin, T. Korte, X. Xiao, D.S. Dimitrov, J.M. Wang, A. Puri, R. Blumenthal, J Virol 74, 6377–6385, 2000*). According to their model, secondary interactions between the V3 loop of gp120 and the polar heads of GSL molecules (Gb3 and, to a lesser extent, GM3) may induce the conformational changes in gp120–gp41 that trigger the fusion process. These data are consistent with our biochemical and biophysical studies and further support the concept that the assembly of the HIV-1 fusion complex takes place in plasma membrane microdomains enriched in specific GSL.