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Characterization of the Functional Properties of *env* Genes from Long-Term Survivors of Human Immunodeficiency Virus Type 1 Infection

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A small number of persons infected with human immunodeficiency virus type 1 (HIV-1) remain clinically and immunologically healthy for more than a decade after infection. Recent reports suggest that these individuals may be infected with an attenuated strain of HIV-1; however, a common genetic basis for viral attenuation has not been found in all cases. In the present study, we examined the functional properties of the HIV-1 *env* genes from six long-term survivors. *env* clones were generated by PCR amplification of proviral *env* sequences, followed by cloning of the amplified regions into expression vectors. Eight to ten clones from each subject were screened by transient transfection for expression of the envelope precursor glycoprotein, gp160. Those clones expressing gp160 were then cotransfected with an HIV-1 luciferase reporter vector, pNL4-3Env(−)LUC(+) and evaluated for their ability to mediate infection of phytohemagglutinin-activated peripheral blood mononuclear cells in single-cycle infectivity assays. Clones expressing gp160 were identified for all six long-term survivors, indicating the presence of proviral *env* genes with intact open reading frames. For two subjects, D and DH, the encoded envelope glycoproteins yielded high levels of luciferase activity when pseudotyped onto HIV-1 virions and tested in single-cycle infectivity assays. In contrast, envelope glycoproteins cloned from four other long-term survivors were poorly processed and failed to mediate infection. Sequencing of the gp120/41 cleavage site and conserved gp41 cysteine residues of these clones did not reveal any obvious mutations to explain the functional defects. The functional activity of *env* clones from long-term survivors D and DH was comparable to that seen with several primary HIV-1 *env* genes cloned from individuals with disease progression and AIDS. These results suggest that the long-term survival of subjects D and DH is not associated with overt functional defects in *env*; however, functional abnormalities in *env* may contribute to maintaining a long-term asymptomatic state in the other four cases we studied.

In most individuals, infection with human immunodeficiency virus type 1 (HIV-1) is marked by a gradual and sustained loss of $CD4^+$ T lymphocytes, increasing levels of circulating virus, and subsequent clinical progression to AIDS. While the underlying pathogenic processes vary considerably from person to person, in general, progression from primary infection to AIDS occurs over 8 to 10 years. However, a small number of infected persons remain clinically healthy, with no evidence of immunological deterioration, for more than a decade after infection. These individuals, termed long-term survivors, are the focus of considerable research efforts aimed at elucidating the viral and host factors which may contribute to the attenuation of disease progression (1, 8, 12, 13, 20, 28, 35).

In previous studies, we have characterized a cohort of 10 long-term survivors (1, 18). These individuals were found to have low-to-undetectable levels of HIV-1 in both plasma and peripheral blood mononuclear cells (PBMC), indicating that they are able to control viral replication extremely well in vivo. This may be explained in part by the presence of a particularly vigorous anti-HIV-1 immune response, including both broadly reactive neutralizing antibodies and potent $CD8^+$ T-cell suppressor activity (1). The strength and breadth of the immune response suggest that they may be continually exposed to viral antigens over the course of infection. However, attempts to isolate HIV-1 from the peripheral blood cells and plasma were unsuccessful in 6 of 10 cases (1), raising the possibility that virus either may be sequestered in certain tissues or may be attenuated because of mutations within the viral genome.

The HIV-1 genome from long-term survivors is currently being studied by several groups to determine a possible genetic basis for viral attenuation (8, 18, 20, 28). Perhaps the strongest evidence stems from recent reports of a cluster of long-term survivors infected with HIV-1 10 to 14 years ago by transfusion of blood from a common infected donor (8, 23, 24). Examination of the viral genome from three of these individuals and the donor revealed the presence of deletions in the *nef* gene and the overlapping U3 region of the long terminal repeat (8). These findings are consistent with the lack of pathogenesis seen in macaques infected with a *nef*-deleted variant of SIVmac239 (19). However, in our own cohort of 10 long-term survivors, *nef* was found to be both genetically and functionally intact (17, 18), suggesting that mutations in other regions of the genome may be involved in viral attenuation. Inactivating mutations have been described in a region encompassing the accessory genes *vif*, *vpr*, *vpu*, *tat1*, and *rev1* from another HIV-1 long-term survivor (28). A detailed analysis of these genes among the individuals in our cohort revealed that the majority have intact open reading frames and few obviously inactivating mutations (39). Taken together, these results suggest that, while some degree of viral attenuation may be present in HIV-1-infected long-term survivors, it is unlikely that a single common genetic determinant will account for the lack of disease progression in all cases.

In the present study, we have focused on defining the functional properties of the HIV-1 *env* genes from six of the long-term survivors in our cohort. The *env* gene of HIV-1 encodes both the surface (gp120) and transmembrane (gp41) glycoproteins which are incorporated into the envelope of budding

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virions. Interaction of HIV-1 gp120 with the CD4 receptor molecule is critical for initiation of the early stages of the virus replication cycle and defines the tropism of HIV-1 for CD4-expressing T lymphocytes and monocytes/macrophages (reviewed in reference 21). In vitro, epitopes on both gp120 and gp41 have been identified as targets for neutralizing antibodies (14–16, 29) and cytotoxic T lymphocytes (27, 33), suggesting that the envelope glycoproteins are important targets for the human immune system.

To define the functional characteristics of HIV-1 envelope glycoproteins from long-term survivors, we screened a panel of 56 *env* clones from six individuals for expression and processing of the precursor glycoprotein, gp160. Clones that expressed gp160 were further examined for their ability to mediate infection of phytohemagglutinin (PHA)-stimulated PBMC by single-cycle complementation assays. The results of these experiments were compared with those from experiments using *env* clones from individuals with disease progression and AIDS.

MATERIALS AND METHODS

Study subjects. PBMC samples from 6 of the 10 long-term survivors in our cohort were available for analysis of the HIV-1 *env* gene. These six individuals have had documented HIV-1 infection for 12 to 15 years and had $CD4^+$ T-cell counts in the normal range at the time of sample collection. The clinical and immunologic characteristics of each have been described in detail elsewhere (1). In addition to the long-term survivors, longitudinal PBMC samples from subject B, who progressed rapidly to AIDS after seroconversion (6, 7), were analyzed. The initial sample was taken when his CD4 cell count was still within the normal range ($677/mm^3$) and the viral load in his PBMC was comparable to that seen in our cohort of long-term survivors (328 HIV-1 DNA copies per 10^6 PBMC) (1, 18). Additional functional analyses were also performed with *env* genes cloned from several primary HIV-1 isolates obtained from individuals with AIDS, including HIV-1_{BA-L} (11), HIV-1_{SF-2} (25), and HIV-1_{JR-FL} (22).

Samples. To clone the HIV-1 *env* genes from the long-term survivors, PBMC were first isolated by centrifugation of blood through Ficoll-Hypaque density gradients. DNA was then extracted by standard methods (34) and used for PCR amplification and cloning of the *env* gene. Because of the limited availability of PBMC from subject D, DNA used for *env* cloning experiments was extracted from cultured PBMC generated by infection of normal donor PBMC with an HIV-1 isolate from subject D.

PCR amplification of viral *env* sequences and cloning. Sequences containing the HIV-1 *env* gene were amplified by nested PCR using primer pairs which recognize conserved sequences in flanking regions of the genome. The outer primers were as follows: ED3, 5′-TTAGGCATCTCCTATGGCAGGAAGAA GCGGA, and R5, 5′-GGTCTGAGGGATCTCTAGTTACCAGAGTC (9). The inner primers were as follows: RC-12, 5′-TATGGCAGGAAGAATTCGAGA CAGCGA (nucleotides 5515 to 5542), and RC-9, 5′-ATGTTTTTCTAGGTCT CGAGATACTGCTCC (nucleotides 8462 to 8433). Restriction enzyme sites for *Eco*RI and *Xho*I were incorporated into the inner primer pair (underlined) to facilitate cloning into the polylinker region of the simian virus 40-based expression vector, pSV7d.

For each sample, 1 to 2 μg of template DNA was added to a PCR mixture (50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, 250 μM deoxynucleoside triphosphate, 20 pmol of each outer primer, and 5 U of *Taq* DNA polymerase) in a final volume of 100 μl. Amplification was carried out for 30 cycles (92°C for 1 min, 55°C for 1 min, 70°C for 6 min). An aliquot of 1.5 μl from the first-round PCR was then transferred to a new reaction mixture containing the inner primer pair, and the second round of amplification was performed for an additional 30 cycles (92°C for 2 min, 55°C for 30 s, 72°C for 5 min).

The resulting PCR products were purified with the Magic PCR Preps DNA purification system (Promega), digested with *Eco*RI and *Xho*I, and ligated into the *Eco*RI and *Sal*I sites of pSV7d. Individual *env* clones were generated by selection and expansion of ampicillin-resistant colonies, following transformation of competent HB101 cells by electroporation. Plasmids were screened for the correct insert by restriction enzyme digestion, and 8 to 10 clones from each study subject were selected for further analysis. Control plasmids containing the *env* genes from the molecular clones HIV-1_{HXB2}, HIV-1_{BA-L}, HIV-1_{SF-2}, and HIV-1_{JR-FL} were generated by single-round PCR amplification and cloning using primers RC-9 and RC-12.

Transfection and Western blot (immunoblot) detection of envelope glycoproteins. To evaluate expression of HIV-1 envelope glycoproteins, 15 μg of *env* plasmid DNA and 5 μg of a plasmid encoding the HIV-1 Rev protein (kindly provided by Nathaniel Landau) were cotransfected by calcium phosphate methods (3) into 293 cells. The 293 cells were prepared by plating 3×10^6 cells per 10-cm-diameter dish in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Twenty-four hours after transfection, the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in 500 μl of Western lysis buffer (10 mM Tris [pH 7.5], 0.15 M NaCl, 2 mM EDTA, 0.5% Nonidet P-40) containing 50 μg of phenylmethylsulfonyl fluoride per ml. Lysates were incubated on ice for 5 min and clarified by centrifugation at $16,000 \times g$ at 4°C for 5 min.

For Western blot analysis, 15 μl of each lysate was mixed with an equal volume of 2× protein sample buffer (0.1 M Tris [pH 6.8], 1% bromophenol blue, 2% glycerol, 4% sodium dodecyl sulfate [SDS]) with 1% β-mercaptoethanol and heated at 95°C for 5 min. The samples were then separated on an SDS–10% polyacrylamide gel, and the proteins were transferred electrophoretically to Immobilon filters (Millipore). The filters were blocked for 1 h with 1% gelatin in TBST buffer (10 mM Tris [pH 8.0], 150 mM NaCl, 0.5% Tween 20), washed once with TBST, and incubated for 1 h at room temperature with a cocktail of three HIV-1 gp120-specific murine monoclonal antibodies. These monoclonal antibodies (B12, B32, and 660-178) recognize nonoverlapping, conserved epitopes in the gp120 molecule (30). The filters were washed three times with TBST and incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G. Following extensive washing with TBST, the filters were developed by the enhanced chemiluminescence method, following the instructions of the manufacturer (Amersham Lifesciences).

Single-cycle *env*-complementation assays. The functional properties of HIV-1 envelope glycoproteins were assessed by single-cycle complementation assays using the vector pNL4-3 Env(−)LUC(+) (2, 5). This construct expresses the HIV-1_{NL4-3} provirus but contains a frameshift at the 5′ end of *env* (nucleotide 5950) that prevents expression of the envelope glycoproteins. The vector also contains the gene for firefly luciferase inserted into the *nef* gene of pNL4-3. Plasmid DNA encoding Env (15 μg) was cotransfected with pNL4-3 Env(−) LUC(+) (5 μg) into 293 cells. Forty-eight hours after transfection, the culture supernatants were collected, clarified by centrifugation at $1,500 \times g$ for 15 min, and filtered (0.45-μm-pore-size filters). A small portion of each supernatant was used for p24 antigen measurements (Abbott Laboratories), and the remaining stocks were stored in aliquots at −80°C.

To measure the infectivity of Env-pseudotyped HIV-1 particles, PHA-stimulated normal donor PBMC (3×10^6) were inoculated with a standardized amount of virus as defined by the p24 antigen content of the transfected stocks. The cultures were incubated for 3 to 5 days at 37°C, after which the cells were washed with ice-cold PBS and lysed in 100 μl of lysis buffer (Promega). The amount of luciferase activity in 10 μl of each lysate was determined with commercially available reagents (Promega) in a Packard Top Count luminometer.

Sequencing. Regions spanning the gp120/41 cleavage domain (10) and the gp41 cysteine residues (37) were sequenced by the Sequenase 2.0 protocol (United States Biochemical Corp.). Predicted amino acid sequences were derived with the MacVector 3.5 sequence analysis program.

RESULTS

Expression of *env* genes from long-term survivors. To examine the functional properties of the HIV-1 *env* genes from long-term survivors, we generated a panel of clones by nested PCR amplification of proviral *env* sequences from each of the study subjects. Visual inspection of the products after the second round of PCR revealed no gross insertions or deletions in the amplified regions, relative to the same region amplified from the molecular clone HIV-1_{HXB2} (data not shown). For more detailed analysis, the second-round PCR products were subcloned into expression vectors and analyzed by transient transfection in 293 cells. Following transfection, expression of gp160 was measured in cell lysates by Western blot with a cocktail of gp120-specific monoclonal antibodies for detection (30). Clones expressing gp160 were identified for all six long-term survivors and longitudinally for the rapid progressor, subject B (B-1, B-2, and B-3 [Table 1]). A representative blot is shown in Fig. 1 for subject LM. In this case, 5 of 10 *env* clones expressed gp160, while only one of the five had detectable gp120. We were able to detect gp160 processing in a fraction of the clones from LM (1 of 5), SF (1 of 3), BO (1 of 4), D (3 of 7), and DH (4 of 7) but were unable to detect gp120 among any of the clones from LSS (0 of 4) (Table 1). To confirm that the conditions of our assay were adequate to detect gp160 processing, control blots were done with *env* clones derived from HIV-1_{HXB2}. In multiple experiments, we were able to easily detect gp160 expression and processing under the same conditions used to test the clones from the long-term survivors (data not shown and Fig. 4). We also identified *env* clones from

TABLE 1. Summary of HIV-1 gp160 expression and processing for a panel of *env* clones

Sample	Env clones (*n*)	No. of clones expressing:	
		gp160	gp160/120
Long-term survivors			
LM	10	5	1
BO	10	4	1
SF	10	3	1
LSS	10	4	0
DH	8	7	4
D	8	7	3
Rapid progressor			
B-1	15	14	9
B-2	19	14	8
B-3	10	8	6

subject B in which gp160 was processed efficiently, including clones from all three sequential time points (Table 1).

Sequence analysis of gp120/41 cleavage domain and gp41 Cys residues. To determine whether the lack of gp160 processing seen with several of the clones from the long-term survivors was due to specific mutations in *env*, we sequenced two regions that have previously been shown to influence the efficiency of gp160 cleavage. The first domain spans the proteolytic cleavage site between gp120 and gp41 (10), while the second region contains two conserved cysteine residues found in gp41 (37). For each of the study subjects, we selected a pair of *env* clones, one in which gp160 was processed to gp120 and the other in which only gp160 was detected (with the exception of LSS, from whom all the *env* clones failed to process gp160; for this subject, we sequenced a pair of gp160-expressing clones). The deduced amino acid sequences for each of the clones are shown in Fig. 2. We found that both the gp120/41 cleavage site (Fig. 2A) and the downstream gp41 cysteine residues (Fig. 2B) were highly conserved among all the *env* clones tested. With the exception of an E-to-Q change in the sequence of clone SF-10, the amino acid sequences of clones that failed to process gp160 were homologous in these regions to those of clones that processed gp160, suggesting that amino acid changes outside these sites must influence the efficiency of proteolytic cleavage.

Evaluation of Env-mediated single-cycle infection. To evaluate the ability of envelope glycoproteins from long-term survivors to mediate infection of activated PBMC, HIV-1 virions were produced by cotransfection of *env* clones with a reporter construct, pNL4-3Env(−)LUC(+) (2, 5). Supernatants containing the pseudotyped particles were standardized for HIV-1 p24 antigen content and used to infect PHA-stimulated, normal donor PBMC. The amount of luciferase activity measured in PBMC lysates 3 to 5 days after inoculation was used as an indirect assessment of viral entry, integration, and transcriptional activity. To minimize the effects of donor cell variability, all of the clones were tested within a single assay using the same donor PBMC. Similar results were obtained with PBMC from multiple different donors.

The results of a representative experiment are shown in Fig. 3. HIV-1 particles generated by transfection of the reporter vector alone failed to produce detectable luciferase activity in cell lysates. However, this defect could be complemented by cotransfection of the *env* gene from HIV-1_{HXB2}, indicating that expression of *env* was necessary and sufficient to render

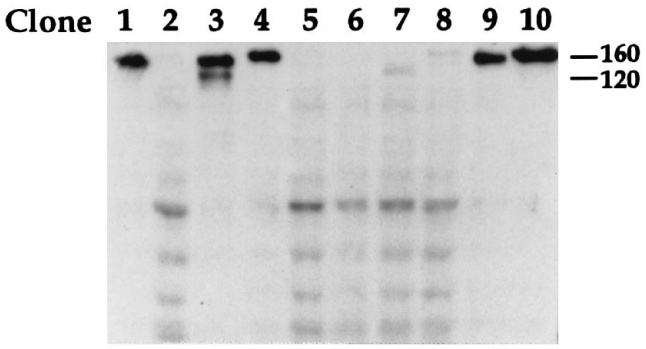

FIG. 1. Expression of HIV-1 envelope glycoproteins from a panel of *env* clones derived from one long-term survivor. *env* clones from subject LM were evaluated in transient-transfection assays for expression of the HIV-1 envelope glycoproteins, gp160 and gp120. Expression of gp160/120 was measured in cell lysates by Western blot using a cocktail of gp120-specific monoclonal antibodies for detection (30). Lanes 1 through 10 represent individual *env* clones.

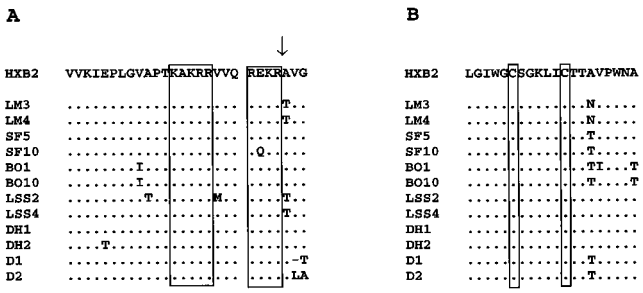

FIG. 2. Predicted amino acid sequences for the gp120/41 cleavage site and gp41 cysteine residues of *env* clones from long-term survivors. Nucleotide sequences were determined for the gp120/41 cleavage site and two conserved gp41 cysteine residues for pairs of *env* clones from each of the long-term survivors. The deduced amino acid sequences are aligned with the same regions of the molecular clone, HIV-1_{HXB2}. The amino acids are designated by the single-letter code; dots indicate identical protein sequences; the arrow indicates the gp120/41 cleavage site. The boxed regions represent the gp120/41 cleavage domains (10) and gp41 cysteine residues (34).

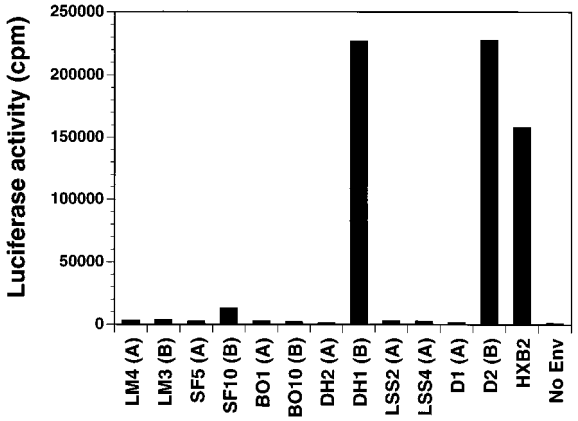

FIG. 3. Functional activity of HIV-1 virions pseudotyped with envelope glycoproteins from long-term survivors. HIV-1 virions pseudotyped with envelope glycoproteins from the long-term survivors were produced by cotransfection of selected *env* clones with a luciferase-expressing reporter vector, pNL4-3Env(−)LUC(+). PHA-activated PBMC were then inoculated with 50 ng of HIV-1 p24 antigen from the transfected stocks, and the level of luciferase activity was measured in cell lysates 3 to 5 days later. Virions pseudotyped with Env from HIV-1_{HXB2} were used as a positive control, while virions produced by transfection of the reporter vector alone were used as a negative control. Luciferase activity is expressed as counts per minute. (A) Clones expressing gp160; (B) clones expressing gp160/120.

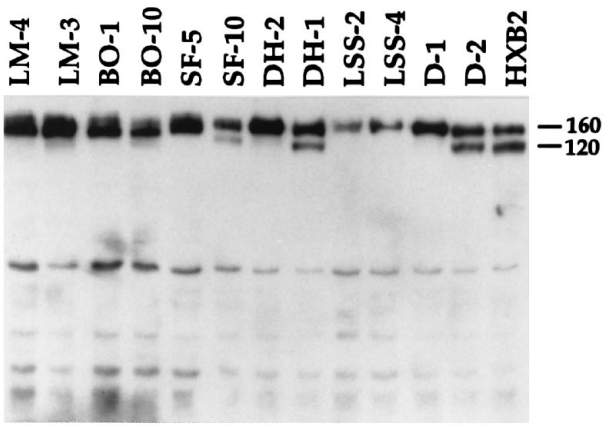

FIG. 4. Expression of HIV-1 envelope glycoproteins in cell lysates following cotransfection of *env* clones with pNL43Env(−)LUC(+). Lysates from 293 cells cotransfected with *env* and the reporter vector, pNL4-3Env(−)LUC(+), were evaluated by Western blot for expression of gp160/120. As a positive control for expression and processing of gp160, a plasmid encoding the *env* gene of HIV-1_{HXB2} was cotransfected with pNL43Env(−)LUC(+) and analyzed under the same conditions. Lanes 1 and 2, *env* clones from subject LM; lanes 3 and 4, clones from subject BO; lanes 5 and 6, clones from subject SF; lanes 7 and 8, clones from subject DH; lanes 9 and 10, clones from subject LSS; lanes 11 and 12, clones from subject D; lane 13, HIV-1_{HXB2}.

the particles infectious. In an analogous manner, we tested pairs of *env* clones, expressing either gp160 (A) or gp160/gp120 (B), from each of the long-term survivors (Fig. 3). No luciferase activity was detected in cell lysates following inoculation of particles pseudotyped with gp160. This result was not unexpected and confirms earlier observations indicating that processing of gp160 is essential for virion infectivity (26).

In contrast, variable levels of luciferase activity were measured in PBMC lysates following inoculation of gp120-pseudotyped particles. While high levels were consistently detected with *env* clones from subjects D (clone D2) and DH (clone DH1), low-to-undetectable levels were found with clones from subjects LM, BO, SF, and LSS. To confirm that *env* was in fact being expressed from these clones, Western blots were performed on lysates from the transfected 293 cells used to generate the pseudotyped particles. We were able to detect gp160 expression for all the *env* clones; however, very low levels of gp120 were seen with clones from subjects LM, BO, and SF, compared with those from DH and D, and no gp120 was detected with clones from LSS (Fig. 4). In repeat experiments, the level of gp160 processing was consistently lower for the *env* clones from subjects LM, BO, and SF and particles pseudotyped with envelope glycoproteins from these subjects yielded undetectable levels of luciferase activity in multiple assays.

In further experiments, we compared the functional activity of the envelope glycoproteins from the long-term survivors with activity of those from patients with disease progression and AIDS (Fig. 5). As in previous assays, luciferase activity was detected with Env-pseudotyped particles from two of the long-term survivors, D and DH, while no activity was detected with particles pseudotyped with envelope glycoproteins from the other long-term survivors, LM, BO, and SF. By comparison, high levels of luciferase activity were detected with several primary HIV-1 *env* clones derived from individuals with AIDS. The levels of luciferase activity measured with these clones were comparable to those from subjects D and DH, suggesting that the long-term survival of D and DH is not the result of functionally defective envelope glycoproteins. Evaluation of *env* clones derived longitudinally from subject B, who progressed rapidly to AIDS, showed a progressive increase in the ability of encoded envelope glycoproteins to mediate infection of activated PBMC (B-1, B-2, and B-3 [Fig. 5]). This suggests that mutations in *env* may increase the efficiency of HIV-1 replication, presumably during the early stages of the virus life cycle. This is consistent with our previous assessment of subject B, whose sequential HIV-1 isolates demonstrated an increase in replication kinetics (6).

DISCUSSION

Recent studies have demonstrated that the viral loads in long-term survivors are considerably lower than those found in patients with disease progression (1, 31). This may be due in part to the presence of a particularly vigorous anti-HIV-1 immune response, including broadly reactive neutralizing antibodies and potent antiviral $CD8^+$ T-cell activity (1, 13). HIV-1 isolated from these individuals often replicates poorly in culture, suggesting that long-term survivors may be infected with a weakened or attenuated strain of the virus (1, 12, 28). Although a common genetic basis for HIV-1 attenuation has not been found, defects have been identified in the *nef* gene (8, 20), in the long terminal repeat region (8), and in regions encoding several of the accessory genes (28, 39). This raises the possibility that specific genetic mutations may contribute to maintaining a long-term asymptomatic state in some individuals.

In the present study, we chose to investigate the functional properties of the HIV-1 *env* gene from six of the individuals in our cohort. Expression of the envelope glycoproteins is essential for HIV-1 infectivity and predicates many of the biological and antigenic properties of the virus. Among a panel of 56 *env* clones generated from six long-term survivors, we found that the majority of clones (46 of 56 [82%]) were defective in either synthesis or processing of the precursor glycoprotein, gp160. The high percentage of defective *env* clones from the long-term survivors is perhaps not surprising given the duration of

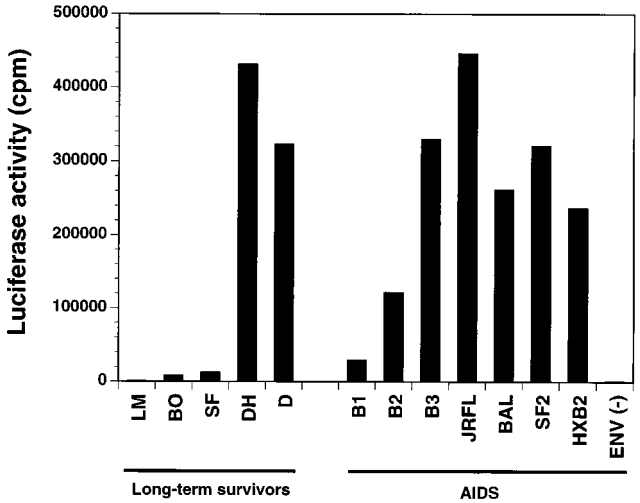

FIG. 5. Comparison of the functional activity of HIV-1 virions pseudotyped with primary HIV-1 Env from individuals with long-term survival of AIDS. HIV-1 virions pseudotyped with envelope glycoproteins from five of the long-term survivors (LM, BO, SF, DH, and D) were compared in single-cycle infectivity assays with virions pseudotyped with Env from individuals with disease progression and AIDS. *env* clones from subject B were derived from PBMC samples obtained at three sequential time points after seroconversion: 31 months (B1), 50 months (B2), and 56 months (B3). Pseudotyped virions were produced as described in the legend for Fig. 4. Luciferase activity is expressed as counts per minute.

infection in these individuals and the probability that a high proportion of infected cells carry defective proviral genomes (4). Nevertheless, clones expressing gp160 were identified for each of the six study subjects, indicating the presence of proviral *env* genes with intact open reading frames.

In many cases, gp160 expressed from these clones was poorly processed. As with other enveloped retroviruses, proteolytic cleavage of the integral membrane proteins is a critical step in the maturation of infectious particles (26, 38). Mutations in the endoproteolytic cleavage domain of *env* result in the release of particles that are morphologically indistinguishable from the wild type but are no longer infectious (38). To address the possibility that some of the *env* clones from long-term survivors may have mutations in the cleavage domain, we sequenced a region spanning the gp120/41 cleavage site by using pairs of clones that differed in their level of gp160 processing. We found this region to be highly conserved among all the clones sequenced. We also found no obvious mutations in a region of gp41 containing two conserved cysteine residues which have previously been shown to influence gp160 processing (37). Although this does not rule out the possibility that mutations elsewhere in *env* may be affecting protein folding and tertiary structure, it does indicate that changes in the regions we sequenced are not directly responsible for the apparent defects in processing that we observed.

We identified envelope glycoproteins from two long-term survivors, D and DH, that consistently generated high levels of luciferase activity in single-cycle infectivity assays. In previous studies, we have shown that these two individuals have a slightly higher viral load than others in the cohort and that cocultures of their PBMC yielded replication-competent virus (1). Our current findings demonstrate that proviral *env* genes cloned from these two subjects encode envelope glycoproteins that are fully capable of mediating the early stages of the viral replication cycle. In fact, the functional activities of *env* clones from D and DH were comparable to those observed with *env* clones derived from individuals with AIDS. It is worth noting, however, that subject D recently developed AIDS after more than 10 years of clinical stability (36), placing him within the normal distribution curve for disease progression. We have also recently observed an increase in the level of plasma viremia for subject DH, suggesting that he too may be progressing, although he is still clinically asymptomatic. While both D and DH fit the criteria for long-term survivors, they can be distinguished from the others in our cohort by the presence of replication-competent virus with fully functional viral envelope glycoproteins and may therefore represent a distinct subset of long-term survivors who progress to AIDS after a prolonged period of clinical stability.

By comparison, envelope glycoproteins cloned from the remaining four long-term survivors (LSS, LM, BO, and SF) were functionally defective. It is possible that we failed to amplify functional *env* genes from these subjects because of the inherently low proviral copy number in their PBMC. However, a number of functional *env* genes were cloned by identical methods from subjects with comparable viral loads in their PBMC (D and DH). Clones expressing gp160 were identified for subjects LSS, LM, BO, and SF, indicating that their *env* genes were not grossly defective in expression of the envelope precursor glycoproteins. However, gp160 expressed from the majority of these clones was inefficiently processed and virions pseudotyped with the envelope glycoproteins failed to mediate infection of activated PBMC. These findings are compatible with our previous results in which we were unable to isolate any infectious virus from LSS, BO, and SF (1). However, we cannot rule out the possibility that mutations elsewhere in the viral genome may account for our inability to recover HIV-1 from these individuals.

The long-term survivors in this cohort have potent and sustained gp120-binding activity, HIV-1-neutralizing responses, and Env-specific cytotoxic T-lymphocyte activity (32), indicating that their immune systems have been continually exposed to viral envelope proteins in vivo. In the present study, we cloned proviral *env* genes that expressed detectable amounts of envelope precursor proteins from all six long-term survivors, although for four subjects the envelope glycoproteins were functionally defective. While sustained infection is clearly not possible in the absence of functional viral envelope glycoproteins, inefficient processing of gp160 may yield sufficient gp120 to permit cell-cell or virus-cell fusion at a low level and stimulate the development of Env-specific antibody responses. In addition, the endogenous expression of defective viral proteins, while compromising particle infectivity, may still allow recognition of infected cells by cytotoxic T lymphocytes. Thus, mutations which reduce HIV-1 infectivity without abrogating recognition of viral proteins by the immune system could contribute to maintaining a long-term asymptomatic state in certain individuals.

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REFERENCES

1. **Cao, Y., L. Qin, L. Q. Zhang, J. T. Safrit, and D. D. Ho.** 1994. Virological and immunological characterization of long-term survivors of HIV-1 infection. N. Engl. J. Med. **332:**201–208.
2. **Chen, B., K. Saksela, R. Andino, and D. Baltimore.** 1994. Distinct modes of human immunodeficiency virus type 1 proviral latency revealed by superinfection of nonproductively infected cell lines with recombinant luciferase-encoding viruses. J. Virol. **68:**654–660.
3. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
4. **Coffin, J. M.** 1995. HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. Science **267:**483–489.
5. **Connor, R. I., B. K. Chen, S. Choe, and N. R. Landau.** 1995. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. Virology **206:**935–944.
6. **Connor, R. I., and D. D. Ho.** 1994. Human immunodeficiency virus type 1 variants with increased replicative capacity develop during the asymptomatic stage before disease progression. J. Virol. **68:**4400–4408.
7. **Connor, R. I., H. Mohri, Y. Cao, and D. D. Ho.** 1993. Increased viral burden and cytopathicity correlate temporally with CD4+ T-lymphocyte decline and clinical progression in HIV-1-infected individuals. J. Virol. **67:**1772–1778.
8. **Deacon, N. J., A. Tsykin, A. Solomon, K. Smith, M. Ludford-Menting, D. J. Hooker, D. A. McPhee, A. L. Greenway, A. Ellett, C. Chatfield, V. A. Lawson, S. Crowe, A. Maerz, S. Sonza, J. Learmant, J. S. Sullivan, A. Cunningham, D. Dwyer, D. Dowton, and J. Mills.** 1995. Genomic structure of an attenuated quasispecies of HIV-1 from a blood transfusion donor and recipients. Science **270:**988–991.
9. **Delwart, E., E. G. Shpaer, J. Louwagie, F. E. McCutchan, M. Grez, H. Rubsamen-Waigmann, and J. I. Mullins.** 1993. Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 env genes. Science **262:**1257–1261.
10. **Dubay, J. W., S. R. Dubay, H.-J. Shin, and E. Hunter.** 1995. Analysis of the cleavage site of the human immunodeficiency virus type 1 glycoprotein: requirement of precursor cleavage for glycoprotein incorporation. J. Virol. **69:**4675–4682.
11. **Gartner, S., P. Markovits, D. M. Markovitz, M. H. Kaplan, R. C. Gallo, and M. Popovic.** 1986. The role of mononuclear phagocytes in HTLV-III/LAV infection. Science **233:**215–219.
12. **Greenough, T. C., M. Somasundaran, D. B. Brettler, R. M. Hesselton, A. Alimenti, F. Kirchhoff, D. Panicali, and J. L. Sullivan.** 1994. Normal immune function and inability to isolate virus in culture in an individual with long-

term human immunodeficiency virus type 1 infection. AIDS Res. Hum. Retroviruses **10:**395–403.
13. **Harrer, E., T. Harrer, S. Buchbinder, D. L. Mann, M. Feinberg, T. Yilma, R. P. Johnson, and B. D. Walker.** 1994. HIV-1-specific cytotoxic T lymphocyte response in healthy, long-term nonprogressing seropositive persons. AIDS Res. Hum. Retroviruses **10**(Suppl. 2)**:**S77–S78.
14. **Ho, D. D., M. S. C. Fung, H. Yoshiyama, Y. Cao, and J. E. Robinson.** 1992. Discontinuous epitopes on gp120 important in HIV-1 neutralization. AIDS Res. Hum. Retroviruses **8:**1337–1339.
15. **Ho, D. D., J. A. McKeating, X. L. Li, T. Moudgil, E. S. Daar, N. C. Sun, and J. E. Robinson.** 1991. Conformational epitope on gp120 important in CD4 binding and human immunodeficiency virus type 1 neutralization identified by a human monoclonal antibody. J. Virol. **65:**489–493.
16. **Ho, D. D., M. G. Sarngadharan, M. S. Hirsch, R. T. Schooley, T. R. Rota, R. C. Kennedy, T. C. Chanh, and V. Sato.** 1987. Human immunodeficiency virus neutralizing antibodies recognize several conserved domains on the envelope glycoproteins. J. Virol. **61:**2024–2028.
17. **Huang, X., L. Zhang, and D. D. Ho.** 1995. Biological characterization of *nef* in long-term survivors of human immunodeficiency virus type 1 infection. J. Virol. **69:**8142–8146.
18. **Huang, Y., L. Zhang, and D. D. Ho.** 1995. Characterization of *nef* sequences in long-term survivors of human immunodeficiency virus type 1 infection. J. Virol. **69:**93–100.
19. **Kestler, H. W., D. J. Ringler, K. Mori, D. L. Panicali, P. K. Sehgal, M. D. Daniel, and R. C. Desrosiers.** 1991. Importance of the *nef* gene for maintenance of high viral loads and for development of AIDS. Cell **65:**651–662.
20. **Kirchhoff, F., T. C. Greenough, D. B. Brettler, J. L. Sullivan, and R. C. Desrosiers.** 1995. Absence of intact *nef* sequences in a long-term survivor with nonprogressive HIV-1 infection. N. Engl. J. Med. **332:**228–232.
21. **Klasse, P. J., J. P. Moore, and B. A. Jameson.** 1993. The interplay of the HIV-1 envelope complex, gp120 and gp41, with CD4, p. 241–266. *In* W. J. W. Morrow and N. L. Haigwood (ed.), HIV molecular organization, pathogenicity and treatment. Elsevier Science Publishers B.V., New York.
22. **Koyanagi, Y., S. Miles, R. T. Mitsuyasu, J. E. Merrill, V. H. Vinters, and I. S. Y. Chen.** 1987. Dual infection of the central nervous system by AIDS viruses with distinct cellular tropisms. Science **236:**819–822.
23. **Learmont, J., L. Cook, H. Dunckley, and J. S. Sullivan.** 1995. Update on long-term symptomless HIV type 1 infection in recipients of blood products from a single donor. AIDS Res. Hum. Retroviruses **11:**1.
24. **Learmont, J., B. Tindall, L. Evans, A. Cunningham, P. Cunningham, J. Wells, R. Penny, J. Kaldor, and D. A. Cooper.** 1992. Long-term symptomless HIV-1 infection in recipients of blood products from a single donor. Lancet **340:**863–867.
25. **Levy, J. A., A. D. Hoffman, S. M. Kramer, J. A. Landis, J. M. Shimabukuro, and L. S. Oshiro.** 1984. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. Science **225:**840–842.
26. **McCune, J. M., L. B. Rabin, M. B. Feinberg, M. Lieberman, J. C. Kosek, G. R. Reyes, and I. L. Weissman.** 1988. Endoproteolytic cleavage of gp160 is required for activation of human immunodeficiency virus. Cell **53:**55–67.
27. **McMichael, A. J., and B. D. Walker.** 1994. Cytotoxic T lymphocyte epitopes: implications for HIV vaccines. AIDS **8:**S155–S173.
28. **Michael, N. L., G. Chang, L. A. D'Arcy, P. K. Ehrenberg, R. Mariani, M. P. Busch, D. L. Birx, and D. H. Schwartz.** 1995. Defective accessory genes in a human immunodeficiency virus type 1-infected long-term survivor lacking recoverable virus. J. Virol. **69:**4228–4236.
29. **Moore, J. P., and D. D. Ho.** 1993. Antibodies to discontinuous or conformationally sensitive epitopes on the gp120 glycoprotein of human immunodeficiency virus type 1 are highly prevalent in sera of infected humans. J. Virol. **67:**863–875.
30. **Moore, J. P., Q. J. Sattentau, R. Wyatt, and J. Sodroski.** 1994. Probing the structure of the human immunodeficiency virus surface glycoprotein gp120 with a panel of monoclonal antibodies. J. Virol. **68:**469–484.
31. **Pantaleo, G., S. Menzo, M. Vaccarezza, C. Graziosi, O. J. Cohen, J. F. Demarest, D. Montefiori, J. M. Orenstein, C. Fox, and L. K. Schrager.** 1995. Studies in subjects with long-term nonprogressive human immunodeficiency virus infection. N. Engl. J. Med. **332:**209–216.
32. **Safrit, J. T.** Personal communication.
33. **Safrit, J. T., A. Y. Lee, C. A. Andrews, and R. A. Koup.** 1994. A region of the third variable loop of HIV-1 gp120 is recognized by HLA-B7-restricted cytotoxic T lymphocytes from two acute seroconversion patients. J. Immunol. **153:**3822–3830.
34. **Sambrook, J., E. F. Fritsch and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 9.14. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Schrager, L. K., J. M. Young, M. G. Fowler, B. J. Mathieson, and S. H. Vermund.** 1994. Long-term survivors of HIV-1: definitions and research challenges. AIDS **8**(Suppl. 1)**:**S95–S108.
36. **Stevens, C.** Personal communication.
37. **Syu, W.-J., W.-R. Lee, B. Du, Q.-C. Yu, M. Essex, and T.-H. Lee.** 1991. Role of conserved gp41 cyteine residues in the processing of human immunodeficiency virus envelope precursor and viral infectivity. J. Virol. **65:**6349–6352.
38. **Willey, R. L., J. S. Bonifacino, B. J. Potts, M. A. Martin, and R. D. Klausner.** 1988. Biosynthesis, cleavage, and degradation of the human immunodeficiency virus 1 envelope glycoprotein gp160. Proc. Natl. Acad. Sci. USA **85:**9580–9584.
39. **Zhang, L., Y. Huang, H. Yuan, S. Tuttleton, and D. D. Ho.** Characterization of *vif*, *vpr*, and *vpu* sequences from long-term survivors of human immunodeficiency virus type 1 infection. Submitted for publication.