Cloning and Characterization of Human Immunodeficiency Virus Type 1 Variants Diminished in the Ability To Induce Syncytium-Independent Cytolysis

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The phenomenon of interference was exploited to isolate low-abundance noncytopathic human immunodeficiency virus type 1 (HIV-1) variants from a primary HIV-1 isolate from an asymptomatic HIV-1-seropositive hemophiliac. Successive rounds of virus infection of a cytolysis-susceptible CD4⁺ cell line and isolation of surviving cells resulted in selective amplification of an HIV-1 variant reduced in the ability to induce cytolysis. The presence of a *PvuII* polymorphism facilitated subsequent amplification and cloning of cytopathic and noncytopathic HIV-1 variants from the primary isolate. Cloned virus stocks from cytopathic and noncytopathic variants exhibited similar replication kinetics, infectivity, and syncytium induction in susceptible host cells. The noncytopathic HIV-1 variant was unable, however, to induce single-cell killing in susceptible host cells. Construction of viral hybrids in which regions of cytopathic and noncytopathic variants were exchanged indicated that determinants for the noncytopathic phenotype map to the envelope glycoprotein. Sequence analysis of the envelope coding regions indicated the absence of two highly conserved N-linked glycosylation sites in the noncytopathic HIV-1 variant, which accompanied differences in processing of precursor gp160 envelope glycoprotein. These results demonstrate that determinants for syncytium-independent single-cell killing are located within the envelope glycoprotein and suggest that single-cell killing is profoundly influenced by alterations in envelope sequence which affect posttranslational processing of HIV-1 envelope glycoprotein within the infected cell.

The human immunodeficiency virus type 1 (HIV-1) exerts profound cytopathic effects upon host CD4⁺ lymphocytes in vitro (2, 33). The cytopathic effects of HIV-1 in vivo may account (in part) for the observed CD4⁺ cell depletion during the progression of acquired immunodeficiency syndrome (14, 53). Supporting evidence for a role of virus-mediated cytopathicity in the progression of acquired immunodeficiency syndrome is the demonstration that the cytopathic phenotypes of sequential HIV-1 isolates correlate with the stage of disease progression (4, 10); isolates obtained during later stages of the disease display greater cytopathic properties in a wider variety of cell types. Two mechanisms appear to account for HIV-1-mediated cytotoxicity in susceptible host cells in vitro, namely, virus-induced cell fusion (23, 40) and syncytium-independent single-cell killing (41, 43). The process of virus-induced cell fusion is well characterized and is facilitated by hydrophobic domains within the amino terminus of HIV-1 gp41 (20) following the binding of gp120 to CD4 (23, 40). Features of HIV-1 responsible for syncytium-independent single-cell killing have not been characterized, but appear to be dependent upon high-level expression of viral antigens (42, 43). In addition, structural determinants of the HIV-1 genome which account for increased cytopathicity of sequential HIV-1 isolates from infected individuals have not been characterized. Presumably, the generation of sequence variability during disease progression (11, 15, 36) directly affects the phenotype of the viral variant, conferring upon it altered biological properties with respect to tropism (13, 21) and cytopathicity (11). Identification of structural determinants of HIV-1 important in governing the cytopathic phenotype would be greatly facilitated by the availability of matched cytopathic and noncytopathic variants from a single

patient, thus allowing the delineation of regions of the HIV-1 genome which contribute to the biological features of the virus.

Here we describe the cloning and characterization of cytopathic and noncytopathic HIV-1 genotypes from a seropositive individual. The noncytopathic variant exhibits similar replication kinetics and syncytium-inducing properties as the cytopathic variant, but is diminished in its ability to induce single-cell killing in susceptible host cells. We provide evidence that the determinant for single-cell killing maps to the envelope glycoprotein. These results demonstrate that minor sequence differences within the envelope glycoprotein of HIV-1 can generate virus variants with markedly different biological properties. These variants may play a role either in the maintenance of persistence or in CD4⁺ cell depletion during disease pathogenesis.

MATERIALS AND METHODS

Isolation and cloning of cytopathic and noncytopathic HIV-1 variants. The primary virus isolate was obtained by cocultivation of peripheral blood lymphocytes from an HIV-1-seropositive asymptomatic hemophiliac with cells of the CD4⁺ cell line CEM. Following infection of CEM cells, the primary isolate was maintained and amplified by sequential passage in CEM cells. Low-abundance noncytopathic HIV-1 variants were isolated and amplified by successive rounds of infection of the human T-cell lymphotropic virus type I (HTLV-I)-transformed leukemic cell line MT-4 (25), and then surviving MT-4 cells were isolated and amplified (see Fig. 1). High-molecular-weight DNA was extracted from third-round-infected MT-4 cells by guanidine thiocyanate lysis and centrifuged in cesium chloride gradients by the procedure of Chirgwin et al. (6). Cellular DNA (150 µg) was digested with *XbaI* and resolved on a 0.8% agarose gel, and

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DNA with a molecular size greater than 10 kilobases (kb) was electroeluted, concentrated, and ligated to *Xba*I-digested λ gem11 (Promega Biotec, Madison, Wis.). Ligations were packaged (Gigapack; Stratagene Inc., La Jolla, Calif.), and 1.2×10^6 recombinant bacteriophage were screened for HIV-1 inserts with an 8.7-Kb proviral HIV-1 probe (*Sac*I-digested fragment of N1G-F [48]). Of 150 HIV-1-positive phage plaques, phage DNA was amplified from 15. These DNAs were digested with *Pvu*II and analyzed by Southern blot hybridization to an HIV-1 *gag*-specific probe. Two clones with *gag*-reactive DNA fragments of 2.2 and 0.6 kb and three clones with *gag*-reactive fragments of 2.8 kb were amplified, digested with *Xba*I, and subcloned into the *Xba*I site of pUC18. Restriction maps of five amplified proviral DNA clones were generated by conventional means with 12 different restriction enzymes.

Virus propagation and virus infection. To obtain virus stocks from cloned virus DNA, we seeded HeLa cells at approximately 30% confluence and transfected them with cloned proviral DNA by the calcium phosphate coprecipitation method (10 μ g/ 10^6 cells). Two days after transfection, cell culture supernatant was removed and filtered (pore size, 0.8 μ m) and added to fresh MT-4 cultures (0.5 ml/ 2×10^6 MT-4 cells). MT-4 cultures were maintained until characteristic multinucleated giant cells were seen and viral antigens were demonstrated by immunofluorescence analysis. For analysis of replication kinetics of cloned viral variants, CEM, Mo-T cells (HTLV-II-transformed CD4⁺ cell line) (3), and MT-4 cells were inoculated with 200 tissue culture infectious units (TCIU) per 10^6 cells. One TCIU is defined as the maximal dilution of the virus inoculum that generates an infected CEM cell culture 2 weeks postinoculation. An inoculum of 200 TCIU typically results in antigen expression in 5 to 10% of CEM cells at 3 to 4 days postinfection. At designated time intervals, culture samples were removed to determine the cell count, viability (trypan blue exclusion), HIV-1 antigen expression (immunofluorescence assay), and reverse transcriptase activity in the culture supernatant. Syncytia were quantitated essentially as described by Sodroski et al. (40).

Reverse transcriptase assay. We measured the DNA polymerase activity [poly(A) dependent] in 50 μ l of 50 mM Tris hydrochloride (pH 7.5)–5 mM dithiothreitol–100 mM KCl–10 mM MgCl₂–10 mM [³H]dTTP–0.1% Triton X-100 containing 2 μ l of poly(A), 0.4 μ g of oligo(dt)_{12–18}, and appropriate amount of virions. The reaction was carried out at 37°C for 1 h, and the polymerized [³H]dTTP was precipitated with 10% trichloroacetic acid, collected on a membrane filter (Millipore Corp., Bedford, Mass.), washed with 10% trichloroacetic acid and ethanol, air dried, and counted in a liquid scintillation counter.

Solution hybridization analysis for viral RNA. HIV-1-specific RNA probes were transcribed in vitro from a transcription plasmid (pGEM3; Promega Biotec) containing the *gag* gene of HIV-1 in the presence of 10 μ M [³²P]CTP (600 Ci/mmol) and excess unlabeled ATP, GTP, and UTP. After 1 h at 37°C, template was digested with RNase-free DNase (1 μ g/ml) for 15 min at room temperature. Following phenol extraction, the RNA probes were purified on a 1-ml Sephadex G50-150 column. HIV-1 RNA in infected cells was quantitated by a modified RNase protection assay in which probe transcribed from linearized transcription plasmid was hybridized to infected-cell lysates in 5 M guanidine thiocyanate under conditions of probe excess, essentially as described by Pellegrino et al. (31).

Immunofluorescence analysis. Infected cells were washed

in phosphate-buffered saline, spotted on glass slides, dried, and fixed in acetone at –20°C for 15 min. Fixed cells were reacted with HIV-1-positive serum from an asymptomatic HIV-1-seropositive hemophiliac (antibody titer, 1:5,160). After a 30-min incubation at 37°C, cells were reacted with fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin G, and virus antigen-positive cells were counted under an epifluorescence microscope (Nikon Inc., Garden City, N.Y.).

Cell surface marker analysis. Cell samples (5×10^5 cells) were washed with phosphate-buffered saline and incubated at 4°C in the presence of unlabeled monoclonal antibody. Cells were then incubated with fluorescein isothiocyanate-conjugated secondary antibody, and fluorescence was quantitated with an Ortho model H cytofluorograph.

Isolation and analysis of unintegrated HIV-1 DNA. Unintegrated HIV-1 DNA was isolated by the method of Hirt (17) with the modifications of Chinsky and Soiero (5). Briefly, aliquots of 2×10^6 cells were lysed at 65°C in 0.6% sodium dodecyl sulfate (SDS), and Hirt supernatants were extracted twice with phenol and once with chloroform, and precipitated with ethanol. DNA extracts were then electrophoresed in 0.8% Tris borate (pH 8.0) agarose gels and transferred to nylon filters by the procedure of Southern. Filters were hybridized at 42°C in a solution containing 5 $\times$ SSC (1 $\times$ SSC is 0.015 M sodium citrate plus 0.15 M NaCl), 50% formamide, 1% SDS, 3 $\times$ Denhardt solution, 100 μ g of denatured salmon sperm DNA per ml, and 5 μ M EDTA (pH 8.0). Hybridization continued for 24 h in hybridization cocktail supplemented with 1×10^6 to 2×10^6 cpm of ³²P-labeled DNA probe per ml (specific activity, 2×10^8 to 5×10^8 cpm/ μ g), and the filters were washed with agitation in 0.2 $\times$ SSC–0.1% SDS at 60°C. Washed filters were blotted dry, wrapped in Saran Wrap, and subjected to autoradiography with X-Omat intensifying screens (Eastman Kodak Co., Rochester, N.Y.) at –80°C. *Hind*III-digested bacteriophage lambda DNA end labeled with the DNA polymerase I large fragment was used as size markers.

Immunoprecipitation analysis. Cells were washed twice with phosphate-buffered saline and labeled for 6 h in RPMI 1640 medium (1×10^6 to 2×10^6 cells per ml) containing 5% dialyzed calf serum and 50 to 100 μ Ci each of L-[³⁵S] methionine and [³⁵S]cysteine per ml (1,000 μ Ci/mmol; Du Pont, NEN Research Products, Boston, Mass.). At the end of the labeling period, cells were washed at 4°C with phosphate-buffered saline containing 0.2 mM phenylmethylsulfonyl fluoride and centrifuged at $3,000 \times g$ for 20 min to remove nuclei. Cell lysates were preabsorbed overnight at 4°C and then incubated with HIV-1-positive serum-treated beads with gentle rotation at 4°C for 12 h. Typically, 100 μ g of Sepharose beads was absorbed with 100 μ l of HIV-1 serum and conjugated with extract from 10^7 cells. Conjugated beads were washed sequentially with immunoprecipitation buffer (0.02 M Tris hydrochloride, 0.12 M NaCl [pH 8.0], 0.2 mM phenylmethylsulfonyl fluoride, 5 μ g of aprotinin per ml, 0.2 mM EDTA, 0.2 mM NaF, 0.2% sodium deoxycholate, 0.5% Nonidet P-40), buffer containing 0.5 M NaCl, and buffer containing 0.1% SDS. Washed beads were suspended in 50 μ l of electrophoresis buffer (0.01 M Tris [pH 8.0], 2% SDS, 5% 2-mercaptoethanol, 25 μ g of bromophenol blue per ml, 10% glycerol). Adsorbed material was eluted by heating beads at 65°C for 30 min. Electrophoresis was performed in a discontinuous polyacrylamide gel (3.5% stacking, 15% resolving). Gels were fixed, dried, and autoradiographed with X-Omat intensifying screens. Protein molecular weight markers were myosin (200,000), phospho-

FIG. 1. Amplification of noncytopathic HIV-1 variants in vitro. A primary HIV-1 isolate was derived from peripheral blood lymphocytes of an HIV-1-seropositive asymptomatic hemophiliac and maintained in CEM cells. Cell-free supernatant from the CEM producer line was used to infect MT-4 cells (first-round infection), which rapidly succumbed to the cytopathic effects of the virus. After 2 weeks, surviving MT-4 cells (approximately 1% of starting cell culture) were isolated by density gradient centrifugation and amplified. Cell-free supernatants from these cultures were used to infect fresh MT-4 cells (second-round infection). Surviving cells were again isolated and amplified, and supernatants from second-round cultures were used to initiate a third-round infection on MT-4 cells. High-molecular-weight DNA from MT-4 cells after first- (a), second- (b), and third (c)-round infection was isolated and digested with *Bgl*II (lanes 1), *Sac*I (lanes 2), *Kpn*I (lanes 3), and *Pvu*II (lanes 4), resolved on 0.8% agarose gels, transferred to nylon filters, and probed with an 8.7-kb *Sac*I fragment of the proviral DNA clone HIV-1 N1G-F (48). Arrowheads indicate positions of *Pvu*II restriction fragment polymorphisms showing the replacement of 2.2- and 0.6-kb restriction fragments with a 2.8-kb restriction fragment by third-round infection.

rylase *b* (97,400), bovine serum albumin (68,000), ovalbumin (43,000), α -chymotrypsin (25,700), β -lactoglobulin (18,400), and lysozyme (14,300) (Bethesda Research Laboratories, Inc., Gaithersburg, Md.).

DNA sequence analysis. DNA sequencing was performed by the chain termination method. All sequence comparisons and identification of potential N-linked glycosylation sites were facilitated by using the HIV-1 sequence data base (28). The GenBank accession number for the HIV-1 mfA sequence is M33943.

Polymerase chain reaction analysis of DNA. DNA samples (1 μ g) were subjected to repeated rounds of amplification (30 cycles) in a 50- μ l reaction mixture containing 100 pmol each of plus- and minus-strand primer (nucleotides 1078 to 1096 and 1337 to 1354, respectively, of the sequence of Ratner et al. [35]), 200 μ M each deoxynucleotide, 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, and 2 U of *Thermus aquaticus* (Taq) DNA polymerase. Each cycle consisted of a 1-min denaturation step (95°C), followed by a 2-min annealing step (61°C), and a 3-min extension at 72°C. Aliquots (20 μ l) of the polymerase chain reaction (PCR) mixture were resolved on 0.5% agarose–2% Nu-Sieve agarose Tris borate gels containing 0.5% μ g of ethidium bromide per ml. Following electrophoresis, gels were denatured, neutralized, and transferred to nylon membranes. Filters were prehybridized in a solution containing 5 $\times$ SSPE (1 $\times$ SSPE is 0.18 M NaCl, 10 mM sodium phosphate [pH 7.7], 1 mM EDTA), 0.1% SDS, and 5 $\times$ Denhardt solution. Hybridization to [γ -³²P]ATP-labeled plus-strand primer probe (see Fig. 3) continued overnight in the same solution, followed by two wash steps at 5°C above the T_m in a solution containing 5 $\times$ SSPE and 0.1% SDS. Filters were autoradiographed overnight at –70°C.

RESULTS

Isolation of noncytopathic HIV-1 variants. A primary HIV-1 isolate was obtained from cultures of peripheral blood from an asymptomatic HIV-1-seropositive hemophiliac who showed an abnormal CD4/CD8 lymphocyte ratio, but lacked evidence of opportunistic infections. The primary HIV-1 isolate was amplified and maintained in CEM cells. For isolation of cytopathic and noncytopathic variants from the primary isolate, cell-free supernatant from CEM producer lines was used to infect MT-4 cells, which are exquisitely sensitive to the cytopathic effects of HIV-1 (16). Infection of MT-4 cells by the primary HIV-1 isolate resulted in a rapid virus-induced destruction of the culture. Cell survivors were isolated by density gradient centrifugation and amplified. Filtered supernatants from amplified survivors were used to infect fresh MT-4 cultures. In the second round of infection the onset of cytopathic effects was delayed, and the extent of cell destruction in this culture was reduced when compared with first-round infection. Again, surviving cells were separated and amplified, and supernatant was used to initiate a third round infection on fresh MT-4 cells. It was evident that virus amplified in this manner was unable to induce cytolysis in host MT-4 cells, and in third-round-infected cultures viability could be maintained at more than 95%, despite an abundance of viral antigen and reverse transcriptase in the culture supernatant. High-molecular-weight DNA was extracted from first-, second-, and third-round-infected cultures, digested with a variety of restriction enzymes, and probed with an HIV-1-specific DNA probe in an attempt to identify possible restriction polymorphisms which may indicate the outgrowth of distinct viral genotypes during the

FIG. 2. Restriction maps of variant HIV-1 proviral DNA clones. High-molecular-weight DNA from third-round-infected MT-4 cultures was initially cloned into the *Xba*I site of the lambda cloning vector, λ gem11. Fifteen HIV-1-positive phage clones were screened by *Pvu*II digestion and Southern blot hybridization to an HIV-1 *gag*-specific probe. Two clones displaying 0.6- and 2.2-kb *gag* fragments and three clones containing 2.8-kb *gag* fragments were further subcloned into the *Xba*I site of pUC18. —, HIV-1 sequences; ---, flanking cellular sequences. Enzyme sites: Xb, *Xba*I; P, *Pvu*II; B, *Bgl*I; S, *Sac*I; H, *Hind*III; Ps, *Pst*I; K, *Kpn*I; E, *Eco*RI; Sa, *Sal*I; Sm, *Sma*I; Ba, *Bam*HI; X, *Xho*I.

isolation procedure. The hybridization profile of high-molecular-weight DNA digested with four different restriction enzymes is shown in Fig. 1. Use of a partial (*Sac*I) genomic HIV-1 probe identified the selective amplification of an HIV-1 variant with a distinct *Pvu*II restriction site polymorphism (Fig. 1). Further analysis with subgenomic HIV-1 probes mapped this polymorphism to the *gag* gene of HIV-1. Thus, the primary HIV-1 isolate which predominated during first-round infection contained *gag*-reactive *Pvu*II restriction fragments of 2.2 and 0.6 kb (Fig. 1a). Upon second- and third-round infections, there was a selective amplification of a variant with a single *gag*-reactive *Pvu*II fragment of approximately 2.8 kb (Fig. 1b and c) which, by third-round infection, was the predominant variant in the infected culture, as indicated by an almost complete absence of 0.6- and 2.2-kb *gag*-reactive *Pvu*II fragments (Fig. 1c).

High-molecular-weight DNA was extracted from third-

round-infected MT-4 cells, digested with *Xba*I, and cloned in the *Xba*I site of the bacteriophage cloning vector λ gem11. Fifteen HIV-1-positive recombinant λ phage clones were amplified, digested with *Pvu*II, and analyzed by Southern blot hybridization to an HIV-1 *gag* probe. Two λ phage clones displaying 0.6- and 2.2-kb *gag*-reactive *Pvu*II fragments and three clones displaying 2.8-kb *gag*-reactive fragments were further subcloned into the plasmid vector pUC18. Restriction maps for these amplified proviral DNA clones are shown in Fig. 2. Restriction maps confirmed a *Pvu*II restriction site polymorphism in the *gag* region of these HIV-1 variants. No other restriction site differences could be observed in these clones when 12 different restriction enzymes were used (Fig. 2).

Cloned HIV-1 variant stocks were obtained following transfection of the human fibroblast cell line HeLa by proviral DNA clones and virus amplification in MT-4 cells.

TABLE 1. Replicative and cytopathic capacity of HIV-1 subclones

HIV-1 clone	Virus production ^a	Presence of syncytia in ^b :			Cytopathic effects in ^c :			% Antigen expression in ^d :		
		MT-4	Mo-T	CEM	MT-4	Mo-T	CEM	MT-4	Mo-T	CEM
mfA	+	+	+	+	+	+	+	>95	>90	5–10
mfB	—	ND ^e	ND	ND	ND	ND	ND	ND	ND	ND
mfC	+	+	+	+	—	—	—	>95	>90	5–10
mfD	+	+	+	+	—	—	—	>95	80–90	5–10
mfF	—	ND	ND	ND	ND	ND	ND	ND	ND	ND

^a Determined following transfection of HeLa cells with 10 μ g of cloned virus and virus amplification on MT-4 cells.

^b Five random fields (approximately 200 cells per field) were examined ($\times 40$ magnification) for the presence of syncytia.

^c Cells were stained (trypan blue) at 7, 10, and 17 days postinfection, and five random fields (200 cells per field) were examined ($\times 40$ magnification) for the number of staining cells which were not incorporated into syncytia.

^d Percentage by immunofluorescence analysis with serum from an HIV-1-seropositive hemophiliac (titer, 1:2,580) at day 5 postinfection.

^e ND, Not done.

FIG. 3. Identification of *PvuII* sequence polymorphism in DNA from primary lymphocytes cocultured by PCR. Total cellular DNA was isolated from primary patient lymphocyte cultures (lane b) and from MT-4 cells infected with HIV-1 mD (lane a) or HIV-1 mA (lane c). Aliquots (1 μ g) were amplified by PCR with primers flanking the *PvuII* restriction polymorphism identified in *gag* (Fig. 2) located at nucleotide 1144 of the sequence of Ratner et al. (35). Following amplification, PCR products were digested with *PvuII* and analyzed by Southern blotting and hybridization to ^{32} P-labeled plus-strand primer probe. Plus-strand (nucleotide 1078 to 1096) and minus-strand (nucleotide 1337 to 1354) primers amplify a 276-base-pair (b.p.) fragment which will be reduced to 210 base pairs after *PvuII* digestion if *PvuII* polymorphism is present in the amplified product.

Transfection of HeLa cells with HIV-1 mA, mC, and mD clones yielded infectious virus after rescue in MT-4 cells. Repeated attempts to rescue virus from HeLa cells transfected with mB or mF clones were unsuccessful (Table 1). To ensure that the genotypes with a *PvuII* polymorphism did not arise as a result of mutation in vitro, the presence of those genotypes in the primary CEM coculture from the donor patient was confirmed by PCR. Primers flanking the *gag PvuII* site were used to amplify a 276-base-pair fragment, and the presence of the *PvuII* polymorphism was evaluated after *PvuII* digestion of amplified products and Southern blot hybridization to ^{32} P-labeled plus-strand primer (Fig. 3). PCR products from cells infected with the HIV mA isolate were completely digested by *PvuII* (Fig. 3, lane c), whereas PCR products from HIV mD-infected cells were resistant to *PvuII* digestion (Fig. 3, lane a). The amplification products of DNA from the primary coculture were partially digested by *PvuII* (Fig. 3, lane b), indicating that both genotypes existed in the patient. However, there appeared to be a far greater proportion of HIV-1 mA genotype, since the majority of the amplified products contained a *gag*-specific *PvuII* site (Fig. 3, lane b).

Characteristic syncytium induction was evident in MT-4 cells infected with cloned HIV-1 mA, mC, and mD isolates (Table 1). There were no differences in the number of syncytia formed between the mA, mC, and mD isolates or in the average number of nuclei per syncytium (data not shown). MT-4 and Mo-T are very sensitive to the cytopathic effects of HIV-1, probably as a consequence of the high level of CD4 expression by these cell types (24). Infection of MT-4 cells by these viral variants resulted in the generation of high levels of reverse transcriptase activity in the culture supernatant (see Fig. 4e), indicating virion production. This was confirmed by a profusion of budding, C-type retrovirus particles with characteristic HIV-1 morphology in electron micrographs of infected MT-4 cells (data not shown). Despite similarities in syncytium induction, the HIV-1 mC and

mD variants induced minimal cytocidal effects on infected CEM, Mo-T, and MT-4 cells, whereas the HIV-1 mA isolate was highly cytopathic for all cell types (Table 1).

Replication kinetics of the mA and mD variants in both MT-4 and CEM cells are shown in Fig. 4. Following infection of MT-4 cells, host cell viability (Fig. 4a), accumulation of viral RNA (Fig. 4d) and viral antigens (Fig. 4b), production of virus particles (Fig. 4e), and host CD4 receptor expression (Fig. 4c) were monitored over a 3-week period. The viability of MT-4 cells infected with the HIV-1 mA isolate rapidly declined (Fig. 4a) and was accompanied by an increase in the level of viral RNA, viral antigens, and reverse transcriptase activity in culture supernatant. In contrast, there was only a slight reduction in the viability of MT-4 cells infected with the HIV-1 mD isolate, despite a rapid accumulation of viral components. This slight viability reduction was due to the syncytium-inducing capabilities of the mD variant. By microscopic observation of trypan blue-stained cultures, the major difference between mA-, mC-, and mD-infected cells was the absence of single-cell killing by mC and mD isolates (Table 1). In HIV-1 mA-infected MT-4 cells, the majority of host cell destruction occurred in cells which were not incorporated into syncytia (Table 1). Thus, at the peak of virus-mediated cytopathic effects, single-cell killing accounted for the majority of cell destruction, whereas syncytia could only be observed in 5 to 10% of the cells in culture. By comparison, cultures infected with HIV-1 mC and mD isolates displayed only syncytium-dependent virus-induced cell death; single-cell killing was almost absent from these cultures (Table 1). Similar results were obtained following infection of Mo-T and CEM cells. After infection with HIV-1 mC (Table 1) and mD (Table 1; Fig. 4a) isolates, cell death was minimal, whereas infection of CEM cells with the HIV-1 mA isolate (Table 1; Fig. 4a) resulted in complete destruction of the culture.

Cytopathic HIV-1 infection in vitro is characterized by the abundance of unintegrated viral DNA which accumulates during superinfection of host cells by HIV-1 and other retroviruses (27, 38, 43, 52). Following infection of MT-4 cells by HIV-1 mA and mD isolates, unintegrated viral DNA was isolated at various times postinfection and analyzed by Southern blot hybridization to an HIV-1-specific DNA probe (Fig. 5). In both mA- and mD-infected cultures, the kinetics of replication, as determined by the increase in the number of antigen-positive cells with time, was similar. There was, however, a marked difference in the level of unintegrated viral DNA accumulation. In HIV-1 mD-infected cells, there were lower levels of extrachromosomal viral DNA, including linear viral DNA (form III) and circular DNA with one and two long terminal repeats (LTRs) (form I) when compared with HIV-1 mA-infected cells. Other viral DNA species were also visible and most probably represented open-circle (form II) and dimeric and trimeric forms of viral form I DNA. For both clones, the highest levels of unintegrated viral DNA could be observed at days 14 to 17 postinfection; however, there was approximately five times more unintegrated viral DNA in HIV-1 mA-infected cultures than in mD-infected cultures (Fig. 5), with the exception that at day 14, there was a greater abundance of (ca. 23-kb) unintegrated multimeric DNA in HIV-1 mD-infected cultures (Fig. 5). The level of unintegrated viral DNA was barely detectable at 21 and 36 days postinfection. In the mD-infected cultures, viability was still better than 95% during the entire experiment, whereas in mA-infected cultures, viability rapidly declined around 17 to 20 days postinfection. The accumulation of unintegrated viral DNA

FIG. 4. Replication kinetics of cytopathic and noncytopathic HIV-1 variants in infected MT-4 and CEM cultures. MT-4 cells ($\blacktriangle$, $\triangle$) and CEM cells ($\bullet$, $\circ$) were infected with HIV-1 mfA ($\triangle$, $\circ$) and mfD ($\blacktriangle$, $\bullet$) isolates at 200 TCID₅₀/10⁶ cells per ml. At designated time points, samples of infected culture were withdrawn for analysis of cell viability by trypan blue exclusion (a), antigen positivity by immunofluorescence analysis (b), accumulation of virus-specific RNA by solution RNA hybridization (d), and virus production by reverse transcriptase activity in culture supernatant (e). Surface CD4 expression was determined by flow cytometry with OKT4 (Becton Dickinson and Co., Parsippany, N.J.) monoclonal antibody (c). Viral RNA and antigen expression and virus production in HIV-1 mfA-infected cultures were not determined at later points postinfection owing to extensive cell death in these cultures.

is greatly influenced by the relative level of CD4 expression in the host cell culture (43). CD4 expression in HIV-1 mfD-infected cultures rapidly decreased (Fig. 4c), whereas a less pronounced and less rapid down modulation of CD4 expression was seen in HIV-1 mfA-infected cultures (Fig. 4c). Differences in accumulation of unintegrated viral DNA between HIV-1 mfA- and mfD-infected cultures (Fig. 5) were consistent with the pattern of CD4 expression in these cultures following infection (Fig. 4c).

Interference during coinfection with cytopathic and noncytopathic HIV-1 isolates. Successful isolation of rare noncytopathic variants of HIV-1 from a background of cytopathic variants within a primary HIV-1 isolate appeared to be dependent upon the ability of noncytopathic variants to persist during amplification in culture. The phenomenon of interference by which HIV-1 is able to restrict expression of the CD4 receptor (18, 43, 44), in addition to altering the outcome of infection, may also restrict coinfection of the cell by more than one viral variant. We therefore determined whether coinfection of CD4 cells by HIV-1 mfA and mfD isolates would consistently lead to the selective amplification of the noncytopathic variant and eventual loss of the cytopathic variant from the coinfecting culture. MT-4 cells were coinfecting with HIV-1 mfA and mfD isolates at 1:1 or 9:1

ratios (based on tissue culture infectious units [TCIU] in the virus inoculum). Unintegrated viral DNA was extracted at various times postinfection and digested with *PvuII* to discriminate between mfA and mfD genotypes. The presence of 0.6- and 2.2-kb fragments was indicative of the mfA isolate, whereas the presence of a 2.8-kb fragment was characteristic of the mfD isolate (Fig. 2). Addition of equal numbers of infectious units of mfA and mfD isolates led to a predominance of the mfD variant by 16 days postinfection (Fig. 6). The selective outgrowth of the HIV-1 mfD isolate in coinfecting cultures was even more dramatic when an excess of mfA isolate was used. At days 6 and 8 postinfection, the predominant isolate was the mfA genotype. However, rapid expansion of the mfD isolate at 11 days postinfection resulted in a predominance of the mfD isolate at 16 days postinfection and an almost complete absence of the mfA isolate (Fig. 6). High-molecular-weight DNA extracted from coinfecting cultures 5 weeks postinfection contained proviral HIV-1 DNA of the mfD isolate, whereas the mfA isolate was undetectable by the Southern blotting procedure used (results not shown). This selective outgrowth by the HIV-1 mfD isolates in coinfecting cultures appeared to be due to the selective destruction of host cells by the mfA isolate, whereas cells infected with the mfD isolate did not succumb

FIG. 5. Accumulation of unintegrated viral DNA in HIV-1-infected MT-4 cells. MT-4 cell cultures were inoculated with 200 TCID₅₀/10⁶ cells. At indicated times postinfection, aliquots of 2×10^6 cells were removed for analysis of antigen expression by immunofluorescence and for isolation of unintegrated viral DNA by a modified Hirt extraction procedure (5). Purified Hirt extracts (from 5×10^6 cells per lane) were resolved on agarose gels, transferred to nylon membranes, and hybridized to a genomic HIV-1-specific DNA probe.

to cytolysis. These results indicate that host cells infected by the mfd isolate could not be coinfecting by the mfa isolate, most probably owing to restricted CD4 expression of infected cells.

Cytopathic and noncytopathic HIV-1 variants differ in

envelope processing. We were interested in determining whether structural determinants in the HIV-1 genome could account for differences in biological properties of the HIV-1 isolates described here. Immunoprecipitation of ³⁵S-labeled whole-cell lysates revealed distinct differences in the relative

FIG. 6. Selective amplification of noncytopathic HIV-1 variant in MT-4 cell cultures. HIV-1 mfa and mfd cloned virus stocks were mixed at ratios of 1:1 and 9:1 (based on TCID₅₀) and added to MT-4 cultures at a total of 200 TCID₅₀/10⁶ cells. At 6, 8, 11, and 16 days postinfection, unintegrated viral DNA was isolated, digested with *Pvu*II, electrophoresed, transferred to nylon membranes, and hybridized with a 2.8-kb *Pvu*II *gag-pol* fragment of HIV-1 mfa. Molecular size markers are provided by ³²P-end-labeled *Hind*III digests of lambda DNA.

FIG. 7. Immunoprecipitation analysis of HIV-1 mfa- and mfd-infected CEM and MT-4 cell cultures. CEM cultures (a, c) and MT-4 cultures (b, d) were infected with HIV-1 mfa isolate (a, b), and HIV-1 mfd isolate (c, d) at 200 TCID₅₀/10⁶ cells. At 7 days postinfection and before the onset of cytopathic effects in mfa-infected cultures, cells were labeled with [³⁵S]methionine and [³⁵S]cysteine. After 6 h, cleared cell lysates were immunoprecipitated with sera from a normal HIV-1-seronegative individual (lanes 1) or two separate HIV-1-seropositive individuals (with titers of 5,160 and 2,580; lanes 2 and 3, respectively).

FIG. 8. Construction of hybrid viruses from HIV-1 mfA and mfD isolates. The hybrid viruses HIV-1 ch-A and ch-B were constructed by exchanging the 5' half of the viral genome containing the 5' LTR, *gag*, *pol*, *vif*, and part of the *vpr* genes. Construction of these hybrids was facilitated by the presence of an additional *Sall* site flanking the 5' *XbaI* site of the proviral DNA clones. The 3.1-kb *Sall*-*XhoI* HIV-1 mfA and mfD fragments containing the entire *vpu*, *tat*, *rev*, and envelope open reading frames were exchanged to form hybrid viruses HIV-1 ch-C and ch-D. Hybrid viruses ch-E and ch-F were constructed by exchanging 3' *XhoI*-*XbaI* fragments containing the HIV-1 *nef*, 3' LTR, and 3' host flanking sequences. Symbols: —, HIV-1 mfA sequences within the hybrid viruses; —, regions of the hybrids derived from mfD. Cytopathicity of the hybrid viruses was confirmed following transfection of recombinant proviral DNA into HeLa cells and amplification of hybrid viruses in MT-4, Mo-T, and CEM cultures.

abundance of precursor gp160 and processed gp120 envelope glycoproteins in the mfA and mfD isolates (Fig. 7). Both MT-4 and CEM cell cultures infected with HIV-1 mfA displayed a greater proportion of gp120 envelope glycoprotein than gp160, as identified by two high-titer sera (Fig. 7a and b). MT-4 and CEM cultures infected with the HIV-1 mfD isolate displayed a greater proportion of gp160 precursor envelope glycoprotein than gp120 glycoprotein (Fig. 7c and d). This indicated that the mfD isolate produced either a more stable precursor envelope glycoprotein or a processed gp120 glycoprotein with a shorter half-life.

A series of viral hybrids were constructed to determine whether altered envelope glycoprotein processing accounted for the biological property of the noncytopathic HIV-1 isolate or whether other, unrelated genetic determinants contributed to this phenotype. Viral hybrids were constructed in which where three different regions of the HIV-1 mfA and mfD genotypes were exchanged (Fig. 8). The 5' LTR *gag* and *pol* genes of these isolates were exchanged in hybrid viruses HIV-1 ch-A and ch-B. The HIV-1 *vpu*, *tat*, *rev*, and envelope coding regions of the mfA and mfD isolates were exchanged to form hybrid viruses ch-C and ch-D, whereas HIV-1 hybrids ch-E and ch-F were formed by interchanging the *nef* coding region and 3' LTR of these isolates. Cloned virus stocks of the hybrid virus genomes were prepared as previously described for parental mfA and mfD virus stocks, and the phenotypes of the hybrid viruses

in CEM, Mo-T, and MT-4 cell cultures were verified. Replication kinetics and cytopathic phenotypes of HIV-1 mfA and mfD isolates were unaffected in hybrids ch-A, ch-B, ch-E, and ch-F. When the *vpu*, *tat*, *rev*, and envelope coding regions of the HIV-1 mfA and mfD isolates were exchanged, as in hybrid viruses ch-C and ch-D, the phenotype of the hybrid virus was reversed (Fig. 8). Sequence differences located within the HIV-1 *vpu*, *tat*, *rev*, or *env* gene were therefore responsible for differences in biological properties of the HIV-1 mfA and mfD isolates.

The complete nucleotide sequence of the *vpu*, *tat*, *rev*, and envelope coding regions of the HIV-1 mfA and mfD isolates exchanged in hybrid viruses ch-C and ch-D was determined, and the amino acid sequence was deduced. There were no amino acid differences between the *vpu*, *tat*, and *rev* coding regions of cytopathic HIV-1 mfA and noncytopathic HIV-1 mfD (data not shown). Only two amino acid differences in the external envelope glycoprotein distinguished the HIV-1 mfA and HIV-1 mfD isolates. The complete amino acid sequence of the HIV-1 mfA and mfD envelope glycoproteins is shown in Fig. 9. For comparison, the envelope sequence of prototype isolate HXB2 (35), together with positions of potential N-linked glycosylation sites (for HXB2 sequence only), is also shown in Fig. 9. At amino acid position 195 (of mfA and mfD), an asparagine residue in the HIV-1 mfA isolate was replaced by an aspartic acid residue in the mfD isolate, whereas at position 276 (of mfA and mfD), a threo-

	signal peptide\gp120	
HIVmfa	MRVKEKYQHLRWGKNGIMLLGLIMICSATEMLMVTYYGVVWKEATTTLFC	54
HIVmfd	-----R--T---M---K-----	
HXB2	-----R--T---M---K-----	
HIVmfa	ASDAKAYDTEVHNVCATHACVPTDPNPQEVILVNVTENFNMWKNMVEQMHEDI	108
HIVmfd	-----W---V-----D-----	
HXB2	-----W---V-----D-----	
HIVmfa	ISLDQNLKPCVKLTPLCVNLKCTDLKNDTNTSSNGRMIMEKGEIKNCSFNIS	162
HIVmfd	-----S-----S-----S-----	
HXB2	-----S-----S-----S-----	
HIVmfa	TSIRNKVQKEYAFFYKLDIRPIN--TSYRLISCNTSVITQACPKVSFEPIPIH	214
HIVmfd	-----A-----D-----	
HXB2	---G-----I---DT---S-T---N-----	
HIVmfa	YCAGAPGAILKCNCKTGFNGTGPCNTVSTVQCTHGIRPVVSTQLLLNGSLAEEEG	268
HIVmfd	-----N-----V-----	
HXB2	-----N-----V-----	
HIVmfa	VIRSANFTDNAKTIIVQLNTSVEINCTRPNNNTRKSIRIQRCGPRAFVTIGKIG	322
HIVmfd	-----A-----R-----	
HXB2	---V---T-----R-----	
HIVmfa	NMRQAHNCISRAKRWSTLTKQIASKLREQFGNNKTIVFKQSSGGDPEIVTHSFNC	376
HIVmfd	-----NN-----I-----	
HXB2	-----NN-----I-----	
HIVmfa	GGEFFYCNSTQLFNSWFTSTWSTEGSNTEGSDTITLPCRIKQFINMMQEVGK	430
HIVmfd	-----I-----K-----	
HXB2	-----I-----K-----	
HIVmfa	AMYAPPISGGIRCSNNTIGLLTRDGGKNNNESEVFRPGGGMDRNRSELYKYK	486
HIVmfd	-----NSN-----I--L-----	
HXB2	-----NSN-----I--L-----	
HIVmfa	VVKIETLGVAPTKAKRRVVRQEKRAVGIGALFLGFLGAAGSTMGAASTMTLVQAR	541
HIVmfd	-----P-----	
HXB2	-----P-----	
HIVmfa	QLLSGIVQQQNLLRAIEAQHLLQLTVNGIKQLQARILAVERYLKDQQLLGIWG	597
HIVmfd	-----	
HXB2	-----	
HIVmfa	CSGKLICTTAVPWNASWSKSLQFNNMTWMEWDREINNYTSLIHSLIDESQNK	652
HIVmfd	-----I--HT-----E-----	
HXB2	-----I--HT-----E-----	
HIVmfa	QEKNEQELLELDKWSLWNNFNITNWLWYIKIFIMIVGGLVGLRIVFAVLSIVNR	707
HIVmfd	-----L-----	
HXB2	-----L-----	
HIVmfa	VRQGYSLPSTFQTHLPNRRGGPRPEGIEEGGERDRDRSVLVNGSLALIWDDLRS	762
HIVmfd	-----IPR-----I-----	
HXB2	-----IPR-----I-----	
HIVmfa	LCLFSYHRLRDLILLIVTRIVELLGRRGWEALKYWNLLQYWSQELKNSAVSLNA	816
HIVmfd	-----rev end -----*	
HXB2	-----rev end -----*	
HIVmfa	TAIAVAEGTDRLVLEVIQAGYRAIRHIPRRIRQGLERILL	854
HIVmfd	-----C-----	
HXB2	-----C-----	

FIG. 9. Complete amino acid sequences of cytopathic and noncytopathic HIV-1 variant envelope glycoproteins. The amino acid sequence was deduced from the nucleotide sequence and is shown by the one-letter amino acid code. Identical amino acid sequences are represented by dashed lines. The envelope amino acid sequence of the prototype HXB2 isolate (35) is given for comparison. The N-linked glycosylation tripeptide consensus sequences asparagine-X-serine (N-X-S) and asparagine-X-threonine (N-X-T) are indicated by an asterisk (for the HXB2 sequence only). Numbering of the amino acid sequences initiates from the methionine codon of the external gp120 envelope glycoprotein of HXB2.

nine residue in the mfa isolate was replaced by an alanine residue in the mfd isolate (Fig. 9). These amino acid differences resulted in the loss of two potential N-linked glycosylation sites from the HIV-1 mfd envelope coding sequence. The high degree of conservation of these potential N-linked glycosylation sites in a number of isolates characterized to date is shown in Fig. 10. In all isolates in which these regions were sequenced, the asparagine-X-serine and asparagine-X-threonine recognition sequences of the oligosaccharide transferase are absolutely conserved. The high

ISOLATE	194	< * >	203	271	< * >	283
HXB2	L T S C N T S V I T Q			V I R S V N F T D N A K T		
MFA	- I - - - - -			- - - - A - - - -		
MFD	- I - - - D - - -			- - - - A - - A - - -		
BRU	- - - - - - - - -			- - - - A - - - -		
MN	- I - - - - -			- - - - E - - - -		
SC	- I N - - - -			- L - E - - - -		
SF2	- I H - - R - - -			- - - D - - - N - - -		
NY5	- I N - - - -			- - - E - - - N - - -		
CDC4	- I N - - - -			- - - E - - - N - - -		
WMJ2	- I N - - - -			- - - E - - - - -		
RF	- I H - - S - - -			- - - E - - - - V - -		
MAL	- I N - - - -			M - - - E - L - - - T - N		
ELI	- I N - - - - A - -			I - - - E - L - N - - - N		
26	- I N - - - - A - -			I - - - E - L - N - - - I		
23	- I N - - - - T - -			I - - - E - I - N - - -		
2321	- I N - - - - A - -			R - - - E - - - - - I		
JY1	- I N - - - - A - -			I - - - E - L - N - V - -		
BR	- I - - - - -			- - - E - - - N - V - -		

FIG. 10. Two potential N-linked glycosylation sites absent from the noncytopathic HIV-1 variant are highly conserved among all HIV-1 isolates. Sequence comparison of currently known HIV-1 isolates was facilitated by information contained within the HIV-1 sequence data base, as compiled by Myers et al. (28). Sequences of the BR isolate were obtained from Anand et al. (1). Numbering is based on the prototype HXB2 sequence with which other amino acid sequences are aligned.

degree of conservation of these two oligosaccharide transferase consensus sequences is highlighted by the variability of flanking amino acids (Fig. 10) and indicates the importance of these sequences in envelope processing.

DISCUSSION

We describe the cloning and characterization of cytopathic and noncytopathic virus variants from an HIV-1-seropositive asymptomatic hemophiliac. Use of an HTLV-I-transformed MT-4 cell line, which is extremely sensitive to the cytopathic effects of HIV-1, was of central importance in the successful isolation of low-abundance noncytopathic HIV-1 variants. Isolation and amplification of surviving MT-4 cells following infection with the primary isolate resulted in the selective amplification of variants which were reduced in their ability to induce host cell cytolysis. This in vitro selection procedure was reproducible with cloned cytopathic and noncytopathic variant virus stocks in that coinfection of MT-4 cells eventually led to the outgrowth of MT-4 cells harboring predominantly virus genotypes that were noncytopathic. It is possible that in the system used here, interference restricted the coinfection of cells with more than one variant. Interference is exhibited by a number of retroviruses (8, 9, 43, 45-47, 50-53), and restriction on virus receptor expression by one variant prevents the coinfection with other variants by the same receptor-mediated route (9, 46, 47).

The phenotype of the noncytopathic HIV-1 variant described in this report appeared dependent upon qualitative, rather than quantitative, differences in viral replication. Phenotypic differences between the cytopathic and noncytopathic virus isolates could not be explained by differences in replication kinetics; CEM and MT-4 cultures infected with both cytopathic and noncytopathic variants displayed similar levels of viral RNA and antigen accumulation. In addition, there were no significant differences in the relative

production of virus particles. Importantly, both cytopathic and noncytopathic variants appeared equally capable of inducing cell fusion, as evidenced by equal levels of syncytium induction following infection of CEM, Mo-T, and MT-4 cells. The noncytopathic variant was, however, markedly attenuated in its ability to induce single-cell killing in all of these cell types. Mounting experimental evidence supports a syncytium-independent mode of virus-mediated cytolysis. Noncytopathic HIV-1 mutants have been constructed by deletion of the C terminus of the HIV-1 envelope glycoprotein (11). These noncytopathic mutants exert minimal cytotoxic effects upon infected host cells, yet are not reduced in their ability to replicate or to induce syncytium formation. Similarly, infection of CD4 cells with the simian immunodeficiency virus SIV_{mac} results in considerable syncytium induction without significant cytolysis (19). Cell destruction in the complete absence of syncytium formation has been demonstrated in wild type HIV-1 isolates (41, 43).

Accumulation of unintegrated viral DNA is indicative of superinfection (43, 52) and can be limited by envelope-mediated CD4 down regulation (43). The ability of the noncytopathic HIV-1 variant to restrict accumulation of unintegrated viral DNA (Fig. 5) may suggest a more efficient virus envelope-mediated CD4 down regulation following virus infection. This was supported by the rapid reduction in CD4 expression observed following infection of cells with the noncytopathic variant (Fig. 4). Immunoprecipitation of HIV-1 proteins revealed distinct differences in the processing of cytopathic and noncytopathic envelope glycoproteins. The predominance of precursor gp160 envelope glycoprotein in cells infected with the noncytopathic variant indicated a more stable precursor envelope glycoprotein or suggested that the processed gp120 envelope glycoprotein had a very short half-life. During HIV-1 envelope-mediated CD4 modulation, the majority of CD4 is complexed to gp160 (54). Thus, a predominance of gp160 within cells infected with the noncytopathic variant may facilitate a more efficient modulation of CD4 receptor expression.

Analysis of the phenotype of viral hybrids confirmed that determinants for syncytium-independent single-cell killing by HIV-1 mapped to a region encoding the *vpu*, *tat*, *rev*, and *env* proteins. No sequence differences between cytopathic and noncytopathic variants were observed in *vpu*, *tat*, or *rev*; therefore, only differences in the envelope glycoprotein could account for changes in single-cell killing observed in the virus variants described here. Two single-amino-acid differences in the envelope glycoprotein of the noncytopathic variant resulted in the loss of two highly conserved N-linked glycosylation sites. These sites are conserved within all current isolates of North American and African origin, indicating their importance in HIV-1 envelope function. The loss of N-linked glycosylation sites within the envelope coding region of the noncytopathic HIV-1 variant may lead to alterations in posttranslational processing of the envelope glycoprotein and subsequently influence the cytopathic potential of that genotype. Such an observation is not without precedent. Differences in posttranslational processing of the envelope glycoprotein account for altered cytopathic potential in diverse clones of feline leukemia virus (30, 34). Posttranslational modification of the HIV-1 envelope glycoprotein is therefore important both in virus-receptor interaction (26) and in the cytopathic potential of the virus (34). It is possible that posttranslational differences within genotypes of HIV-1 may alter the ability of the envelope glycoprotein to interact with CD4 and affect its ability to modulate CD4 receptor expression and thus induce

interference. Alternate explanations for the influence of posttranslational modifications on virus-mediated cytopathicity are plausible. The amphipathic nature of the HIV-1 envelope glycoprotein may impart upon it a direct cytotoxic property. Fusogenic domains within envelope glycoprotein mediate virus-cell membrane fusion following CD4-HIV-1 envelope glycoprotein binding (20). Similarly, the envelope glycoprotein may be directly able to interfere with host cell membrane function and stability (22). It is possible that a high concentration of envelope glycoprotein upon the surface of an infected cell profoundly alters membrane function by, for example, reducing the selective permeability of the membrane, thus inducing osmotic instability in a manner similar to that displayed by paramyxoviruses (7, 12, 29, 37). During establishment of superinfection resistance by the envelope glycoprotein of reticuloendotheliosis virus, envelope-cellular receptor interactions restrict translocation of the envelope glycoprotein to the cell surface (8). Consequently, differences in posttranslational envelope processing may affect its interaction with the cellular receptor and eventual localization of viral envelope glycoprotein on the cell surface.

The results presented here have a bearing on how minor changes within the envelope glycoprotein of HIV-1 can dramatically alter the biological properties of the virus. We demonstrate here that subtle differences in the coding sequence of the envelope glycoprotein lead to alterations in posttranslational modification which are sufficient to influence the cytopathic properties of the virus. Although other mechanisms may contribute to CD4 cell depletion in vivo (32, 39, 49), it is clear that the determinants for direct virus-mediated cytotoxicity (i.e., syncytium induction and single-cell killing) map to the envelope glycoprotein. It is possible that sequence variability in the envelope leads to a spectrum of viral phenotypes, although the role of noncytopathic variants in disease pathogenesis remains to be determined.

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