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## Quantitative Studies of Binding between Synthetic Galactosyl Ceramide Analogues and HIV-1 Gp120 at Planar Membrane Surfaces**

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The primary cellular receptor for the HIV-1 virus is CD4, a protein abundantly expressed on T cells. The HIV-1 viral envelope glycoproteins, gp120 (120 kD) and gp41 (41 kD) mediate the recognition and infection of CD4 + cells.[1–3] However, in the early 1990s, it was discovered that HIV-1 can also infect CD4 − cell lines, such as neural cells and epithelial cells.[4, 5] Galactosyl ceramide (GalCer), a glycosphingolipid (GSL) that is highly expressed in neural and intestinal tissues, is thought to be the alternate receptor to which gp120 binds to initiate the infection of CD4 − cells.

Although the interaction between CD4 and gp120 has been extensively studied, relatively limited information has been obtained about the interaction between GSLs and gp120.[6–14] In an effort to elucidate the relationship between gp120 binding activity and GSL structure, we previously investigated recombinant gp120 (rgp120) binding to several naturally occurring GSLs reconstituted into planar supported lipid bilayers by using total internal reflection fluorescence microscopy (TIRF).[13] The presence of a carbohydrate on ceramide was shown to be required for rgp120 binding. Among the GSLs studied, galactosyl ceramide (GalCer) was the preferred receptor with respect to binding affinity, although rgp120 also recognized lactosyl ceramide (LacCer) and glucosyl ceramide (GlcCer). No attempt was made to investigate whether structural variations in the noncarbohydrate portion of the GSL (i.e. the ceramide) affected rgp120 binding.

Previous studies of protein binding to water-soluble receptors tethered to planar membranes have established that the structure of the nonreceptor portion of lipid is an important variable.[15] For example, the efficiency of streptavidin binding to a biotin-conjugated lipid at a planar membrane surface is strongly influenced by the length of the spacer arm linking the biotin moiety to the lipid.[16, 17]

Herein we have assessed the influence of steric accessibility in rgp120 – galactosyl recognition at a membrane surface. GalCer analogues with a water-soluble spacer arm of variable length between the galactosyl receptor and the lipophilic portion of the molecule were designed and synthesized. The analogues Gal-**3**, Gal-**4**, and Gal-**5** were prepared by using tri(ethylene glycol), tetra(ethylene glycol), and penta(ethylene glycol) linkers, respectively. Each ethylene glycol unit adds approximately 3 Å to the length of the spacer arm. The lipophilic portion of each molecule is dioleoyl maleate. The

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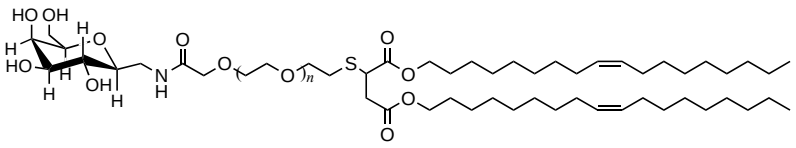

Gal-**3, 4, 5** $n = 1, 2, 3$

presence of unsaturated linkages ensured that the analogues would not undergo phase segregation when incorporated into a fluid bilayer membrane.[18]

To study the interaction of rgp120 with these analogues at a membrane-mimetic surface, they were incorporated into planar lipid bilayers deposited on fused silica substrates.[13, 19] The outer leaflet of the bilayer was composed of Gal-**3**, Gal-**4**, or Gal-**5** mixed with 1,2-dioleoylphosphatidylcholine (DOPC; 5 mol%). TIRF microscopy[20] was used to quantitatively measure the binding of fluorescein-tagged rgp120 to each type of membrane.

In Figure 1 are shown representative TIRF isotherms for rgp120 binding to membranes doped with Gal-**3**, Gal-**4**, and Gal-**5**, and to a pure DOPC membrane, which served as a control.[21] Attempts to fit the binding data to a conventional

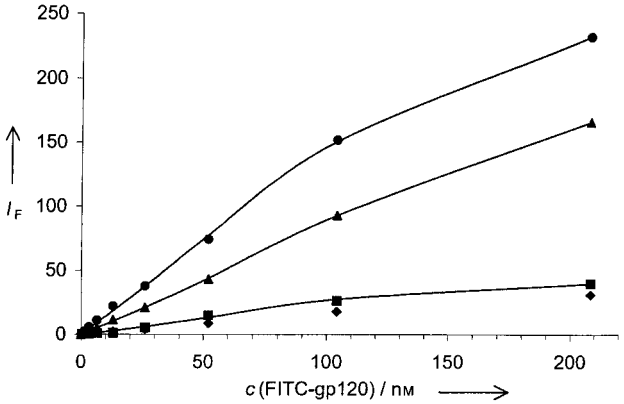


Figure 1. TIRF isotherms of FITC–rgp120 adsorption to DOPC membranes, either pure (◆) or doped with 5% (mol mol$^{-1}$) of Gal-**3** (■), Gal-**4** (●), or Gal-**5** (▲). Experimental fluorescence intensities were normalized to the respective rgp120 surface coverages (Table 1). The solid lines through the data represent fits obtained with a cooperative binding model in the form of $\omega^{(F/F_{max})}K_a[rgp120] = F/(F_{max} - F)$, where $K_a$ is the binding constant, $\omega$ is a coefficient that accounts for cooperativity in the binding process ($\omega > 1$ for cooperative binding, $\omega = 1$ for noncooperative (Langmuir-type) binding, and $\omega < 1$ for negative cooperativity), and $F_{max}$ is the fluorescence intensity in the plateau region of the isotherm (presumably where all the receptors are occupied by protein molecules).[22, 23]

Langmuir model, for which the binding sites are assumed to be equivalent, produced unsatisfactory results. Satisfactory results were obtained by fitting the data sets to a cooperative model in which rgp120 binding promotes adsorption of additional protein molecules.[22, 23] The association constants ($K_a$) obtained for Gal-**3**, Gal-**4**, and Gal-**5** as well as the respective cooperativity coefficients ($\omega$) are listed in Table 1. The $K_a$ values are in the range of $2 \times 10^6$ to $6 \times 10^6$ for all three compounds and are statistically indistinguishable. Cooperativity coefficients of approximately 1.5 suggest that a moderate increase in rgp120 affinity occurs over the concentration range studied.

Comparable $K_a$ and $\omega$ values were measured previously for cooperative rgp120 binding to 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) membranes that bear glucosyl, galactosyl, and lactosyl receptors.[13] In that study, isolates of naturally occurring GSLs were used,

Table 1. Summary of rgp120 binding behavior to planar DOPC membranes, either pure or doped with Gal-**3**, -**4**, or -**5** (5 mol%) in the outer leaflet.[a]

| | DOPC | Gal-**3** | Gal-**4** | Gal-**5** |
|---|---|---|---|---|
| $K_a \times 10^6$ | – | $5.4 \pm 1.8$ | $3.8 \pm 0.57$ | $2.5 \pm 0.57$ |
| cooperativity coefficient ($\omega$) | – | $1.44 \pm .20$ | $1.50 \pm 0.28$ | $1.59 \pm 0.11$ |
| surface coverage $[\times 10^{-14}$ mol cm$^{-2}]$[b] | $3.1 \pm 0.49$ | $5.9 \pm 2.1$ | $18.5 \pm 0.92$ | $13.2 \pm 0.71$ |

[a] All values listed are the mean and standard deviation of two experimental trials. [b] Surface coverages were determined at a dissolved rgp120 concentration of 208 nM, by using a modification of the method described by Hlady et al.[26]

in which the lipophilic chains on most of the molecules are saturated. Upon mixing these GSLs with a fluid-phase lipid such as POPC, phase segregation may occur, producing GSL-rich domains.[24] It was not clear from the earlier study whether the cooperative binding observed could be due to the laterally heterogeneous distribution of GSL receptors in the membrane. The results obtained here show that the rgp120–GSL interaction is also cooperative when the receptors appear to be uniformly distributed in the membrane (at optical resolution, when examined by epifluorescence microscopy).[18a] Thus the cooperative nature of rgp120–GSL binding cannot necessarily be attributed to the formation of GSL domains.[18b, 25]

The $K_a$ and $\omega$ values listed in Table 1 do not provide any information on the amount of protein bound at membranes that bear Gal-**3**, -**4**, and -**5**. To address this question, an in situ calibration method[26] was used to determine the surface coverage of rgp120 bound to these membranes, as well as the amount of protein nonspecifically adsorbed on a pure DOPC membrane (Table 1). The rgp120 surface coverage obtained on Gal-**3** was only twice the value measured on a pure DOPC membrane. However, when the spacer was extended to four ethylene glycol units (versus three units in Gal-**3**), the protein surface coverage increased threefold to approximately $18 \times 10^{-14}$ mol cm$^{-2}$. A further increase in the linker length to five ethylene glycol units in Gal-**5** resulted in a moderate decrease in rgp120 surface coverage (to $13 \times 10^{-14}$ mol cm$^{-2}$).

These results show that a spacer arm of three ethylene glycol units is inadequate for highly efficient rgp120 recognition of a galactosyl receptor present on the surface of a DOPC membrane. A significant fraction (ca. 50%) of the protein binding observed at a Gal-**3**-bearing membrane is nonspecific in nature. The sharp increase observed with Gal-**4** indicates that a spacer arm of four ethylene glycol units is necessary to allow the galactosyl receptor to access the carbohydrate binding region on rgp120. The cause of the lower surface coverage observed with Gal-**5** is not yet clear, but it is possible that: 1) the longer spacer arm may have a

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higher probability of adopting conformation(s) unfavorable for rgp120 recognition; or 2) the degree of nonspecific rgp120 adsorption on a Gal-**5**-bearing membrane may be less than that on a Gal-**4**-bearing membrane.

In summary, we have prepared synthetic GalCer analogues that bind to rgp120 cooperatively when incorporated uniformly into a planar fluid membrane at 5 mol%. A critical spacer arm length necessary to promote efficient binding has been identified. These results should aid efforts to design anti-HIV-1 agents based on membrane-tethered, carbohydrate-based receptors for rgp120.

### *Experimental Section*

DOPC was purchased from Avanti Polar Lipids and used as received. Rgp120 expressed in baculovirus, labeled with fluorescein isothiocyanate (FITC; FITC/rgp120 4:1, molar), was purchased from Intracel Corporation and used without further purification. Dextran (10000 $M_W$) labeled with FITC (1:2.9, molar) was obtained from Molecular Probes.

A convergent approach was used to synthesize the galactosyl lipids.[27] Each component, the galactosyl moiety, the hydrophilic spacer, and the hydrophobic maleic ester, were synthesized and then linked together. The galactose methyl amine was obtained by reaction of the peracetylated galactose with TMS cyanide, followed by reduction to the amino-substituted *C*-galactose. Each spacer group was prepared from readily available oligo(ethylene glycol) chains. Each diol was differentiated and modified to yield a thiol and a carboxylic acid at opposite ends of the spacer molecule. The lipid portion was obtained by reaction of maleic anhydride with oleoyl alcohol. A Michael addition of the nucleophilic thiol of the spacer to the maleic ester then gave an intermediate lipid with an available carboxylic acid. This acid was coupled with the galactose methyl amine to form the final amide link in compounds **3**, **4**, and **5**.

The procedures used to measure rgp120 binding isotherms were similar to those described previously.[13] Briefly, Gal-**3**, Gal-**4**, or Gal-**5** was incorporated into the upper leaflet of a supported planar DOPC bilayer at a concentration of 5 mol%. The asymmetric bilayer was deposited at 35 mN m$^{-1}$ on a fused silica substrate by using the Langmuir–Blodgett–Schaefer technique.[19] A series of FITC–rgp120 standard solutions was prepared in phosphate buffer (10 mM, pH 7.2, containing 150 mM NaCl). To measure TIRF isotherms the solutions were injected sequentially from lowest to highest concentration into a ca. 50-μL TIRF flow cell. After each injection, the solution was equilibrated in the cell for 30 min before the fluorescence intensity was measured.

An in situ calibration procedure was performed to determine the amount of rgp120 protein adsorbed from the most concentrated protein solution (208 nM) on each type of membrane. After protein adsorption was allowed to proceed for 30 min, aqueous surfactant solution (PCC-54, Pierce; 2%, v/v) was injected into the flow cell to dissolve the lipid bilayer and associated protein. After 20 min, the cell was flushed with phosphate buffer to re-establish the optical baseline. A series of four FITC–dextran standard solutions (range of 5–25 μg mL$^{-1}$, prepared in phosphate buffer) was injected, sequentially from lowest to highest concentration, into the flow cell. Fluorescence intensities were measured by TIRF to generate a calibration curve. Protein surface coverages were calculated according to Equation (8) in Hlady et al.,[26] using 173 nm as the penetration depth of the evanescent wave and assuming equivalent quantum yields for FITC–rgp120 and FITC–dextran.

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[18] a) To examine membrane morphology by using epifluorescence microscopy, membranes doped with 1 mol% tetramethylrhodamine-phosphatidylethanolamine were prepared. A homogeneous film morphology, indicative of a uniformly fluid phase, was observed for monolayer membranes composed of Gal-**5** mixed with DOPC at ratios of 5 and 50 mol%; b) since phase separation in a uniformly fluid membrane could conceivably occur by means of self-association of Gal moieties, we cannot eliminate the possibility that Gal nanodomains may exist in these membranes, and that cooperative binding of rgp120 may be correlated with the presence of these domains.
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