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Galactosyl ceramide or a derivative is an essential component of the neural receptor for human immunodeficiency virus type 1 envelope glycoprotein gp120

(AIDS/demyelination/sulfatide/glycolipid)

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ABSTRACT **This report demonstrates that galactosyl ceramide (GalCer) or a molecule derived from it may serve as an alternative receptor for human immunodeficiency virus in the nervous system. Recombinant gp120, an envelope glycoprotein of human immunodeficiency virus type 1, specifically binds to GalCer and its derivatives. This specificity was studied by inhibiting binding of radioiodinated gp120 to GalCer with antibodies to GalCer, antibodies to gp120, and an excess of unlabeled gp120. Binding activity was also removed by absorbing gp120 with liposomes containing GalCer. In addition, studies using natural and semisynthetic lipids indicate that the linkage between galactose and ceramide is essential for binding. The significance of an alternative receptor for human immunodeficiency virus in the nervous system is discussed.**

The CD4 molecule of lymphoid cells, including lymphocytes, monocytes, and macrophages, is the main receptor for human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) (1–8). However, the existence of an alternate pathway has been strongly suggested by the susceptibility of many CD4-negative cells of diverse tissue origins to HIV infection (9–17). Recently, we showed that antibodies to galactosyl ceramide (GalCer) inhibit uptake and infection of HIV-1 in two neural cell lines, U373-MG and SK-N-MC, suggesting that GalCer may mediate HIV entry in these cells (18). To further refine these findings, we studied the interaction between gp120, the viral receptor-binding protein, and various glycolipids, including GalCer. Recombinant gp120 specifically binds to GalCer or a molecule derived from it (Table 1) but does not bind to other related or unrelated lipids. In addition, these studies indicate that the linkage between galactose and ceramide is essential for gp120 binding.

MATERIALS AND METHODS

Binding Assay. The high-performance thin-layer chromatography (HPTLC) binding assay for ^{125}I-labeled gp120 was done as described (18). In brief, lipids were separated on aluminum-backed silica gel G-60 HPTLC plates (EM Science) with chloroform/methanol/water (60:35:8). After chromatography, the plates were coated with 0.1% poly(isobutyl methacrylate) (Polysciences) in hexane for 90 sec. The dried plates were then incubated with 1% gelatin in 50 mM Tris·HCl, pH 7.4/0.15 M NaCl (GTS buffer) for 45 min and subsequently incubated with ^{125}I-labeled gp120 or ^{125}I-labeled glycoprotein D from herpes simplex virus ($\approx 1.25 \times 10^6$ dpm/ml) in GTS buffer for 1 hr at room temperature. Recombinant gp120/BH10 produced in a bacculovirus expression system (MicroGenesys, West Haven, CT) and other proteins were radioiodinated by using Bolton–Hunter reagent (ICN) according to the manufacturer's instructions. The specific activity of each of the labeled proteins varied from 10 to 16×10^3 dpm/ng. After binding, the plates were washed three times for 10 min each with phosphate-buffered saline/0.3 M NaCl. The plates were exposed to x-ray film for 16 hr (Fig. 1) and 6 hr (Fig. 2) at −70°C. For quantitation, either autoradiograms were analyzed with a scanning densitometer (Hoefer) or the lipid-associated radioactivity was counted with a γ spectrometer. The lipids were identified by exposure to iodine vapors. In most quantitation experiments, ^{125}I-labeled gp120 bound to glucosyl ceramide (GlcCer) was considered nonspecific binding. Binding of ^{125}I-labeled gp120 to GalCer was confirmed by analyzing the ^{125}I-labeled gp120–GalCer complex with SDS/PAGE.

Liposomes. Liposomes were prepared by dissolving 5 mg of lipids in chloroform/methanol (2:1) and making a thin film on the walls of a test tube by evaporation under nitrogen. The lipid film was suspended in phosphate-buffered saline solution containing bovine serum albumin at 1 mg/ml and then subjected to ultrasonication. ^{125}I-labeled gp120 (1.25×10^6 dpm) was incubated with GalCer, sphingosine, or GlcCer liposomes in 100 μl for 16 hr at 23°C and then centrifuged at $10,000 \times g$ for 10 min to remove bound gp120. The supernatant was used in the binding assay, as described above.

Purified Glycolipids and Lipid Fractions. Total lipids were extracted from human brain (autopsy specimens from the Hospital of the University of Pennsylvania) and human erythrocytes. Alkali-stable total, neutral, and Folch lower-phase lipids were prepared as described (19). Binding of ^{125}I-labeled gp120 to these lipids was assessed after separation by HPTLC. Various purified lipids and glycolipids were obtained from Sigma, and G_{M4} was provided by Robert Yu (Medical College of Virginia).

RESULTS AND DISCUSSION

Recombinant gp120 Binds to GalCer and Its Derivatives. We studied the interaction of gp120 and various lipids by using a HPTLC binding assay (18). Lipids were separated by HPTLC on silica gel G-60 and then directly incubated with radioiodinated recombinant gp120. Autoradiograms of the HPTLC plates were then analyzed for binding activity. Fig. 1*B* shows that gp120 bound to GalCer and galactosyl sulfatide (sulfated GalCer, Table 1) but did not bind to GlcCer, lactosyl ceramide, or neutral glycolipids isolated from human erythro-

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Abbreviations: HIV, human immunodeficiency virus; GalCer, galactosyl ceramide; GlcCer, glucosyl ceramide; HPTLC, high-performance thin-layer chromatography.

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Table 1. Structure of glycolipids used to determine specificity of gp120 binding

Glycolipid	Structure
Ceramide	Cer
GalCer*	Gal-Cer
Galactosyl sulfatide*	SO_4-Gal-Cer
G_{M4}*	NeuAc(α2-3)Gal-Cer
GlcCer	Glc-Cer
Lactosyl ceramide	Gal(β1-4)Glc-Cer
Ceramide trihexoside	Gal(α1-4)Gal(β1-4)Glc-Cer
Globoside	GalNAc(β1-3)Gal(α1-4)Gal(β1-4)Glc-Cer
G_{M1}	Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc-Cer \| NeuAcα
G_{D1a}	Gal(β1-3)GalNAc(β1-4)Gal(β1-4)Glc-Cer | | NeuNAc NeuNAc

Gal, D-galactose; Glc, D-glucose; Cer, ceramide; GalNAc, *N*-acetylgalactosamine; NeuNAc, *N*-acetylneuraminic acid (sialic acid). Other lipids used were as follows: psychosine*, GalCer without fatty acid; sphingosine, galactose of GalCer replaced by phosphocholine; palmitoyl-*, steroyl-* and nervonoyl* GalCer, fatty acids in GalCer are palmitic, steric, and nervonic acids, respectively. *gp120 binds to these lipids.

cytes. Radioiodinated proteins, such as recombinant glycoprotein D of herpes simplex virus (Fig. 1*C*) or bovine serum albumin (data not shown), did not bind to any of these lipids.

The Binding of gp120 Is Specific. Specificity of gp120 binding to GalCer was examined by three different approaches. (*i*) Antibodies to GalCer and gp120 were tested for their ability to inhibit binding of ^{125}I-labeled gp120 to GalCer. Incubation of ^{125}I-labeled gp120 with antibodies to either gp120 or GalCer reduced binding, but polyclonal antibodies to neural cell-adhesion molecule (NCAM) (20) (Table 2) and a monoclonal antibody to complex gangliosides (A_2B_5) (21) had no effect (data not shown). (*ii*) The ability of liposomes containing GalCer to block binding of gp120 to GalCer was tested. After incubation of ^{125}I-labeled gp120 with GalCer-containing liposomes, the gp120–GalCer liposome complexes were removed by centrifugation. The supernatant containing residual ^{125}I-labeled gp120 was incubated with GalCer in the HPTLC binding assay. Because gp120 did not bind to GlcCer (Fig. 1), GlcCer-containing liposomes were used as control. As shown in Table 2, GalCer-containing liposomes inhibited gp120 binding, compared with GlcCer-containing liposomes. (*iii*) An excess of unlabeled gp120 was incubated with ^{125}I-labeled gp120. At a 160-fold excess of unlabeled gp120, binding of ^{125}I-labeled gp120 decreased by 90% (Table 2). As expected, from the biological evidence (14) recombinant soluble CD4 did not inhibit the binding of gp120

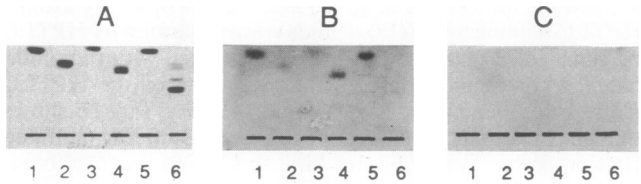

FIG. 1. Binding of ^{125}I-labeled gp120 to GalCer and other lipids. (*A*) Lipids stained with orcinol. (*B*) ^{125}I-labeled gp120. (*C*) ^{125}I-labeled glycoprotein D from herpes simplex virus. Lanes: 1, GalCer; 2, lactosyl ceramide; 3, GlcCer; 4, galactosyl sulfatide; 5, *N*-oleoyl GalCer; 6, neutral lipids from erythrocytes, which include GlcCer, lactosyl ceramide, ceramide trihexoside, and globoside. For HPTLC separation, 10 μg of lipids was used.

Table 2. Effect of polyclonal antisera, liposomes, and glycosides on binding of ^{125}I-labeled gp120 to GalCer

Effector	Concentration	Binding, %
Anti-gp120*	5.0 μl/ml	38 ± 5
Anti-gp120*	10.0 μl/ml	11 ± 1
Anti-gp120*	20.0 μl/ml	6 ± 5
Anti-GalCer*	5.0 μl/ml	48 ± 11
Anti-GalCer*	10.0 μl/ml	32 ± 8
Anti-GalCer*	20.0 μl/ml	22 ± 5
Anti-NCAM*	5.0 μl/ml	98 ± 6
Anti-NCAM*	10.0 μl/ml	104 ± 8
GlcCer liposomes†	500 μg	100
GalCer liposomes†	500 μg	24 ± 10
Sphingosine liposomes†	500 μg	97 ± 11
None		100
Unlabeled gp120‡	160 nM	11 ± 3
Methyl β-D-galactopyranoside‡	100 mM	108 ± 8
Methyl β-D-glucopyranoside‡	100 mM	101 ± 8

Ten micrograms of GalCer and GlcCer were used for binding assay. Percent binding was calculated as (cpm of test-blank)/(cpm of control-blank) × 100. Values are mean ± SD from three experiments. NCAM, neural cell adhesion molecule.

*In these experiments normal rabbit serum in equivalent concentrations was used as control (100% binding). Polyclonal anti-gp120 was from MicroGenesys, West Haven, CT. Anti-GalCer was produced by injecting rabbits with GalCer liposomes with bovine serum albumin (22).

†^{125}I-labeled gp120 was incubated with GalCer, sphingosine, or GlcCer liposomes in 100 μl for 16 hr at 23°C and then centrifuged. The supernatant was used in the binding assay.

‡Sugars or unlabeled gp120 were added with ^{125}I-labeled gp120 during binding assay.

to GalCer (data not shown). These experiments demonstrate that the interaction is stable and specific.

Gp120 Binds to GalCer and Sulfatide Isolated from Human Brain. Alkali-stable Folch lower-phase lipids isolated from human brain were analyzed for binding activity. As shown in Fig. 2, gp120 bound to GalCer and sulfatide. Preliminary analysis suggests that the lower band in lane 3 of Fig. 2 is G_{M4}.

The Galactose–Ceramide Linkage Is Essential for Binding of gp120 to GalCer. The fine specificity of the interaction between gp120 and GalCer was delineated by using related glycolipids (Table 1) and sugars. As shown in Table 3 and Fig. 1, gp120 bound to GalCer and galactosyl sulfatide but not to

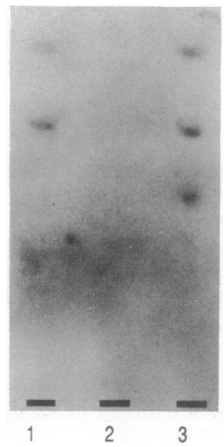

FIG. 2. Binding of gp120 to Folch lower-phase lipids from human brain. Alkali-stable Folch lower-phase lipids from human brain were separated on HPTLC and then gp120 binding occurred, as described. Lanes: 1, GalCer (upper band, 5 μg) and sulfatide (lower band, 5 μg); 2, GlcCer (5 μg); 3, alkali-stable Folch lower-phase lipids from human brain. For relative positions of lipids, see Fig. 1*A*. Preliminary analysis indicates that the lower band in lane 3 is G_{M4}.

Table 3. Binding of ^{125}I-labeled gp120 to lipids

Lipid	Binding, %
GalCer	100
Galactosyl sulfatide	109 ± 11
Psychosine	95 ± 14
N-Palmitoyl GalCer	115 ± 6
N-Steroyl GalCer	99 ± 6
N-Nervonyl GalCer	100 ± 25
Lactosyl ceramide	19 ± 6
Ceramides	9 ± 3
GlcCer	5 ± 2
G_{D1a}	10 ± 7
G_{M1}	11 ± 6

Concentration of each lipid was 5 μg. Binding assay was done as described. Values are mean ± SD from three experiments.

GlcCer, ceramide, or lactosyl ceramide. In addition, gp120 bound to semi-synthetic variants of GalCer, such as psychosine (GalCer lacking the fatty acid), *N*-palmitoyl GalCer, *N*-steroyl GalCer, *N*-oleoyl GalCer, and *N*-nervonyl GalCer, compounds in which the fatty acids of native GalCer were replaced by the respective fatty acids. In contrast, lipid-free methyl glucosides and galactosides did not inhibit binding of gp120 to GalCer (Table 2). Incubation of ^{125}I-labeled gp120 with sphingosine liposomes also had no effect on binding (Table 2).

These experiments demonstrate that the fatty acid moiety on the GalCer molecule has little effect on gp120 binding. The linkage of galactose to ceramide is essential for binding: gp120 did not bind to ceramide (Table 3); nor did it bind to lactosyl ceramide, in which galactose is linked to ceramide through an intervening glucose.

We found that the 3′-hydroxyl position in galactose tolerated considerable substitution, as seen with gp120 binding to sulfatide (Fig. 1 and Table 3) and to purified G_{M4} (data not shown). Sulfatide contains a sulfate group, and G_{M4} contains an acetylneuraminic group linked to the 3′-hydroxyl group of galactose. In contrast, gp120 did not bind to GlcCer, in which glucose is the epimer of galactose at the C-4 position. A schematic representation of the binding of gp120 to GalCer is shown in Fig. 3.

Our studies (18) showed that ≈1% of U373-MG and 20% of SK-N-MC cells express GalCer on their surface. Alkali-stable Folch lower-phase glycolipids isolated from cultured U373-MG and SK-N-MC cells showed the presence of GalCer with specific antibodies and HPTLC binding assay, whereas HeLa cells, which are not infected by HIV, had no GalCer (S.B., unpublished results). In addition ^{125}I-labeled gp120 binds to a band in lipids extracted from U373-MG and SK-N-MC cells, which comigrates with authentic GalCer (S.B., unpublished results).

The binding of gp120 to GalCer is saturable and has a K_d of 11.4 nM (18). This study, together with our studies using anti-GalCer inhibition of HIV internalization and infection (18), demonstrate that GalCer can serve as a receptor for gp120.

Most of the binding studies reported here were done with bovine GalCer. However, the binding of gp120 to human GalCer (Fig. 2) suggests that HIV may make use of these lipids as receptors *in vivo* to enter cells.

Attachment to host cells is an important initial event of the viral infection process. Most known viral receptors are glycoproteins. However many bacteria and their toxins can make use of neutral glycolipids and gangliosides as receptors (for review, see ref. 23). Well-documented examples include ganglioside receptors for cholera toxin, tetanus toxin, shiga toxin, delta toxin, heat-labile toxin, and botulinum toxin. Sendai virus also uses gangliosides as receptors (24). Our studies indicate the involvement of a cell-surface glycolipid in mediating the entry of a retrovirus.

HIV frequently causes neurological dysfunction (see ref. 25), including demyelination (25–30), and is present in the central nervous system of AIDS patients. However, in the brain the virus is found mostly in cells of macrophage–monocyte lineage and infrequently in astrocytes and oligodendrocytes (30, 31). The lack of definitive detection of HIV in neural cells may be due to difficulties in detecting retroviruses, as has been noted with murine retroviral infections (32).

Oligodendrocytes in the central nervous system and Schwann cells in the peripheral nervous system express GalCer and galactosyl sulfatide (33, 34). Therefore, HIV infection of oligodendrocytes and Schwann cells, if any, could be mediated by GalCer or galactosyl sulfatide, two lipids specific to these cell types (33). Alternatively, the virus could mediate demyelination without actual infection, by binding at the cell surface of these myelinating cells, as seen with the anti-GalCer effect in tissue cultures of oligodendrocytes (35, 36).

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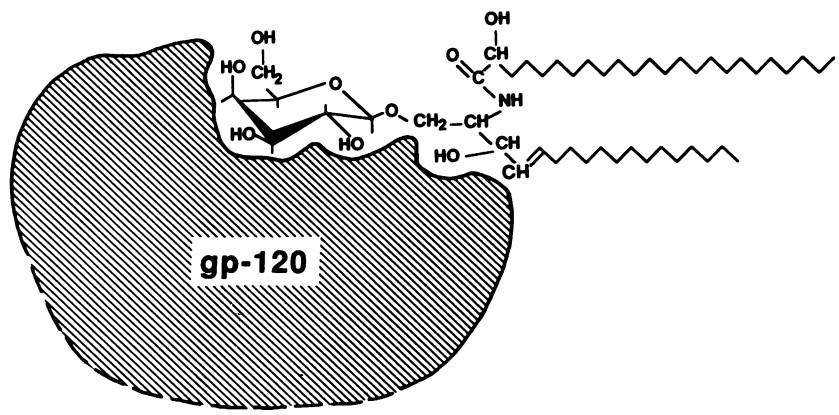

FIG. 3. Schematic representation of the binding region of GalCer. 3′-Hydroxyl of galactose appears uninvolved in binding (see text for details).

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