[3] R. W. Alder, S. P. East, *Chem. Rev.* **1996**, *96*, 2097.
[4] R. W. Alder, *Tetrahedron* **1990**, *46*, 683.
[5] R. W. Alder, A. G. Orpen, R. B. Sessions, *J. Chem. Soc. Chem. Commun.* **1983**, 999.
[6] R. W. Alder, N. C. Goode, T. J. King, J. M. Mellor, B. W. Miller, *J. Chem. Soc. Chem. Commun.* **1976**, 173; R. W. Alder, R. J. Arrowsmith, A. Casson, R. B. Sessions, E. Heilbronner, B. Kovac, H. Huber, M. Taagapera, *J. Am. Chem. Soc.* **1981**, *103*, 6137.
[7] R. W. Alder, E. Heilbronner, E. Honegger, A. B. McEwen, R. E. Moss, E. Olefirowicz, P. A. Petillo, R. B. Sessions, G. R. Weisman, J. M. White, Z.-Z. Yang, *J. Am. Chem. Soc.* **1993**, *115*, 6580.
[8] J. G. Verkade, *Acc. Chem. Res.* **1993**, *26*, 483; J. G. Verkade, *Coord. Chem. Rev.* **1994**, *137*, 233; X. Liu, J. G. Verkade, *J. Org. Chem.* **1999**, *64*, 8058, and references therein.
[9] Y. T. Chen, K. K. Baldridge, D. M. Ho, R. A. Pascal, Jr., *J. Am. Chem. Soc.* **1999**, *121*, 12082.
[10] Gaussian 98, Revision A.7, M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, V. G. Zakrzewski, J. A. Montgomery, Jr., R. E. Stratmann, J. C. Burant, S. Dapprich, J. M. Millam, A. D. Daniels, K. N. Kudin, M. C. Strain, O. Farkas, J. Tomasi, V. Barone, M. Cossi, R. Cammi, B. Mennucci, C. Pomelli, C. Adamo, S. Clifford, J. Ochterski, G. A. Petersson, P. Y. Ayala, Q. Cui, K. Morokuma, D. K. Malick, A. D. Rabuck, K. Raghavachari, J. B. Foresman, J. Cioslowski, J. V. Ortiz, A. G. Baboul, B. B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R. Gomperts, R. L. Martin, D. J. Fox, T. Keith, M. A. Al-Laham, C. Y. Peng, A. Nanayakkara, C. Gonzalez, M. Challacombe, P. M. W. Gill, B. Johnson, W. Chen, M. W. Wong, J. L. Andres, C. Gonzalez, M. Head-Gordon, E. S. Replogle, J. A. Pople, Gaussian, Inc., Pittsburgh, PA, **1998**.
[11] A. E. Reed, L. A. Curtiss, F. Weinhold, *Chem. Rev.* **1988**, *88*, 899.
[12] R. W. Alder, C. Ganter, M. Gil, R. Gleiter, C. J. Harris, S. E. Harris, H. Lange, A. G. Orpen, P. N. Taylor, *J. Chem. Soc. Perkin Trans. 1* **1998**, 1643.

Gp120 Binds Cooperatively to Several Biologically Relevant Glycosphingolipids: Quantitative Measurements at Equilibrium by Total Internal Reflection Fluorescence Microscopy**

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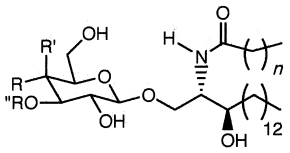
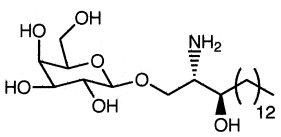
Understanding the molecular events responsible for HIV-1 viral recognition and binding to receptors expressed on the host cell is critical to developing treatment strategies based on HIV-1 inhibition. The virus is encapsulated by a lipid bilayer which supports the envelope glycoprotein gp160. This protein is comprised of two noncovalently linked subunits: 1) a 41-kD transmembrane protein (gp41) which anchors the assembly in the viral membrane and 2) a 120-kD protein (gp120) which coats the outer surface of the viral particle and is responsible for initial recognition and attachment to the host cell.[1]

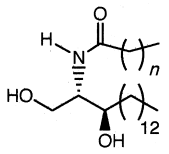
HIV-1 infection of a host cell can be initiated by gp120 binding to galactosyl ceramide (GalCer), a glycosphingolipid (GSL) expressed in human vaginal epithelial, colonic epithelial, and sperm cells.[2–7] The literature on gp120-GSL binding activity is surprisingly inconsistent. Harouse et al.[3, 4] and Bhat et al.[8] used high-performance thin-layer chromatography (HPTLC) and enzyme-linked immunosorbent assays (ELISA) to study the binding of several glycolipids to rgp120 including GalCer (**1**), galactosyl sulfatide (GalS, **2**), glucosyl ceramide (GlcCer, **3**), lactosyl ceramide (LacCer, **4**), psychosine (**5**), and ceramide (Cer, **6**), and found that **1**, **2**, and



R=H, R'=OH, R"=H: GalCer **1**
R=H, R'=OH, R"=SO_3^-: GalS **2**
R=OH, R'=H. R"=H: GlcCer **3**
R=*O*-β (1-4)-D-Gal, R'=H, R"=H: LacCer **4**



Psychosine **5**



Ceramide **6**

5 bound. Latov and co-workers used immunospot assays on nitrocellulose and TLC plates as well as ELISA to test the binding affinity of **1** and **2** for recombinant gp120 (rgp120).[5] They reported that in both the nitrocellulose immunospot assay and ELISA **2** bound to rgp120 but **1** did not. However, in the immunospot TLC assay, both **1** and **2** bound. Long et al.[9] studied interactions of rgp120 and liposomes doped with various glycolipids (**1**–**5** and sphingomyelin). They reported that **1** bound to rgp120 strongly, **2**–**4** were less efficient binders, and **5** was inactive. More recently, McReynolds et al.[10] found that rgp120 binds to **1**, **3**, and **4**, with **4** being the preferred receptor. In contrast, Hammache et al. have reported minimal binding to **3** and **4**.[11]

It is evident that considerable confusion exists regarding the relative affinities of gp120 for GalCer and similar lipids. Furthermore, quantitative studies of gp120–glycolipid binding have not been performed, and thus the structural variations among these GSLs that are responsible for the apparent differences in affinity for gp120 are not known. The confusion may be in part a result of the inherent limitations of the binding assays used to study gp120–GSL interactions to date. Employing a heterogeneous solid-phase assay (for example, ELISA) to quantitatively characterize protein ligand–receptor binding processes that occur in vivo at the extracellular surface of a plasma membrane is fraught with difficulties. Although such methods are useful in screening for such interactions, proper geometric presentation of a mem-

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brane-bound receptor at a defined surface coverage in a bioactive conformation is not possible. Furthermore, none of these methods can provide a quantitative measurement of the binding affinity at equilibria, which makes comparisons of binding data obtained in different laboratories problematic.

Total internal reflection fluorescence (TIRF) microscopy is a well-established technique for the quantitative studies of the behavior of proteins at interfaces.[12–15] Total internal reflection at a planar fused-silica–water interface spatially confines the excitation energy to the near surface region, which minimizes the contribution from dissolved fluors (that is, unbound ligands) to the measured fluorescence signal. In combination with the availability of methods to deposit supported phospholipid bilayers on planar fused silica substrates, a diverse array of behaviors at model membrane surfaces can be examined under equilibrium conditions.[15–19]

In an effort to elucidate the chemical and structural properties important in gp120-GSL interactions, we have measured rgp120 binding to **1**, **3**, **4**, and **6** when incorporated into the upper leaflet of a supported planar bilayer of 2-oleoyl-1-palmitoylphosphatidylcholine (POPC). Representative binding curves are plotted in Figure 1. Glycosylation of ceramide is clearly required for gp120 recognition. There is minimal adsorption of rgp120 to **6**, which lacks the terminal carbohydrate group, and virtually no binding to a pure POPC bilayer. More significantly, the structure of the carbohydrate linked to ceramide apparently has little influence on the binding activity in the high rgp120 concentration range (25–200 nM). The respective affinity constants (K_a) for rgp120 at POPC membranes bearing **3**, **4**, and **1** are 3.8×10^6, 3.6×10^6, and 7.5×10^6, respectively.[20] These results are contrary to studies that report strong binding to **1** and minimal interaction with **3** and **4**,[11] but are consistent with the ELISA results obtained by McReynolds et al.[10]

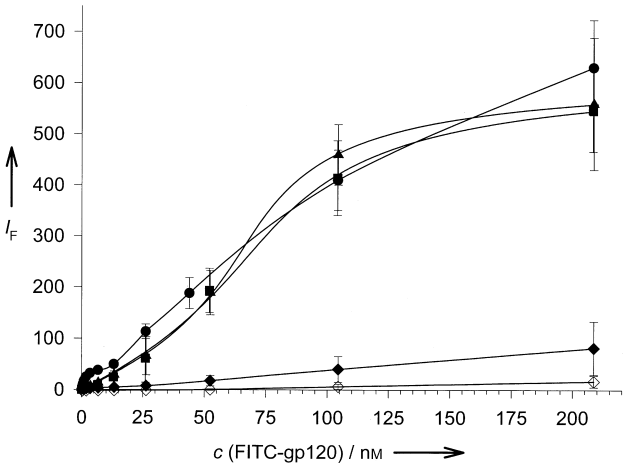

Figure 1. TIRF isotherms of FITC–rgp120 adsorption to POPC membranes, either pure (◇) or doped with 5% (mol mol^{-1}) of GalCer (●), GlcCer (■), LacCer (▲), or Cer (◆). The data plotted represent the mean and standard deviation of three independent measurements for each sample. The solid lines through the data of GlcCer and LacCer represent fits obtained with a cooperative Langmuir isotherm of the form $\omega^{F/n} K_a[\text{gp120}] = \frac{F}{1-F}$, where $F = \frac{F}{F_{max}}$ is the normalized fluorescence intensity, K_a is the association constant, and ω is an additional term to account for cooperativity in the binding process ($\omega > 1$ is cooperative, $\omega = 1$ is noncooperative, $\omega < 1$ is negative cooperativity).[21, 22] An average number of four protein-bound neighbor receptors was assumed ($n = 4$). For GlcCer and LacCer, the fitted parameters are $K_a = 3.6 \pm 0.1 \times 10^6$, $\omega = 2.0 \pm 0.3$, and $K_a = 3.8 \pm 0.3 \times 10^6$, $\omega = 1.8 \pm 0.2$, respectively. For GalCer the sum of two independent Langmuir isotherms of the form $K_a[\text{gp120}] = \frac{F}{1-F}$, where $F = \frac{F}{F_{max}}$, were used to fit the data. The isotherms were considered independent as a result of a difference of three orders of magnitude in the recovered binding constants. The K_a obtained at high concentrations of gp120 was $7.5 \pm 1.8 \times 10^6$. The solid lines through the data for Cer and POPC are shown only as a guide to the eye.

Considerably different behavior is observed at low concentrations of rgp120 (0.2–20 nM). As shown in Figure 2 **1** is clearly the stronger receptor, with a K_a of 1.6×10^9, whereas bilayers doped with **3** and **4** show only a small, linear increase in protein adsorption in this concentration regime. The strength of rgp120 binding to **1** at a POPC membrane is comparable to the binding of antibodies to surface-bound antigens ($K_a = 10^5 - 10^7$),[13, 16] and of avidin and streptavidin to a biotin-bearing lipid bilayer ($K_a = 10^7 - 10^8$).[21–23]

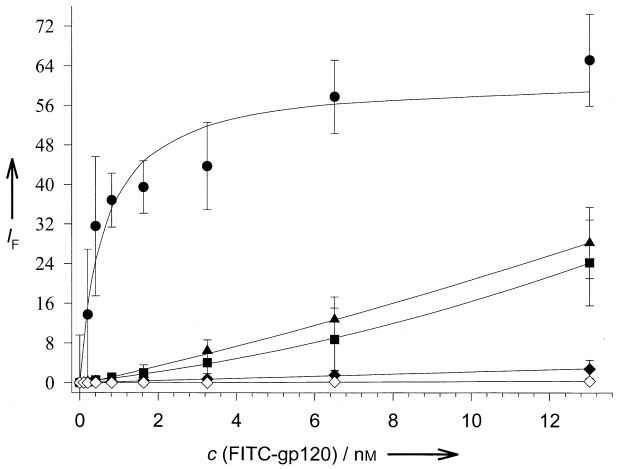

Figure 2. TIRF isotherms of FITC–rgp120 adsorption to POPC membranes, either pure (◇) or doped with 5% (mol mol^{-1}) of GalCer (●), GlcCer (■), LacCer (▲), or Cer (◆). The data plotted represent the mean and standard deviation of three independent measurements for each sample. The solid lines through the data of GlcCer and LacCer represent fits obtained with a cooperative Langmuir isotherm (see caption to Figure 1). For GalCer a single Langmuir isotherm of the form $K_a[gp120] = \frac{F}{1-F}$, where $F = \frac{F}{F_{max}}$, was used to fit the data. The association constant (K_a) at low concentrations of gp120 (0.2–20 nM) was determined to be $1.6 \pm 0.2 \times 10^9$. The solid lines through the data for Cer and POPC are shown only as a guide to the eye.

Another important difference is the mode of binding. Two models were used in fitting the isotherms plotted in Figures 1 and 2: 1) a Langmuir-like model that includes a cooperative binding process (that is, rgp120 binding promotes the binding of additional protein molecules);[21, 22] and 2) a conventional Langmuir model (that is, noncooperative, in which all binding sites are equivalent). For **3** and **4**, the data are fitted satisfactorily only when the cooperative model is used, which explains the minimal protein adsorption at low concentrations and the subsequent sharp increase at higher concentrations.

The behavior of **1** is more complex. Strong, Langmuir-like binding occurs in the low concentration regime, whereas a weaker (but also Langmuir-like) binding process is observed

at rgp120 concentrations greater than 20 nM. Comparison of these data with those for **3** and **4** suggests that the initial strong binding of gp120 to GalCer triggers the further adsorption of rgp120 at higher concentrations (namely, the binding of **1** to rgp120 at higher concentrations is cooperative). This proposal would explain the similar behavior observed for all three GSLs at high concentrations of rgp120. In other words, the strong binding event at low protein concentrations differentiates the activity of **3** and **4** from **1**.[24]

The fact that this complex binding behavior has not been observed previously is likely a consequence of the limitations of the methods that have been used to study gp120–receptor interactions in the past. However, such complex behavior is not unexpected. Previous studies have suggested the occurrence of a conformational change in gp120 upon binding to a receptor, which results in the exposure of a hydrophobic region in the protein,[25] and possibly stimulating the cooperative adsorption of additional protein. The occurrence of adsorption-induced conformational changes is a probable explanation for the similar binding observed for **1**, **3**, and **4** at high gp120 concentrations. The mechanism of cooperative binding is currently unclear. Specifically, we do not know if, in the high concentration range, additional gp120 molecules bind to glycosphingolipids, or to membrane-bound gp120 molecules, or to a combination thereof. This issue will be the subject of future investigations.

In summary, the results presented here represent the first quantitative measurements of the complex binding process between rgp120 and glycosphingolipid receptors at a membrane surface. At low concentrations of rgp120, GalCer is the preferred receptor, with an affinity constant of about 10^9, which is at the upper range of measured protein–receptor interactions at membrane surfaces. At higher concentrations, cooperative binding occurs, regardless of the chemical structure of the carbohydrate on Cer. Finally, we emphasize that the results presented here cannot be obtained from ELISA and other nonequilibrium based techniques, although they do complement and further substantiate the results reported by McReynolds et al.[10]

Experimental Section

POPC and cerebrosides were purchased from Matreya Lipids and Sigma and used as received. Rgp120 expressed in baculovirus and labeled with fluorescein isothiocyanate (FITC) was obtained from Intracel Corporation and used without further purification. The FITC:rgp120 labeling ratio was determined to be about 4:1 by UV/Vis and fluorescence spectroscopy.

Phospholipid bilayers composed of pure POPC in the inner leaflet and 19:1 POPC:cerebroside (mol mol^{-1}) in the outer leaflet were deposited on fused silica slides (Dynasil) at a surface pressure of 35 mN m^{-1} (58 Å^2 molecule^{-1}) using the Langmuir–Blodgett–Schaefer technique.[26] Nano-Pure water with a resistivity of 18 Mohms cm was used as the subphase. All depositions were carried out at 25 °C. Substrates were cleaned by sonication in 1:1 isopropanol:water, rinsing in water, soaking in 30% H_2O_2:70% concentrated H_2SO_4 for 30 min, and rinsing in water.

Supported bilayers were maintained in an aqueous environment during mounting in a micro-TIRF flowcell. The micro cell (volume of approximately 50 μL) was constructed to minimize the amount of gp120 needed to perform binding studies; details of its construction will be provided in a later publication. The flowcell was mounted on a Nikon Diaphot inverted microscope. FITC–rgp120 was injected into the flowcell and allowed to equilibrate for 30 min prior to each measurement, which was sufficient to achieve a steady-state response. Fluorescence emission was back-collected through the quartz slide with a 10X objective, optically filtered, and detected with a photomultiplier tube.

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[1] J. A. McKeating, R. L. Willey, *Aids* **1989**, *3*, S35-S41.
[2] N. Yahi, S. Baghdiguian, H. Moreau, J. Fantini, *J. Virol.* **1992**, *66*, 4848–4854.
[3] J. M. Harouse, C. Kunsch, H. T. Hartle, M. A. Laughlin, J. A. Hoxie, J. A., B. Wigdahl, F. Gonzalezscarano, *J. Virol.* **1989**, *63*, 2527–2533.
[4] J. M. Harouse, S. Bhat, S. L. Spitalnik, M. Laughlin, K. Stefano, D. H. Silberberg, F. Gonzalezscarano, *Science* **1991**, *253*, 320–323.
[5] T. McAlarney, S. Apostolski, S. Lederman, N. Latov, *J. Neurosci. Res.* **1994**, *37*, 453–460.
[6] A. Brogi, R. Presentini, D. Solazzo, P. Piomboni, E. Constantino-Ceccarini, *AIDS Res. Hum. Retroviruses* **1996**, *12*, 483–489.
[7] A. Brogi, R. Presentini, P. Piomboni, G. Collodel, M. Strazza, D. Solazzo, E. Costantino-Ceccarini, *J. Submicrosc. Cytol. Pathol.* **1995**, *27*, 565–571.
[8] S. Bhat, S. L. Spitalnik, F. Gonzalezscarano, D. H. Silberberg, *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 7131–7134.
[9] D. Long, J. F. Berson, D. G. Cook, R. W. Doms, *J. Virol.* **1994**, *68*, 5890–5898.
[10] K. D. McReynolds, M. J. Hadd, J. Gervay-Hague, *Bioconjugate Chem.* **1999**, *10*, 1021.
[11] D. Hammache, G. Pieroni, N. Yahi, O. Delezay, N. Koch, H. Lafont, C. Tamalet, J. Fantini, *J. Biol. Chem.* **1998**, *273*, 7967–7971.
[12] J. S. Burmeister, L. A. Olivier, W. M. Reichert, G. A. Truskey, *Biomaterials* **1998**, *19*, 307–325.
[13] E. Kalb, J. Engel, L. K. Tamm, *Biochemistry* **1990**, *29*, 1607–1613.
[14] V. Hlady, D. R. Reinecke, J. D. Andrade, *J. Colloid Interface Sci.* **1986**, *111*, 555–569.
[15] N. L. Thompson, C. L. Poglitsch, M. M. Timbs, M. L. Pisarchick, *Acc. Chem. Res.* **1993**, *26*, 567–573.
[16] M. L. Pisarchick, N. L. Thompson, *Biophys. J.* **1990**, *58*, 1235–1249.
[17] M. L. Pisarchick, D. Gesty, N. L. Thompson, *Biophys. J.* **1992**, *63*, 215–223.
[18] M. M. Timbs, C. L. Poglitsch, M. L. Pisarchick, M. T. Sumner, N. L. Thompson, *Biochim. Biophys. Acta* **1991**, *1064*, 219–228.
[19] T. H. Watts, H. E. Gaub, H. M. McConnell, *Nature* **1986**, *320*, 179–181.
[20] FITC–streptavidin (Pierce) was used as a negative control. The adsorption of FITC–streptavidin to a membrane doped with 5% (mol mol^{-1}) of **1** was minimal (that is, comparable to streptavidin adsorption to an undoped membrane). Thus a protein lacking a carbohydrate recognition site does not nonspecifically adsorb to a GSL-bearing membrane.
[21] S. L. Zhao, W. M. Reichert, *Langmuir* **1992**, *8*, 2785–2791.
[22] S. L. Zhao, D. S. Walker, W. M. Reichert, *Langmuir* **1993**, *9*, 3166–3173.
[23] The experimental methods described herein were used to measure the binding constant for streptavidin at a dioleoylphosphatidylcholine (DOPC) bilayer doped with 2.5% (mol mol^{-1}) biotin-capped dioleoylphosphatidylethanolamine (DOPE). The binding constant was $5.2 \pm 0.9 \times 10^7$ and the protein surface coverage at the "plateau" region of the isotherm was 2.8×10^{-12} mol cm^{-2}, which are consistent with published data for avidin binding at a membrane surface.[21, 22].
[24] Gp160 is thought to exist as a trimer on the HIV-1 surface, which also suggests that cooperative binding behavior may be observed. The estimated surface density of gp120 on the surface of the HIV-1 particle is 1.2×10^4 Å^2 molecule^{-1}, assuming a spherical particle with a diameter of 100 nm and 72 protein complexes per particle, with an average of 3.5 gp120 molecules per complex. See: a) J. A. Levy, *Microbiol. Rev.* **1993**, *57*, 183–289; b) D. C. Chan, D. Fass, J. M. Berger, P. S. Kim, *Cell* **1997**, *89*, 263–273.
[25] M. Kowalski, J. Potz, L. Basiripour, T. Dorfman, C. G. Wei, E. Terwilliger, A. Dayton, C. Rosen, W. Haseltine, J. Sodroski, *Science* **1987**, *237*, 1351–1355.
[26] H. M. McConnell, T. H. Watts, R. M. Weis, *Biochim. Biophys. Acta* **1984**, *864*, 95–106.

  1433-7851/00/3916-2884 $ 17.50+.50/0