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Human Immunodeficiency Virus Type 1 Infection of SK-N-MC Cells: Domains of gp120 Involved in Entry into a CD4-Negative, Galactosyl Ceramide/3′ Sulfo-Galactosyl Ceramide-Positive Cell Line

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The primary receptor for human immunodeficiency virus (HIV) is the CD4 molecule; however, in vitro evidence suggests that a neutral glycolipid, galactosyl ceramide (GalCer) or a derivative molecule, 3′ sulfo-galactosyl ceramide (GalS), may serve as an alternative receptor for HIV type 1 (HIV-1) in cells of neural and colonic origin. Biochemical studies have demonstrated that recombinant gp120 envelope protein binds to GalCer/GalS in both solid-phase enzyme-linked immunosorbent assay and high-performance thin-layer chromatography overlays. We have used the SK-N-MC cell line, a CD4-negative, GalCer/GalS-positive cell line previously characterized as susceptible to HIV-1 infection, to identify virus isolates with either a positive infection phenotype, HIV_{HxB2}, or a negative infection phenotype, HIV-$1_{89.6}$. Using a solid-phase virus binding assay, we determined the level of restriction in HIV-$1_{89.6}$ infection to be at the level of virus-glycolipid binding. Furthermore, using HIV-1_{HxB2}–HIV-$1_{89.6}$ chimeras, we have identified a 193-amino-acid fragment from the envelope region of HIV-1_{HxB2} containing the V3, V4, and V5 regions which confers a positive infection phenotype on the HIV-$1_{89.6}$ background. Recombinant viruses which separate this 193-amino-acid fragment into two distinct chimeras are each able to confer a positive infection phenotype on the background of $HIV_{89.6}$, suggesting that a stable GalCer/GalS-envelope interaction is dependent on the conformation of the envelope protein in the context of the viral membrane. Alternatively, the GalCer/GalS-gp120 bond may involve multiple sites on the oligomeric envelope protein.

The CD4 molecule is the principal receptor for human immunodeficiency virus type 1 (HIV-1) and is responsible for entry of the virus into its main host cells: $CD4^+$ lymphocytes, monocytes, and microglia (18, 33, 46). However, a number of studies have demonstrated that cell lines of neural, gastrointestinal, fibroblastic, and cervical origin can be infected in vitro by a number of HIV-1 strains, in spite of undetectable levels of CD4 protein or mRNA (11, 14, 15, 25, 30, 49, 52, 56). Recent in vivo findings indicate that CD4-negative cells in the brain, including astrocytes, endothelial cells, and neurons, harbor HIV sequences (37, 50). Furthermore, although expression of the CD4 protein through recombinant methods can render many human cell lines that are normally CD4 negative permissive to HIV infection, the human CD4 protein is unable to mediate entry of HIV-1 into nonprimate cells (16, 33, 53). Therefore, it has been proposed that there are alternative receptors for HIV entry which are responsible for CD4-independent entry into neural and endothelial cells in the brain and that in $CD4^+$ cells, the CD4 molecule acts in concert with a coreceptor that is present only in primate cells.

Although many have been suggested, no single protein has been established as either a coreceptor or an alternative receptor for HIV-1 infection (1, 9). In some human cells of neural and gastrointestinal origin, galactosyl ceramide (GalCer), a glycolipid primarily of neural origin, or its sulfated form (GalS) may serve as an alternative receptor, since anti-GalCer antibodies inhibit infection (24, 56), gp120 binds tightly to these glycolipids (6, 51), and high expression of GalCer is associated with greater infectivity (22). Similar glycolipids have been proposed as alternative receptors in cervical cells and in sperm (3, 23). Additionally, experiments in which the incorporation of lipid extracts from human cells rendered $CD4^+$ mouse cells permissive for HIV entry have suggested that some as yet undefined glycolipids may be important as coreceptors in conjunction with CD4 (21).

Identification of the domains of the HIV cellular attachment protein, gp120, responsible for CD4-independent entry in Gal Cer/GalS-positive cells would provide additional insights into the biology of the early events in glycolipid-mediated infection and may provide information regarding the entry of HIV into CD4-negative cells in vivo. This may be particularly useful in understanding the pathophysiology of HIV dementia, in which astrocytes and neurons have been recently found to be infected by using sensitive techniques (37, 50). As a first step toward defining this region, Cook et al. used a panel of gp120 monoclonal and monospecific antibodies in a gp120-GalCer binding assay (20) to demonstrate that antibodies against V3 blocked GalCer binding. However, antibodies directed against the CD4 binding domain also inhibited binding between gp120 and Gal Cer, although to a lesser extent, which is consistent with a proposed conformational relationship between the CD4 binding and V3 domains (35). Using a gp120-GalCer liposome binding assay, Long et al. (32) demonstrated that heat denaturation of gp120 abolished binding to GalCer, emphasizing the conformational dependence of gp120 binding to GalCer.

We have used the SK-N-MC cell line (31), a GalCer/GalS-

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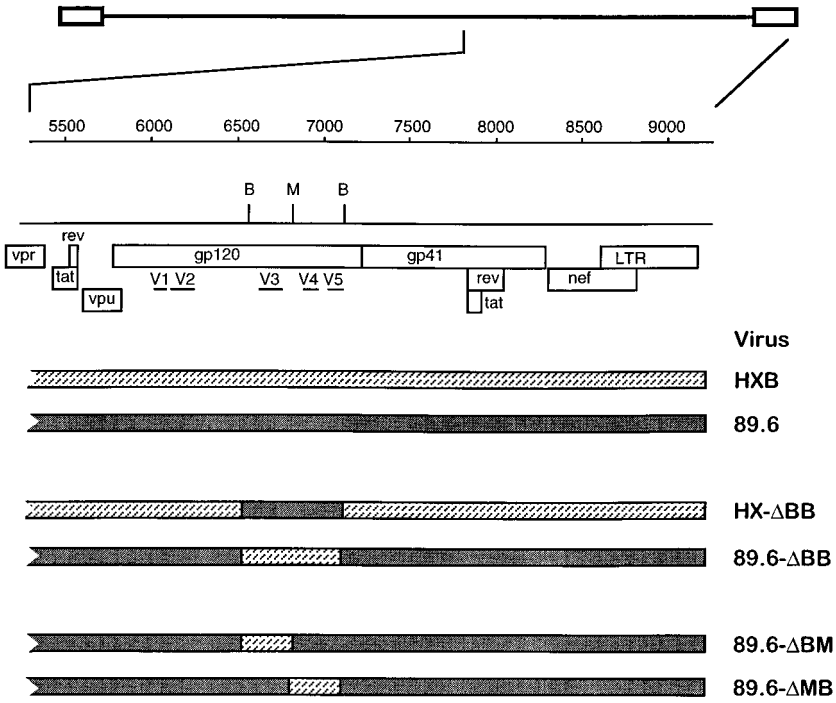

FIG. 1. Viruses used in these experiments. Chimeric viruses incorporating regions of the HIV-1_{HxB2} (HXB) *env* on the background of HIV-$1_{89.6}$ have been described elsewhere (28). Restriction sites used to construct the chimeras: B, *Bgl*II; M, *Mst*II.

positive HIV-1-infectable cell line, and a panel of chimeric viruses derived from parental strains with a positive and negative infection phenotype to extend the antibody inhibition studies. The results indicate that the domain of the envelope involved in CD4-independent entry encompasses a relatively large area in the carboxy-terminal half of gp120; we were unable to subdivide this region by using smaller chimeras. These findings suggest a model whereby the binding between HIV and GalCer/GalS involves conformational regions of gp120 including V3, V4, and V5.

MATERIALS AND METHODS

Cells. SK-N-MC is a CD4-negative, GalCer/GalS-positive peripheral neuroblastoma cell line obtained from the American Type Culture Collection; CEMx174 is a CD4-positive T-cell/B-cell hybrid line (26). HeLa is a cervical carcinoma cell line, and HeLaCD4-6C is a CD4-positive stable HeLa transfectant cell line (both a gift of B. Chesebro [12]). All cell lines were maintained in RPMI supplemented with 10% fetal calf serum and glutamine.

Expression of CD4 in SK-N-MC cells. To generate a CD4-expressing SK-N-MC cell line, we used a retroviral vector, SCX, containing full-length CD4 gene and a neomycin gene for eukaryotic selection (a gift from Richard Morgan, National Institutes of Health, Bethesda, Md.). SK-N-MC cells were exposed to retroviral supernatants containing SCX or a control vector, G1N, containing the neomycin gene only, supplemented with 5 μg of protamine sulfate per ml, and incubated for 12 h at 37°C. Following transduction, the retroviral inoculum was aspirated and the cells were selected in medium containing Geneticin (G418; 1 mg/ml; GIBCO BRL, Gaithersburg, Md.). Cell surface expression of the CD4 molecule was determined by flow cytometry analysis (see below).

Flow cytometry staining and analysis. The following monoclonal antibodies were used: OKT4 and OKT4A (Ortho Pharmaceuticals), both anti-CD4; W632, a positive control antibody that recognizes a constant domain of the class I major histocompatibility complex (Accurate Scientific); and a control hybridoma which recognizes a bunyavirus protein. Monolayer SK-N-MC and SK-N-MC/CD4 cells were removed by using EDTA and pelleted in a tabletop centrifuge at 1,500 rpm for 10 min. The cells were washed in ice-cold staining buffer (SB; 10% fetal calf serum, phosphate-buffered saline [PBS]) and incubated for 30 min on ice to block nonspecific binding. SB was then removed, and the cells were incubated with a primary antibody diluted in SB for 30 min on ice. After washing in SB, the cells were incubated for 45 min on ice with a 1:10 dilution of the secondary antibody, fluorescein isothiocyanate-conjugated goat anti-mouse $F(ab')_2$. The cells were then washed, fixed in 500 μl of PBS–2% paraformaldehyde, and stored at 4°C until analysis by flow cytometry.

Viruses. Wild-type viral stocks were prepared from the infectious molecular clones of pHxB2 (42) and p89.6 (17). Proviral DNA was transfected into rhabdomyosarcoma (RD) cells with calcium phosphate (5′-3′, West Chester, Pa.). After transfection, infectious virus was rescued by cocultivation with CEMx174 cells and then propagated in CEMx174 cells for 2 to 3 weeks. High-titer cell-free viral supernatants were then filtered and stored in 1.0-ml aliquots at −80°C until use. The 50% tissue culture infectious dose ($TCID_{50}$) was determined by inoculation of CEMx174 cells with 10-fold dilutions of virus stocks and observing the cells for cytopathological changes (45); the endpoint was calculated by the method of Reed and Muench (43). HIV-1 primary isolates contributed by the WHO Network for HIV Isolation and Characterization were obtained from the AIDS Reagent Program and amplified in interleukin-2-stimulated peripheral blood mononuclear cells (54a). Viral $p24^{gag}$ antigen in the virus stocks was quantitated by antigen capture enzyme-linked immunosorbent assay (ELISA; DuPont).

Recombinant viruses were prepared as described previously (28). The 3′ hemigenome of either pHxB2 or p89.6 was subcloned by using a conserved *Sal*I restriction site (nucleotide [nt] 5332). Chimeric 3′ hemi-genomes were then created between pHxB2 and p89.6 by using shared restriction sites (Fig. 1). To obtain infectious recombinant viruses, equal amounts of the appropriate 5′ and 3′ hemi-genomes were first digested with *Sal*I to produce compatible termini and then cotransfected into RD cells. Chimeric viruses were amplified, harvested, and quantitated as described above. Each chimeric virus was confirmed by sequence and restriction enzyme analysis (28).

Infections and DNA isolation. After overnight adherence in 24-well plates, the cells were washed with RPMI and incubated in media with and without 10 μM zidovidine (AZT) for an additional 18 h. For antibody inhibition experiments, the cells were also incubated with monoclonal antibodies for 30 min just prior to infection. The cells were then incubated for 1 to 6 h with equivalent infectious doses of DNase I-treated (30 min at 36°C) virus. The multiplicity of infection (MOI) was 0.1 to 0.2 $TCID_{50}$ per cell unless otherwise indicated. The inocula were removed, and the cells washed with PBS, treated with 100 μl of tissue culture-grade trypsin (Sigma), replated, and refed with 1.0 ml of medium with or without 10 μM AZT. At 18 to 48 h postinfection, the cells were washed twice in serum-free medium and their DNA was extracted.

To extract DNA for PCR, the cells were suspended in 500 μl of S-EDTA (0.1 M NaCl, 0.05 M Na_2EDTA) and transferred to microcentrifuge tubes. Sodium dodecyl sulfate (SDS; final concentration, 1%) and proteinase K (0.1 mg/ml) were added, and the samples were incubated at 56°C. DNA was extracted in equal volumes of phenol-chloroform-isoamyl alcohol (25:24:1; Ameresco). The aqueous phase was removed, and the DNA was precipitated by the addition of 2 volumes 100% ethanol at −80°C and pelleting in a microcentrifuge for 10 min.

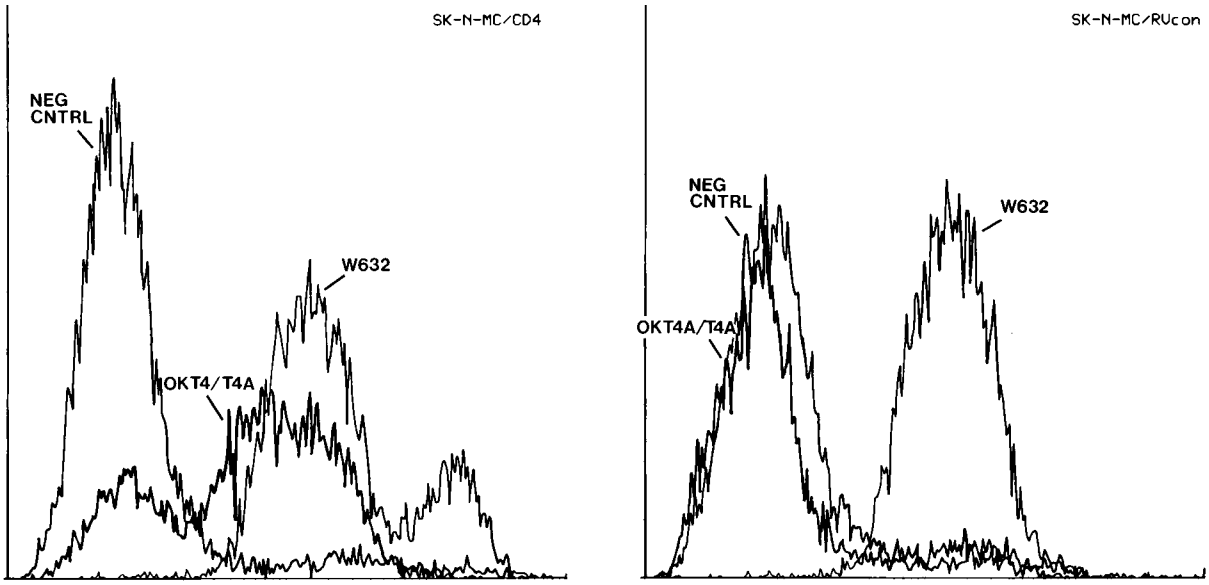

FIG. 2. SK-N-MC/CD4 cells express the CD4 molecule. SK-N-MC/CD4 cells were prepared as described previously (45) and analyzed by flow cytometry. Fluorescence with a combination of monoclonal antibodies OKT4 and OKT4A (CD4), positive control antibody W632 (HLA class I), or a negative control (NEG CNTRL) antibody is shown in the flow cytometry tracings.

The DNA was resuspended in 400 μl of distilled H_2O and treated with 3 U (10 μg) DNase-free RNase (36°C, 60 min). The nucleic acid extraction was repeated, and the DNA was recovered by centrifugation, washed in 70% ethanol, vacuum dried, and suspended in distilled H_2O.

PCR. Each PCR sample contained 10 ng of DNA (in 10 μl of distilled H_2O), 400 μM each deoxynucleoside triphosphate, 10 mM Tris-HCl, 50 mM KCl, 2.5 mM Mg_2Cl, 1 μM each primer, and 1.25 U of AmpliTaq DNA polymerase (Perkin-Elmer). The reaction mix (total volume of 50 μl) was subjected to thermocycling under the following conditions: for the long terminal repeat (LTR) LTRU31 (ACAAGCTAGTACCAGTTGAGCC, nt 139 to 159 of 89.6)-LTRU32 (GCACACACTACTTGAAGCACTC, nt 562 to 541 of 89.6) primer pair, 30 cycles of amplification (94°C for 30 s, 58°C for 30 s, and 72°C for 60 s) followed by a 5-min extension at 72°C (45). Reaction mixtures that contained the GAG04 (CATICTATTTGTTCITGAAGGGTACTAG, nt 1081 to 1054 of HxB2)-GAG06 (GCITTIAGCCCIGAAGTIATACCCATG, nt 822 to 848 of HxB2) primer pair were subjected to three cycles of 97°C for 1 min, 55°C for 2 min, and 72°C for 1 min followed by 37 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 1 min and finally 5°C for 5 min (39, 40). Primers PC04 (nt 54 to 73) and GH20 (nt −195 to −176) of the β-globin gene (obtained from Perkin-Elmer) were used as DNA controls and cycled under the same conditions as the LTRU31-LTRU32 primer pair. Twenty microliters of each reaction mix was then analyzed by gel electrophoresis (4% NuSieve GTG–agarose, 1:1; FMC, Rockland, Maine), transferred to Zetaprobe GT (Bio-Rad), and probed with a γ-^{32}-P-labeled internal oligonucleotide (2).

Competitive PCR. Increasing dilutions of competitive *gag* sequence containing an 80-bp internal deletion (39, 40) were added to the PCR mixtures containing DNA from infected cells. The number of copies of viral DNA per 10^6 cells was then quantitated on the basis of the relative intensities of the two different PCR products, using PhosphorImager (Molecular Dynamics) analysis (39, 40).

Virus binding. GalS (1 mg/ml) was dissolved in methanol and plated on ELISA plates (100 μl), and the solvent was allowed to evaporate overnight. Recombinant soluble CD4 (sCD4) was plated at 1 μg/ml in $NaHCO_3$ (pH 8.7) overnight at 4°C. The plates were washed with PBS then blocked with 10% fetal calf serum in PBS (1 h at room temperature), and twofold dilutions of stock virus (89.6 = 0.64 μg of p24 per ml; HxB2 = 0.63 μg of p24 per ml) in blocking medium were added. After incubation with virus for 3 h at room temperature, the wells were washed extensively with PBS. The virus was disrupted by incubation with 0.5% Triton X-100–PBS for 10 to 20 min, and the concentration of p24 in the lysate was measured with an antigen capture assay (DuPont).

RESULTS

Generation of a CD4-positive SK-N-MC cell line, SK-N-MC/CD4. SK-N-MC is a GalCer-positive, CD4-negative cell line that was derived from a peripheral neuroblastoma and can be infected with several HIV-1 strains (31). In this series of experiments, it was used to define the regions of gp120 important for entry using this glycolipid pathway. To ensure that our infectivity protocol for recombinant viruses reflected GalCer-dependent entry, and not cellular tropism due to postentry events, we prepared an SK-N-MC line expressing the CD4 receptor to be used as comparison. SK-N-MC were transduced with a retroviral vector expressing the CD4 molecule as described in Materials and Methods. After selection in G418, the level of surface expression of CD4 was determined by flow cytometry using monoclonal antibodies OKT4 and OKT4A (Fig. 2). Thirty-five percent of the SK-N-MC/CD4 cells expressed CD4 on the surface, and immunoprecipitation of the radiolabeled protein showed that it had appropriate mobility in SDS-polyacrylamide gel electrophoresis (data not shown).

To ascertain that the expressed protein could serve as a functional HIV-1 receptor, both the parental SK-N-MC and the SK-N-MC/CD4 cells were infected with HIV-1_{HxB2}, and virus production was monitored by assay of the culture supernatants for $p24^{gag}$. The SK-N-MC/CD4 line produced approximately 10-fold-greater levels of p24 than the SK-N-MC line, consistent with the greater efficiency of the primary HIV-1 receptor (Fig. 3). Pretreatment of the SK-N-MC/CD4 cells with Leu3A, a monoclonal antibody that blocks the interaction between gp120 and CD4, led to a marked decrease in p24 production, but there was a residual low level (around 30 pg), consistent with the view that these cells can be infected by two pathways (Fig. 3). As expected, infection of the SK-N-MC cell line was not inhibited by Leu3A (31).

Restriction of HIV-$1_{89.6}$ in the SK-N-MC cell line is at the level of viral entry. We then tested a small panel of HIV-1 isolates for the ability to infect the SK-N-MC cells (Table 1). Several isolates, including HIV-1_{HxB2} and HIV-1_{NDK}, infected the SK-N-MC line readily, whereas others did not, but we could not discern any pattern based on existing classifications. The results were consistently negative for HIV-$1_{89.6}$, a macrophage-tropic virus initially isolated from peripheral blood mononuclear cells (17), whether we used production of p24, PCR (Fig. 4A), or cocultivation with CEMx174 cells as the assay for infectivity (not shown). In contrast, HIV-$1_{89.6}$ could infect the SK-N-MC/CD4 line, as shown by PCR amplification

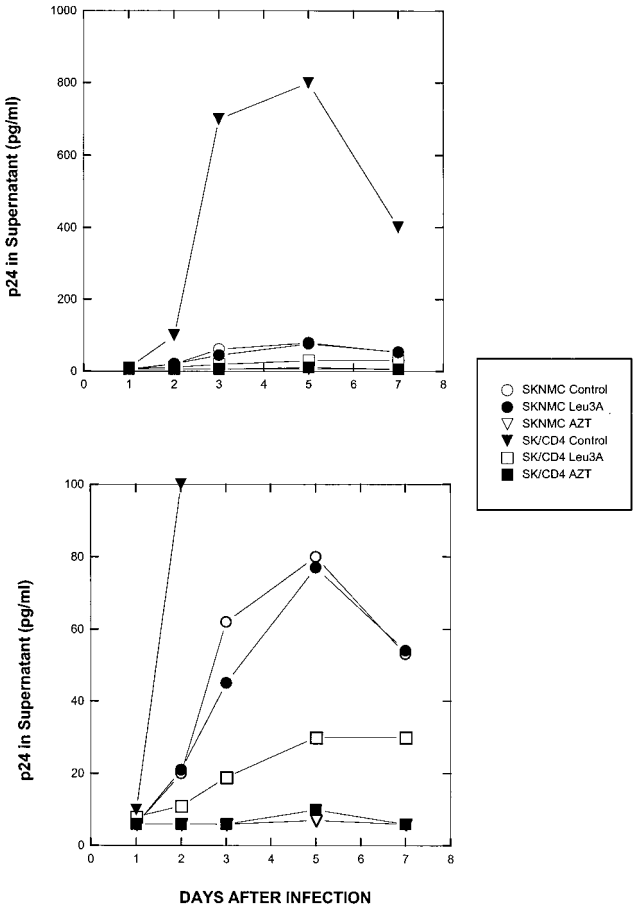

FIG. 3. HIV-1 infection of SK-N-MC and SK-N-MC/CD4 cells. Approximately 10^5 cells were pretreated with Leu3A (100 μg/ml), a control monoclonal antibody (100 μg/ml), or AZT (10 μM) and infected with HIV-1_{HxB2} at an MOI of 0.1 $TCID_{50}$ per cell. $p24^{gag}$ antigen in the supernatant was measured by antigen capture assay. The data are presented in two scales to recognize the higher level of $p24^{gag}$ production in the SK-N-MC/CD4 cells. The results are representative of two independent experiments.

TABLE 1. Infection of SK-N-MC cells by a panel of HIV isolates

HIV-1 strain	Phenotype[a]	Clade	SK-N-MC infectability[b]
HxB2	T/SI	B	+
BR/92/021	SI	B	+
RW/92/009	NSI	A	+
SF162	M/NSI	B	+
NDK	T/SI	D	+
SF2	T/SI	B	+
UG/92/021	SI	D	+
89.6	M/T[c]/SI	B	−
ADA	M/NSI	B	−
BR/93/029	ND	F	−
CDC451	T	B	−
CDC/C	T	A	−
THA/92/022	NSI	E	−
Z6	T/SI	D	−

[a] M, replicates in monocyte-derived macrophages and peripheral blood lymphocytes; T, replicates in peripheral blood lymphocytes and in transformed cell lines; SI, syncytium inducing. NSI, non-syncytium inducing; ND, not done.
[b] Defined by the ability to detect products of reverse transcription by PCR and/or the ability to rescue virus by cocultivation.
[c] Replicates in monocyte-derived macrophages, peripheral blood lymphocytes, and cell lines CEMx174 and MT-2.

with primers amplifying the LTR U3/R region (Fig. 4A; see Materials and Methods) or *gag* sequences (sk38-sk39 and GAG04-GAG06; data not shown). These results suggested that the block to HIV-$1_{89.6}$ infection in the GalCer-positive SK-N-MC line is due to the inability of HIV-$1_{89.6}$ to use the glycolipid for entry.

To determine whether other primary isolates could infect SK-N-MC cells, we tested a limited panel of viruses from different clades (Fig. 4B). Isolates RW/92/009, BR/92/021, and UG/92/021 gave a positive PCR signal and could be rescued by cocultivation with peripheral blood mononuclear cells (data not shown).

An endpoint titration with HIV-1_{HxB2} and HIV-$1_{89.6}$ was then performed in each of the two cell lines. Eighteen hours following inoculation with 10-fold dilutions of virus, DNA was amplified by using *gag*-specific primers (see Materials and Methods), and the signal was quantified with a PhosphorImager following Southern analysis. As shown in Fig. 5, there was a 50-fold difference between SK-N-MC and SK-N-MC/CD4 entry with HIV-1_{HXB2}. HIV-$1_{89.6}$ did not register a significant signal at the lowest dilution of virus used in the SK-N-MC cells.

Binding between viruses and GalS. A virus-GalS binding assay was then used to confirm that the failure of HIV-$1_{89.6}$ to infect SK-N-MC cells represented a block at the level of entry. GalS is more soluble in methanol than GalCer and can be plated for ELISA more readily (see Materials and Methods). Dilutions of stock virus with equivalent $TCID_{50}$s and $p24^{gag}$ concentrations were incubated with GalS or sCD4, and the plates were washed extensively. After disruption of the virus with 0.5% Triton X-100, the amount of $p24^{gag}$ was measured as an index of bound virus. The results (Fig. 6) clearly demonstrate that HIV-1_{HxB2} bound the immobilized glycolipid whereas HIV-$1_{89.6}$ did not. In contrast, both viruses bound sCD4.

Identification of viral determinants for entry into SK-N-MC cells. A series of chimeric viruses constructed with the molecular clones of HIV-1_{HxB2} and HIV-$1_{89.6}$ was then obtained and used to identify the domains of gp120 important for SK-N-MC entry. A 580-bp fragment of the *env* gene flanked by conserved *Bgl*II sites (Fig. 1) translates into 193 amino acids that encompass the V3, V4, and V5 domains of gp120. Insertion of this region in the background of HIV-$1_{89.6}$ yielded recombinant 89.6ΔBB, which replicated well in SK-N-MC/CD4 cells as well as in the B-cell/T-cell hybrid line CEMx174 that was used to

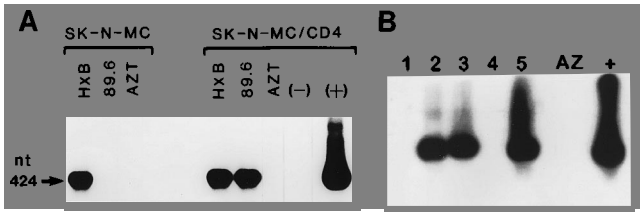

FIG. 4. (A) SK-N-MC or SK-N-MC/CD4 cells were infected with HIV-1_{HxB2} or HIV-$1_{89.6}$, and DNA was extracted 48 h after infection and amplified with LTR primers as described in Materials and Methods. AZT (10 μM) was used as control for newly synthesized viral DNA, and positive [(+)] and negative [(−)] plasmid controls were also included. HIV-$1_{89.6}$ did not infect the SK-N-MC cells but gave a positive signal in the SK-N-MC/CD4 cells. (B) SK-N-MC cells were infected with primary isolates, and DNA was extracted 18 h after infection and amplified with the GAG04-GAG06 primer pair as described in Materials and Methods. Lanes 1, THA/92/022; 2, RW/92/009; 3, BR/92/021; 4, BR/93/029; 5, UG/92/021; AZ, cells pretreated with AZT and infected with HIV-1_{HXB2}; +, positive control.

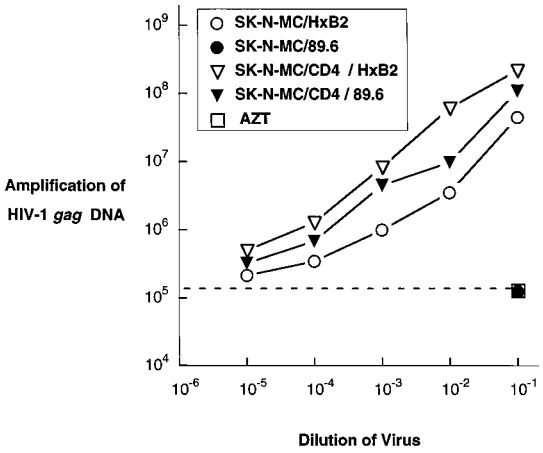

FIG. 5. SK-N-MC or SK-N-MC/CD4 cells were infected with dilutions of stock virus, and DNA was isolated 18 h later, amplified with the GAG04-GAG06 primer pair, probed, and analyzed with a PhosphorImager. The results are expressed in energy emissions per integrated volume, with the signal obtained from cells pretreated with AZT taken as background.

propagate all of the viruses. Moreover, in contrast to the parental HIV-$1_{89.6}$, this virus was now able to infect the SK-N-MC cells (Fig. 7A). Pretreatment of the cells with R-MAb, a monoclonal antibody that recognizes GalCer/GalS, inhibited entry of 89.6ΔBB (as well as the parental HxB2) (Fig. 7B). These results identified a 580-bp sequence of the HxB2 *env* gene that encodes viral determinants that are sufficient to mediate entry into GalCer-positive cells.

We then used a pair of recombinant viruses that divide the 580-bp region enclosed by 89.6ΔBB into two domains: 89.6ΔBM, which includes the V3 region, and 89.6ΔMB, which includes V4 and V5 (Fig. 1). Chimeric viruses that contained either the 91-amino-acid fragment of HxB2 containing the V3 loop (89.6ΔBM) or the 101-amino-acid fragment of HIV-1_{HxB2} encoding the V4 and V5 domains including regions implicated in CD4 binding (89.6ΔMB) on the HIV-$1_{89.6}$ background were able to enter the GalCer/GalS-positive SK-N-MC cells (Fig. 8). Cocultivation of infected SK-N-MC with CEMx174 cells resulted in productive infection with each chimera (1 to 7 ng of p24 per ml), and nucleotide sequencing of PCR amplified DNA showed the expected genotype. While these results suggested that gp120 conformational determinants and not linear amino acid sequence are involved in SK-N-MC entry, quantitative differences would not have been detected by this assay.

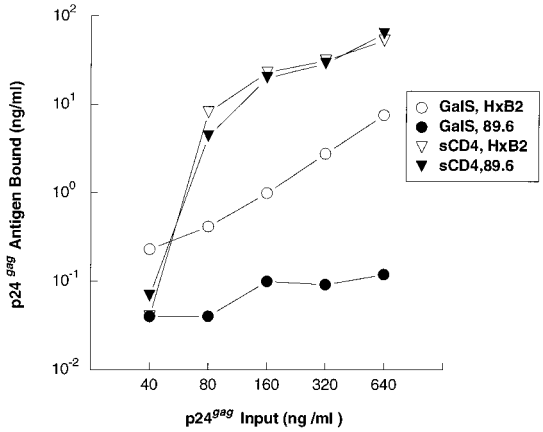

FIG. 6. Binding of GalS by HxB2 and 89.6. Dilutions of stock virus were incubated with GalS or sCD4 immobilized on ELISA plates for 3 h at room temperature, washed extensively with PBS, and lysed with 0.5% Triton X-100–PBS. The lysate representing bound virus was assayed for p24 content. The figure shows the input p24 concentration (*x* axis) and the bound p24 concentration ($\log_{10}$, *y* axis).

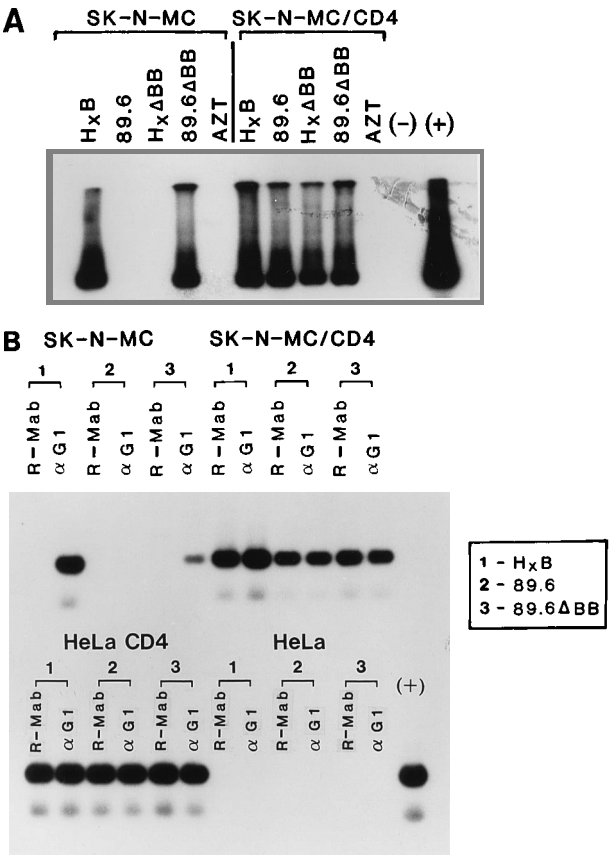

FIG. 7. Infection with HIV-1_{HxB2}, HIV-$1_{89.6}$, HxΔBB, and 89.6ΔBB. (A) SK-N-MC or SK-N-MC/CD4 cells were exposed to each parental virus or the ΔBB recombinants containing 580 bp (193 amino acids) of the reciprocal parent (e.g., HxΔBB contains the *Bgl*II-*Bgl*II *env* region from HIV-$1_{89.6}$). Forty-eight hours after infection, DNA was extracted and PCR amplified, and Southern blotting was performed as indicated in Materials and Methods. Neither HIV-$1_{89.6}$ nor a recombinant containing 193 amino acids from the HIV-$1_{89.6}$ *env* (HxΔBB) infected the SK-N-MC cells, though they both infected the SK-N-MC/CD4 cells. AZT-treated and positive [(+)] and negative [(−)] controls were included. (B) Infection of SK-N-MC or SK-N-MC/CD4 cells with HIV-1_{HxB2}, HIV-$1_{89.6}$, or 89.6ΔBB was performed with or without pretreatment with R-MAb, a monoclonal antibody directed against GalCer/GalS (4, 41). Infection of SK-N-MC was blocked by this antibody. αG1 is a monoclonal antibody directed against a viral glycoprotein used as a control. HeLa and HeLa CD4 cells were used as negative and positive controls.

Two experiments were then performed to determine if there were quantitative differences not detected by conventional PCR techniques. In the first, infections with the recombinant and parental viruses were performed at different MOIs. At an MOI of 0.01 $TCID_{50}$ per cell (determined by cytopathology in CEMx174 cells), there was no PCR signal after infection with recombinant 89.6ΔMB. The other recombinants, as well as HxB2, showed a positive PCR signal at that MOI (data not shown).

The second series of experiments was performed at an MOI of 0.1, using a quantitative competitive PCR analysis with a *gag* probe (39, 40) (see Materials and Methods). SK-N-MC and SK-N-MC/CD4 DNAs were extracted 18 to 24 h after infection

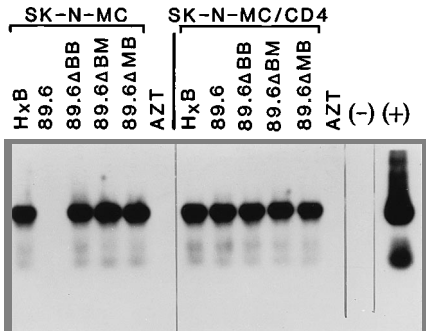

FIG. 8. Infection with recombinant viruses. SK-N-MC or SK-N-MC/CD4 cells were infected with recombinant viruses prepared from parental HxB2 and 89.6 molecular clones as described previously (28). DNA was extracted 48 h later and amplified by PCR (see Materials and Methods). Recombinants containing regions of the HxB2 envelope, 89.6ΔBB, 89.6ΔBM, and 89.6ΔMB, all infected SK-N-MC cells, whereas parent 89.6 virus had a negative phenotype. The results are representative of five independent experiments. (−) and (+) represent negative and positive controls.

at an MOI of 0.1 and amplified in the presence of an increasing copy number of a competitive *gag* template (see Materials and Methods). After Southern blot with a ^{32}P-labeled synthetic oligonucleotide, the amplified target and competitive signals were quantified with a PhosphorImager. The ratio between the two bands was determined, and the number of copies of HIV DNA in the infected cells was calculated.

As shown in Fig. 9, infection with recombinant 89.6ΔBM yielded fivefold more DNA copies than infection with recombinant 89.6ΔMB in the SK-N-MC cells. Both viruses were less efficient than virus 89.6ΔBB, which includes both regions. No significant differences were noted in infections of SK-N-MC/CD4 (Fig. 9). Thus, although the region of the viral envelope involved in CD4-independent entry is relatively large, the region including the V3 loop is of paramount importance and can by itself confer a phenotype close to that of the wild-type HIV-1_{HxB2}.

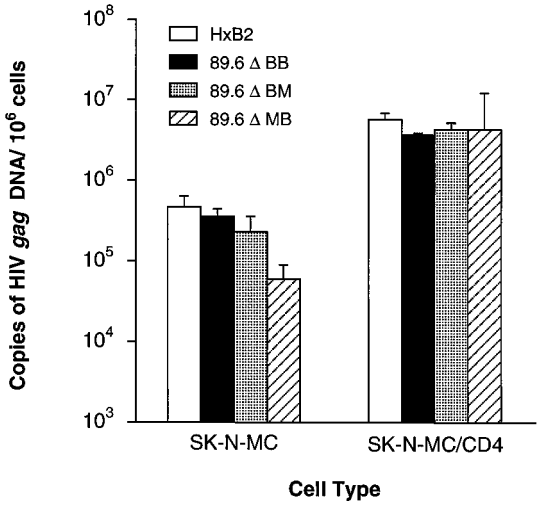

FIG. 9. Quantitative competitive PCR analysis of SK-N-MC and SK-N-MC/CD4 infection. Quantitative differences in HIV-1 entry among the recombinants were analyzed by determination of the number of copies of HIV *gag* DNA present in the infected cells 24 h after infection. Quantitative competitive PCR was performed as described in Materials and Methods. The bars represent the means and standard deviations of three independent experiments, each analyzed with independent curves.

DISCUSSION

In this series of experiments, we have defined the regions of the HIV-1 envelope that are critical for viral entry into the SK-N-MC cell line. Using chimeras obtained between viruses that infect or do not infect SK-N-MC, we have identified a 193-amino-acid region encompassing V3, V4, and V5 of the HIV-1_{HxB2} gp120 that confers the ability to infect SK-N-MC to a phenotypically negative isolate, HIV-$1_{89.6}$. Although HIV-$1_{89.6}$ is a macrophage-tropic virus (17, 19, 45), experiments with a small panel of HIV-1 isolates showed that macrophage tropism bore no clear relationship to SK-N-MC infectability (Table 1). Interestingly, when this 193-amino-acid region was subdivided into two pieces, one of which contained the V3 loop, the other more distal regions, either half allowed the 89.6 virus to infect SK-N-MC, although quantitative competitive PCR identified quantitative differences among the recombinants. This finding indicates that tropism for this cell line is dependent on a complex interaction involving multiple regions of the carboxyl half of gp120, which may act either in an independent fashion or in concert to form a stable gp120-glycolipid bond.

We believe that these results with recombinant viruses relate to the interaction between gp120 and GalCer/GalS, since the infectivity of each chimera in SK-N-MC was compared with the infectivity in an SK-N-MC line engineered to express CD4. This paradigm should differentiate entry from postentry restriction to viral replication. More direct evidence that the restriction in HIV-$1_{89.6}$ entry is at level of binding comes from solid-phase ELISA in which HIV-1_{HxB2} bound GalS and HIV-$1_{89.6}$ did not. These results will be compared with experiments now in progress in which the gp120 of each isolate is being expressed in a recombinant system.

Our results are consistent with previous experiments which demonstrated the inhibition of gp120-GalCer binding either by V3 antibodies or by some conformational antibodies directed against the CD4 binding domain (20). Furthermore, multimeric peptides derived from the V3 loop can bind to GalCer, and block infection of HT-29 cells (57), as can antibodies against the V3 loop (20). Long et al. (32) have postulated that GalCer binding by gp120 is due to a network of hydrogen bonds, on the basis of the resistance of the gp120-GalCer complex to urea and KSCN, which would be expected to destabilize the binding if it were due to ionic or hydrophobic protein interactions. Our model, which involves a large region of gp120, is compatible with these results. The HIV-1 gp120 is also known to bind heparan sulfate and other sulfated polysaccharides that could serve as accessory molecules in conjunction with CD4. The V3 region has been implicated in binding to those molecules as well (5, 8, 44).

Initial studies of HIV-1 tropism for macrophages and CD4$^+$ cultured cells stressed the importance of the V3 loop. For example, the V3 loop determines the tropism of an HIV-1_{GUN} isolate for a CD4$^+$ brain-derived cell line (47) and is a component of the regions that determine the tropism of HIV-1_{NL4-3} for HUT-78 cells (10). The V3 loop is also a critical element in the macrophage tropism of several HIV isolates, including HIV-1_{JRFL}, HIV-1_{ADA}, and HIV-1_{BaL} (13, 27, 38, 54). However, as the number of isolates studied has grown, there is increasing evidence suggesting that other regions of gp120 are important for cell tropism (7). Concomitantly, there has been a new appreciation for the interactions between the V3 loop and other domains of gp120. Amino acid changes in the gp120 C4 region have been shown to affect the binding of monoclonal antibodies against the V3 loop (55), and vice versa, antibodies binding to conformation-sensitive areas of C4

have decreased binding to mutants with alterations in the V3 loop (36).

In the case of HIV-$1_{89.6}$, the V3 loop is not the predominant determinant of macrophage tropism. Recombinant 89.6ΔBM, which contains the V3 loop of HIV-1_{HxB2} (which is not macrophage tropic), had markedly decreased replication in macrophages, but replication was not abolished (28). Recombinant 89.6ΔBB, which contains a larger piece of HIV-1_{HxB2} on the background of the macrophage-tropic HIV-$1_{89.6}$, replicated well in macrophages, suggesting an interaction between V3 and V4/V5 in this chimera. This interaction is also evident in the current experiments, but in the case of SK-N-MC, the presence of the two halves is not as critical for a positive phenotype.

Other evidence supporting a role for multiple domains of the envelope gene in tropism have been provided by macrophage-tropic HIV-1_{IIIB} variants obtained from an infected laboratory worker, in which case the determinants of tropism are scattered throughout the *env* gene (34). O'Brien et al. also suggested a contribution from the regions of gp120 involved in CD4 binding in the tropism of HIV-1_{JRFL} for monocyte-derived macrophages (38). Using a panel of monoclonal antibodies, Koito et al. have demonstrated that mutations in V2 can alter the conformation of the V3 region (29).

Taken together, these studies all indicate that in the oligomeric HIV-1 envelope there is structural proximity between the V3 loop and the C4 domain as well as other domains, as described by Moore and coworkers (36). Our results with this model of CD4-independent infection indicate that there is a cooperative interaction between these regions in SK-N-MC infection, but that some of the domains can be substituted. Although no linear sequences have been identified, our data suggest that there is a conformation which can be acquired by different combinations of primary sequences among these two viruses. Furthermore, it has been postulated that GalCer may be present in the plasma membrane in patches or microdomains (48), and perhaps the gp120-GalCer interaction involves multiple bonds. In this interpretation, the differences between the recombinant viruses would reflect a quantitative relationship to the number of binding sites.

Although microglia, which like macrophages are thought to be $CD4^+$, are the most highly infected cell type in the brain, recent evidence obtained by using in situ PCR has implicated astrocytes, endothelial cells, neurons, and oligodendrocytes in the pathogenesis of HIV dementia (30, 57). As none of these cell types express CD4, understanding CD4-independent entry may become very important in discerning the pathophysiology of HIV dementia. These experiments should provide some insights into this question.

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