Differences in the Fitness of Two Diverse Wild-Type Human Immunodeficiency Virus Type 1 Isolates Are Related to the Efficiency of Cell Binding and Entry

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The ability of one primary human immunodeficiency virus type 1 (HIV-1) isolate to outcompete another in primary CD4⁺ human lymphoid cells appears to be mediated by the efficiency of host cell entry. This study was designed to test the role of entry on fitness of wild-type HIV-1 isolates (e.g., replicative capacity) and to examine the mechanism(s) involved in differential entry efficiency. The gp120 coding regions of two diverse HIV-1 isolates (the more-fit subtype B strain, B5-91US056, and less-fit C strain, C5-97ZA003) were cloned into a neutral HIV-1 backbone by using a recently described yeast cloning technique. The fitness of the primary B5 HIV-1 isolates and its *env* gene cloned into the NL4-3 laboratory strain had similar fitness, and both were more fit than the C5 primary isolate and its *env*/NL4-3 chimeric counterpart. Increased fitness of the B5 over C5 virus was mediated by the gp120 coding region of the *env* gene. An increase in binding/fusion, as well as decreased sensitivity to entry inhibitors (PSC-RANTES and T-20), was observed in cell fusion assays mediated by B5 gp120 compared to C5 gp120. Competitive binding assays using a novel whole virus-cell system indicate that the primary or chimeric B5 had a higher avidity for CD4/CCR5 on host cells than the C5 counterpart. This increased avidity of an HIV-1 isolate for its cell receptors may be a significant factor influencing overall replicative capacity or fitness.

Human immunodeficiency virus type 1 (HIV-1) entry begins with the interaction of the viral envelope glycoprotein gp120 with the cellular CD4 receptor that induces a conformational shift in gp120 and exposes the conserved coreceptor-binding site (48, 53, 61). After interactions with either the CCR5 or CXCR4 coreceptor (2, 14, 18, 20–22), further conformational changes promote the gp41-mediated fusion of the viral and cellular membranes (12, 60). High mutation frequencies coupled with plasticity of functional *env* glycoproteins have now resulted in extreme *env* diversity observed among different subtypes (>15% predicted amino acid diversity) and between isolates of the same subtype (10 to 15%) (30). Although this diversity may have been shaped by immune response (47, 59), it is difficult to refute that some of this variability would affect the multistep process of HIV-1 entry and that divergent HIV-1 isolates may not all enter with identical efficiencies.

Most studies of HIV-1 fitness tend to focus on particular regions in the genome that are the target of antiretroviral drugs and mutate under drug pressure (8, 24, 38, 62). Many mutations conferring drug resistance to reverse transcriptase (RT) and protease inhibitors typically have deleterious effects on replicative capacity and thus confer decreased fitness (8, 24, 38, 62). These findings imply that fitness is related to the region

of the genome subject to the greatest selective pressure. In the absence of drug pressure the HIV-1 *env* gene may be under the greatest selective pressure due not only to the humoral immune response (47, 59) but also to factors that affect virus entry into the host cell such as coreceptor tropism (2, 14, 18, 20–22), coreceptor expression (58), interference by host chemokines (15), and host polymorphisms (16, 33, 49). However, the impact of any HIV-1 gene on replication efficiency must be considered in the context of the entire virus due to the interplay between gene products in the life cycle and the extreme diversity between HIV-1 isolates of even the same subtype (6, 43).

Until recently, few studies have compared the relative replicative capacity of “wild type” HIV-1 isolates of the same subtype let alone different subtypes (6, 41, 54). We have now performed thousands of dual HIV-1 competitions in human peripheral blood mononuclear cells by using over fifty different primary “wild-type” HIV isolates of different groups (M and O), group M subtypes (A, B, C, D, and E/CRF01), and types (HIV-1 and HIV-2) (3, 6). These experiments have proposed a relative order in replicative fitness (HIV-1 group M > subtype C > HIV-2 >> group O) but have failed to identify the viral genetic elements responsible for these intrinsic differences in replication efficiency. Preliminary studies comparing fitness differences to genetic elements by using phylogenetic neighbor-joining trees and phyletic fitness trees suggest that fitness maps more closely to the *env* gene than *gag* or *pol* genes (6). Recent studies by Rangel et al. (44) suggest that the *env* gene

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and not the PR-RT coding region of wild-type HIV-1 isolates may have a greater impact on replication efficiency.

Primary subtype C isolates appear to be at least 10- to 100-fold less fit than subtype B isolates in PBMC, CD4⁺ T cells, and macrophages (6). By tracking all of the retroviral replication steps mediated by nucleic acids, it appeared that the "winner" of several dual virus competitions was already determined within 8 to 24 h after virus exposure. From these findings we presumed that the competition between HIV-1 pairs was occurring at the level of entry and that other steps in the retroviral life cycle (reverse transcription, integration, and viral mRNA transcription) had minimal impact on the fitness of wild-type HIV-1 isolates. In the present study we tested this hypothesis by first by using a novel yeast recombination system to clone the gp120 coding regions of two divergent *env* genes into a eukaryotic expression vector and then into a neutral HIV-1 backbone (NL4-3) to produce chimeric viruses (35). Complementation of the NL4-3 gp41 with B5 or C5 gp120 coding regions did not affect gp120/gp41 expression on virus or in cells. We then competed the parental wild-type isolates and chimeric viruses (NL4-3_{B5 gp120} and NL4-3_{C5 gp120}) against each other or a set of primary subtype B and C HIV-1 isolates. Competitive cell fusion assays further confirmed that entry and not another point in the HIV-1 life cycle was the major determinant of fitness. Finally, we developed and used a whole virus-cell competitive binding assay to calculate virus avidity to the host cell (expressing coreceptors and CD4) in the absence of fusion. Reduced fitness of the primary C5 isolate compared to the B5 HIV-1 isolate correlates to weaker cell surface binding, poor entry efficiency, and greater sensitivity to CCR5 agonists and fusion inhibitors.

MATERIALS AND METHODS

Cells and viruses. PBMC from HIV-seronegative blood donors were obtained by Ficoll-Hypaque density gradient centrifugation of heparin-treated venous blood. Prior to HIV-1 infection, the cells were stimulated with 2 μ g of phytohemagglutinin (PHA; Gibco-BRL)/ml for 2 to 3 days and maintained in RPMI 1640 supplemented with 10% fetal bovine serum. Non-syncytium-inducing (NSI) HIV-1 isolates, three subtype B (93US076 or B4, 91US056 or B5, 91US714 or B6) and four subtype C HIV-1 isolates (97ZA012 or C3, 97ZA003 or C5, 96USNG58 or C8, 93MW959 or C9), were obtained from the NIH Research and Reference Reagent Program. The 50% tissue culture infective dose(s) (TCID₅₀) was determined in PHA-interleukin-2 (IL-2)-treated peripheral blood mononuclear cells (PBMC) as described previously (36). Titers were expressed as infectious units (IU) per milliliter. It is important to note that the TCID₅₀ assay and subsequent dual virus competitions were performed with PBMC from the same donor and blood draw.

For Western blot analysis and virus avidity studies, the chimeric NL4-3_{B5 gp120} and NL4-3_{C5 gp120} viruses were purified on a 65% sucrose cushion and then concentrated by centrifugation at 40,000 $\times$ g for 1 h to remove extraneous cleaved glycoproteins and cell debris from the virus stock. These viruses were then titered as described above.

Adherent U87 (human glioma) cells expressing CD4 and CCR5 and GHOST cells (human osteosarcoma) expressing CD4, CCR5, and green fluorescent protein (GFP) under the control of the HIV-2 long terminal repeat (LTR) were obtained through the AIDS Reagent Project. U87.CD4-CCR5 cells were grown in complete Dulbecco modified Eagle medium (DMEM) containing 300 μ g of Genetecin/ml (G418) and 1 μ g of puromycin/ml (Life Technologies, Inc.) to maintain CD4 and coreceptor expression, respectively. GHOST cells were grown in complete DMEM containing 500 μ g of Genetecin/ml (G418), 100 μ g of hygromycin (Invitrogen)/ml, and 1 μ g of puromycin/ml. The suspension CEM-SS and CEM-NKR-CCR5 cell lines were obtained from the NIH AIDS Research and Reference Reagent Program and were cultured in complete RPMI medium containing 10% fetal calf serum and antibiotics. Finally, QT6 cells (quail cell line) were maintained in DMEM supplemented with 10% fetal calf serum.

For the competitive fusion assay, 293T cells stably transfected with pEXP B5 gp120 or pEXP C5 gp120 vectors (Fig. 1) were maintained in complete DMEM supplemented with 240 μ g of zeocin/ml. Stable expression of the B5 and C5 gp120 in the 293T cells was verified by both mRNA and by intracellular flow cytometry specific for the common HXB2 gp41 tethered to both B5 and C5 gp120 (see below).

Construction of chimeric viruses containing the HIV-1 *env* gene of primary HIV-1 isolates. The B5 and C5 viral chimeras were constructed through the use of a yeast recombination system as previously described in Fig. 1 and by Marozsan and Arts (35). It is important to note that the original pREC-*env*/URA3 vector was constructed with the HXB2 EcoRI to XhoI fragment instead of NL4-3 that did not contain the SalI site. HXB2 and NL4-3 are almost identical in the gp41 coding sequences (9 nucleotide [nt] substitutions in a 1,040-nt coding sequence). Thus, to avoid confusion, all NL4-3 vectors containing a B5 (or C5) gp120/HXB2 gp41 coding region were referred to as pNL4-3/B5 (or C5) gp120, inferring that B5 gp120 is the only foreign DNA introduced into the NL4-3 backbone.

HIV-1 infections and growth competition assays. All HIV-1 isolates listed above were used in monoinfection and dual infection studies in PBMC. Virus was added alone or in pairs to 200,000 PHA-IL-2-treated PBMC per well at a multiplicity of infection (MOI) of 0.0005 (MOI, [IU/cell]) in a 24-well plate. After an 8-h incubation at 37°C at 5% CO₂, cells were washed three times with phosphate-buffered saline (PBS) and then resuspended in complete medium (10⁶ cells/ml). All monoinfection and dual infection-competition experiments were performed in PBMC from one donor in duplicate. As described in Quinones-Mateu et al. (43) and in Ball et al. (6), the dual infection-competition assay involved the addition of two HIV-1 isolates (MOI of 0.0005) and was performed alongside the monoinfections. Cell-free supernatants were assayed for RT activity (36) at days 1, 2, 5, 10, and 15 postinfection. Aliquots of cells were removed at day 10 in the relative fitness dual infection assays and at 8, 24, 48, 110, and 240 h for the time course dual infection assays. Supernatants and two aliquots of cells were stored at -80°C for subsequent analysis.

HIV-1 DNA PCR and heteroduplex tracking assays. For both the fitness and time course competition experiments, proviral DNA was extracted from lysed PBMC by using the QIAamp DNA blood kit (Qiagen). Viral DNA was PCR amplified by using a set of external primers, *env*B (23)-ED14 (gp120-coding region of *env*, ~1.7 kb), followed by nested amplification with the E80-E125 primer pair (50) (C2-V3 *env* region, 0.48 kb) under conditions described previously (6, 43). The same genomic regions (C2-V3) were amplified with radiolabeled primers from a subtype E HIV-1 *env* clone (E-pTH22) and a subtype D *env* clone (D-pUG38) (17) for use as DNA probes. Reaction mixtures containing DNA annealing buffer (100 mM NaCl, 10 mM Tris-HCl [pH 7.8], 2 mM EDTA), 0.5 pmol of PCR-amplified DNA from the competition culture, and ca. 0.1 pmol of radioactive probe DNA were denatured at 95°C for 3 min and then rapidly annealed at 4°C. After 30 min on ice, the DNA heteroduplexes were resolved on 8% nondenaturing polyacrylamide gels and then analyzed by using a Bio-Rad phosphorimager as described previously (6, 43).

Estimation of viral fitness. In HIV-1 competition experiments, the final ratio of two viruses produced from a dual infection was determined by heteroduplex tracking assay (HTA) and compared to production in the monoinfections. Production of individual HIV-1 isolates in a dual infection (f_d) was divided by the initial proportion in the inoculum (i_0) and is referred to as the relative fitness ($w = f_d/i_0$) (43). The ratio of the relative fitness values of each HIV-1 variant in the competition is a measure of the fitness difference (W_D) between both HIV-1 strains ($W_D = w_M/w_L$), where w_M and w_L correspond to the relative fitnesses of the more and less fit viruses, respectively (43).

Fusion assays. Two fusion assays were performed in the present study: a kinetic fusion assay using QT6 cells transiently transfected with *env* expression constructs and a competitive fusion assay using stably transfected 293T cells. The kinetics of fusion mediated by HIV-1 envelope glycoproteins was determined in a β -lactamase reporter cell-cell fusion assay based on that recently described (32). QT6 cells, cotransfected with *env* and β -lactamase expression constructs and infected with a vaccinia virus encoding T7 polymerase (vTF1.1) to drive *env* and β -lactamase expression, were added to CD4/CCR5⁺ HeLa cells (JC53s) labeled with CCF2-AM as previously described (46). Cell-cell fusion was detected at 10 to 240 min (at 10-min intervals) by assaying for the shift from green to blue fluorescence, indicating β -lactamase cleavage of CCF2. Fluorescence was quantitated with a CytoFluor Series 4000 Fluorescence multiwell plate reader (PerSeptive Biosystems), and the results are expressed as the ratio of blue to green fluorescence obtained with *env*-transfected effectors minus the ratio of background blue to green fluorescence obtained with empty-vector-transfected cells.

To perform the competitive fusion assay, U87 target cells were mixed and

FIG. 1. Use of yeast recombination/gap repair for cloning of divergent HIV-1 *env* genes. The gp120 coding regions of HIV-1 *env* genes from B5-91US056 and C5-97ZA003 share <82% nucleotide sequence identity (A) and differ at 11 of 46 amino acid positions within and flanking the V3 loop sequence (B). The entire HIV-1 gp120 coding sequence (aside from the leader sequence) was PCR amplified from primary B5 and C5 HIV-1 isolates and then transfected into yeast, along with the pREC-*env*(HXB2) vector. (C) Schematic illustration of the yeast recombination/gap repair cloning technique used to introduce diverse HIV-1 genes into expression (pEXP-*env*) or subcloning vectors (pREC-*env*) without the need or use of unique restriction enzyme sites. Details of the technique are provided by Marozsan and Arts (35).

pelleted with various levels of 293T effector cells expressing B5 gp120/HXB2 gp41 or C5 gp120/HXB2 gp41. A total of 6×10^5 or 6×10^4 293T cells expressing B5 gp120 or C5 gp120 were added together (for competition) or separately to 6×10^6 U87.CD4.CCR5 target cells with or without G418-puromycin-zeocin selection. Control experiments involved the addition of U87 cells to 293T cells that do not express *env* in the presence or absence of selection. Pelleted target and effector cells were incubated for 1 h at 37°C and 5% CO₂ and then resuspended in selection medium and plated in triplicate. On day 3 after plating, cells were washed twice with PBS and removed from the plate with 3 mM EDTA. DNA was then extracted from the cells (see below), PCR amplified with the E80-E125 primer set (C2-V3 *env* region), and then used in an HTA analysis as described above. Mitochondrial DNA was also PCR amplified by using the MTA2-MTS2 primer pair as described previously (5). Calculations to determine relative fusion efficiency of B5 gp120 compared to C5 gp120 in these competitions were as described above for relative fitness.

Ghost cell fusion assay. Sensitivity of 293T cells expressing B5 and C5 envelope glycoproteins to PSC-RANTES and T-20 (1 or 100 nM) was evaluated in the

context of a Ghost cell fusion assay. For each condition, 7.5×10^4 Ghost cells were mixed with 1.0×10^5 293T cells expressing B5 gp120, C5 gp120, or neither. Cells were incubated for 48 h at 37°C and 5% CO₂ and then analyzed for GFP expression by fluorescence microscopy. Images were captured as five 100- μ m² images/well, and the mean GFP fluorescence per well was then measured by using Quantity One software (Bio-Rad).

Western blot analyses of HIV-1 proteins. Western blots were performed on the B5 and C5 viruses and their chimerics, as well as subtype controls. Viruses were clarified from cell debris by centrifugation at $3,000 \times g$ for 20 min and then pelleted by centrifugation at $32,000 \times g$ for 1 h. Some virus pellets were resuspended in PBS and purified on a 60% sucrose cushion (4). Virus pellets were resuspended in sodium dodecyl sulfate (SDS) lysis buffer (40 mM Tris-HCl [pH 6.8], 10% glycerol, 10% β -mercaptoethanol, 1% SDS) to equilibrate for TCID₅₀ values. The TCID₅₀ values (prior to virus pelleting) were also compared to RT activity in each virus stock (36). Lysed virus samples were serially diluted 1:5 and 1:25, separated by SDS-10% polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane, blocked with 5% milk, and then incubated with pri-

mary antibody. To detect gp120, blots were probed with the B13 antibody (provided by George Lewis, Institute of Human Virology, Baltimore, MD, and Bruce Chesebro, NIAID, Hamilton, MT) that recognizes a highly conserved linear epitope in the C2 region of gp120 (1, 63). gp41 was detected with the antibody Chessie 8, which recognizes a gp41 epitope found in most subtype B isolates but not in subtype C (1). Detection of p24 was performed by using a mouse monoclonal antibody (Fitzgerald Industries International, Inc., Concord, MA). Blots were incubated with horseradish peroxidase-conjugated secondary antibody, detected with the ECL Plus Western blotting detection system (Amersham Biosciences, Piscataway, NJ), and exposed to film.

Whole virus-cell competitive binding assay. CEM-SS and CEM-NKR-CCR5 cells were washed twice in serum-free RPMI and 10^4 cells were used for each binding assay condition. Viruses B5, C5, NL4-3^{B5 gp120}, and NL4-3^{C5 gp120} were purified, added to the cells at an MOI of 0.0001 and were competed off the cells by the addition of NSI isolate B2 (92BR017) or the SI isolate D1 (92UG021) at fivefold MOI intervals from 0.0001 to 0.0625. HIV-1 D1 acts as a control and does not directly compete with the B5 or C5 viruses for binding to CCR5. Viruses were added to cells in binding buffer (50 mM HEPES, 5 mM MgCl₂, 1 mM CaCl₂, 5% bovine serum albumin [BSA], 0.1% NaN₃) on ice and then incubated for 1 h at 15°C (26). The cell-virus mix was added to filter tubes (Ultrafree-MC; Millipore) and then centrifuged through the filter at $300 \times g$ for 2 min. Cells on the filter were washed twice with 300 μ l of wash buffer (50 mM HEPES, 5 mM MgCl₂, 1 mM CaCl₂, 500 mM NaCl) by centrifugation at $300 \times g$ for 2 min and then lysed in the buffer AVL containing carrier RNA (Qiagen) (26). RNA was extracted by using a Qiagen viral RNA minikit and was reversed transcribed by using M-MLV RT (Invitrogen) and the E105 primer (50). cDNA was then amplified with an external set of primers (ED33 and ED5) and a nested set of primers (E80 and E125) (C2-V3 *env* region) for HTA analysis (see above).

Flow cytometry. 293T cells stably transfected with pEXP *env*/B5 gp120 or pEXP *env*/C5 gp120 vectors (Fig. 1) were maintained in complete DMEM supplemented with 240 μ g of zeocin/ml. Cells were washed in PBS containing 1% BSA and 0.1% sodium azide and then permeabilized with FACS Perm (Becton Dickinson) for 10 min. Cells were incubated with 150 μ l of Chessie 8 mouse α -human gp41 immunoglobulin G1 monoclonal antibody supernatant from a hybridoma cell line for 30 min (NIH AIDS Research and Reference Reagent Program), washed, and then incubated with 2 μ l of goat anti-mouse-fluorescein isothiocyanate (FITC)-conjugated immunoglobulin (BD Biosciences). An FITC-conjugated immunoglobulin G1 isotype control antibody to the kappa chain was also used to label the 293T cells as a negative control (Pharmingen). After incubation with a secondary antibody, the cells were washed and then fixed in 1% paraformaldehyde-PBS. Cells were analyzed by using a FACSCalibur flow cytometer with CellQuest software (BD Biosciences).

Quantitative PCR with radiolabeled primers and real-time PCR. The number of HIV-1 viruses bound to CEM cells at the end of the binding assay was quantitated from RNA extracted from cell-bound viruses. The RNA was reverse transcribed with primer E105 specific for the viral *env* gene and primer ABA-7 (6) for the cellular β -globin gene by using M-MLV RT (Invitrogen). β -Globin cDNA was then amplified with a [γ -³²P]ATP-labeled sense primer, SBA-7, and the antisense primer ABA-7 (6). The *env* cDNA was amplified with the γ -³²P-labeled E110 and E125 primer pair and was quantitated relative to standards containing equal known copy numbers of pNL4-3^{B5 gp120} and pNL4-3^{C5 gp120} plasmid DNA (10 to 10^8 copies) in 10-fold serial dilutions (4). Quantitation of copy numbers of viruses was performed by using a phosphorimager (Bio-Rad) as described above.

For the time course competition experiments, the viral DNA load derived from reverse-transcribed viral RNA in the culture supernatant or from lysed cells was measured by real-time PCR with the primers sU3-1 (GCAGTGTCTTTT GCCTGTACTGG) and a U5-2 (AGTCACACAACAGACGGGCAC). Cellular DNA input was also measured by real-time PCR with primers for β -globin, SIBG (GGATCTGTCCACTCTGATG) and AIBG (GTGCAGCTCACTCAGT GT). All reactions were performed on an ABI 7900HT Real-Time PCR machine under the following conditions: 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

RESULTS

Relationship between host cell entry and viral fitness. In a preliminary study, we found that NSI/R5 subtype B and C HIV-1 isolates shared similar intrasubtype fitness but that subtype B isolates were the clear winners over subtype C isolates in head-to-head intersubtype competitions in PBMC (6). In

time course competitions, analyses of HIV-1 DNA at various steps of the retroviral life cycle with HTA revealed that entry and not RT, proviral integration, or transcription appeared to have the greatest influence on the ability of one virus to out-compete another. The study presented herein was designed to test the role of entry on fitness of wild type HIV-1 isolates (e.g., replicative capacity) and to examine the mechanism(s) involved in differential entry efficiency.

To confirm that entry is playing a dominant role in determining fitness, ideally the *env* genes of primary HIV-1 isolates should be removed from the autologous HIV-1 genome and placed into a neutral genomic backbone (e.g., NL4-3). However, this task is extremely difficult using conventional cloning techniques due to the extreme heterogeneity in the *env* gene (e.g., Fig. 1A) and the lack of conserved/convenient restriction enzyme sites. Thus, we have adopted a yeast recombination/gap repair cloning system to rapidly exchange, within a yeast vector (pREC-*env*/URA3), the gp120 coding region in place of the URA3 gene through conserved flanking sequences found in both (Fig. 1C) (35). Colonies are selected on minimal media plates lacking leucine but containing FOA (5-fluoro-2-dUMP), i.e., toxic to yeast carrying a functional URA3 gene (11). The pREC-*env* vector can then be used to construct the pEXP-*env* vector or for cloning into the pNL4-3 vector (Fig. 1C). 293T cells stably transformed with the pEXP-*env* can be used as effector cells in fusion assays containing a CD4⁺/CCR5⁺ (CXCR4⁺) target cell line, whereas transfection of 293T cells with the pNL4-3-*env* chimeric vector can result in production of infectious, replication-competent, chimeric virus particles (Fig. 1C).

For the present study, we focused our attention on the B5 and C5 HIV-1 isolates due to the modest but significant fitness difference in direct competition (8- to 10-fold in favor of B5) but compared the fitness of these B5 and C5 primary and *env* chimeric viruses against numerous other primary HIV-1 isolates and in a variety of entry and/or binding assays. Sequence differences between the C2-C3 regions of B5 and C5 *env* are shown in Fig. 1A (6). Only the gp120 coding region of B5 and C5 was introduced into pEXP-*env*, which was subsequently subcloned to produce pEXP-B5 gp120/HXB2 gp41, pEXP-C5 gp120/HXB2 gp41, pNL4-3/B5 gp120, and pNL4-3/C5 gp120 (Fig. 1C). The successful cloning of these products, recombination breakpoint analyses, and initial *env* functional analyses has been previously described (35). As described below, we observed no significant complementation defect in terms of proper Env surface expression between the heterologous B5 or C5 gp120 and HXB2 gp41. The chimeric NL4-3^{B5 gp120} and NL4-3^{C5 gp120} viruses were used in direct competitions alongside the parental B5 versus C5 viruses in PHA-IL-2-treated PBMC. Total HIV-1 DNA was quantified by reverse transcription-PCR (RT-PCR) (Fig. 2B and C) but relative amounts of B5 and C5 DNA was analyzed by HTA (Fig. 2A). In direct competitions, the NL4-3^{B5 gp120} chimeric virus outcompeted the NL4-3^{C5 gp120} virus as early as 8 h postinfection and maintained its dominance for 10 days (Fig. 2A). These results are nearly identical to that observed in the parental virus competitions (Fig. 2C). This apparent increase in B5 or NL4-3^{B5 gp120} entry over the C5 counterparts was detected even at relatively low levels of initial infection. It is important to note that W_D of B5 versus C5 *env* chimeras was greater than observed with the

FIG. 2. Direct time course competitions between parental and *env* chimeric primary HIV-1 isolates. Primary HIV-1 isolates, B5 and C5 or their chimeric counterparts, NL4-3_{B5 gp120} and NL4-3_{C5 gp120}, were used in head-to-head competitions. Time course competitions in these PBMC were analyzed by using HTA at 8, 24, 48, and 120 h after dual infection (chimeric viruses in panel A). The heteroduplex representing relative production of the NL4-3_{B5 gp120} and NL4-3_{C5 gp120} chimeras in monoinfections are indicated in the right hand lanes and provide a reference for the heteroduplex migrations in the dual infections time course samples in the left hand lanes. The relative B5 and C5 virus (or NL4-3_{B5 gp120} and NL4-3_{C5 gp120}) production derived from the HTA are plotted as a function of peak HIV-1 DNA production. In panels B and C, the solid line and closed circle represent the total HIV-1 DNA detected at each time point by real-time PCR relative to β -globin DNA, also amplified by real-time PCR. The relative B5 and C5 virus production at each time point was measured relative to the highest level of HIV-1 DNA copies detected for each time course. In panel B, ratios of B5 and C5 chimeric HIV-1 DNA (detected by HTA [panel A]) were plotted as relative to peak HIV-1 DNA production at 120 h.

primary isolates. Decreased fitness of the C5 gp120 chimera as compared to the C5 primary isolate may be due to compensation by other regions or gene products in the C5 genome. The C5 gp120 was, however, expressed at equal levels on the viral

FIG. 3. Fitness of parental and *env* chimeric viruses relative to various primary HIV-1 isolates. (A) The parental and *env* chimeric B5 HIV-1 isolates were competed against C3, NL4-3_{C5 gp120}, C5, C8, C9, B4, and B6 primary HIV-1 isolates. The fitness difference (W_D) was calculated as the relative fitness value of B5 or NL4-3_{B5 gp120} divided by the relative fitness value of the various competitor viruses. Relative fitness values were calculated by the formula, $W = f_0/i_0$, where f_0 is the production of individual HIV-1 isolates in a dual infection and i_0 is initial proportion of the respective isolate in the inoculum. (B) Same competitions as in panel A but with parental and *env* chimeric C5 HIV-1 isolates.

surfaces compared to the gp120 levels on the NL4-3_{B5 gp120}, NL4-3_{C5 gp120}, and B5 primary HIV-1 isolates.

Comparing the fitness of the *env* chimeric and parental HIV-1 isolates. To further confirm the role of the gp120 coding region on the fitness of B5 and C5 isolates, we competed both parental strains (B5 and C5) and chimeric NL4-3_{B5} (or C5) gp120 viruses against five other primary NSI/R5 subtype B and C HIV-1 isolates in PBMC cultures (Fig. 3). The primary B5 isolate and NL4-3_{B5 gp120} chimera could outcompete both the C5 parental isolate and the NL4-3_{C5 gp120} chimeric virus (Fig. 3A). In addition, the fitness differences (W_D) obtained from competitions between the primary B5 HIV-1 isolates or NL4-3_{B5 gp120} with the wild type C8 and C9 were quite similar and, with B4 primary isolates, only slightly different (Fig. 3A). Fitness difference is a ratio of relative fitness values of each isolate in a dual virus competition (see the discussion of estimation of viral fitness in Materials and Methods). This observation was not limited to B5 considering C5, and its chimeric counterpart (NL4-3_{C5 gp120}) also competed with nearly equal efficiencies against the C8, C9, and B4 isolates (Fig. 3B). All competitions were performed in duplicate, and the differences in relative

fitness values were <10% of the mean value. Only competitions between B6 and B5 or NL4-3_{B5 env} resulted in disparate fitness differences. The reasons for this 100-fold difference when competed against B6 are not clearly understood but is the subject of ongoing study. The chimeric B5 env virus was slightly but significantly more fit than the primary B5 virus in two of seven competitions (against NL4-3_{C5 env} and B6) and slightly less fit in two of four competitions (against C3 and B4). In contrast, the fitness of the C5 primary and of NL4-3_{C5 env} were nearly identical in all competitions. The exceptions being that C5 had a higher fitness than NL4-3_{C5 env} when competed against the primary C3. The opposite was evident when competed against B6, i.e., C5 had a lower fitness than NL4-3_{C5 env}. Overall, these results confirm the preliminary findings in our previous report that the efficiency of host cell entry (of at least the B5 and C5 HIV-1 isolates) may have the greatest influence on replicative capacity (6).

Relating relative levels of viral gp120 expression and relative fitness. Addition of a divergent gp120 coding region of B5 or C5 to the HXB2 gp41 coding region in the *env* gene could have an effect on the expression and stability of envelope glycoprotein on the viral membrane. However, the nearly identical fitness of the primary B5 or C5 HIV-1 isolates and their respective gp120 chimeric viruses suggest that an incompatibility between gp120 and gp41 was unlikely. Since the NL4-3_{C5 gp120} and NL4-3_{B5 gp120} had identical genomic sequences aside from the gp120 coding region, we were able to probe the virus in cell-free supernatant for various viral proteins (gp41 and p24 capsid) and compare protein levels to infectious titer (i.e., TCID₅₀ values) (Fig. 4B and C). Using Western blot analyses, we detected similar levels of gp41 or p24 for equal titers of both chimeric viruses (Fig. 4B). To detect gp120, blots were probed with the B13 antibody that recognizes the epitope TQLLL in the C2 region of gp120 (1), which is highly conserved across HIV-1 subtypes (63). There was only a 1.2-fold increase in B5 gp120 expression over C5 gp120 on virus particles of the same titer, i.e., similar to that difference in gp41 expression. In contrast, p24 levels were actually slightly less in NL4-3_{B5 gp120} compared to the NL4-3_{C5 gp120} virus (Fig. 4B). Even with the amplification via multiple rounds of infection (ca. four rounds over 10 days) during the head-to-head competitions, it is unlikely that these minor differences could account for a 100-fold increase in NL4-3_{B5 gp120} over NL4-3_{C5 gp120} replication (Fig. 2B). Furthermore, a slight difference in gp120 expression on these parental and chimeric B5 and C5 viruses likely did not account for differences in total relative fitness values derived from the addition of relative fitness values from seven dual virus competitions (Fig. 4A). The total relative fitness values of B5 and NL4-3_{B5 gp120} were 9.5 and 10.5, respectively, and significantly lower than the total relative fitness values of C5 (4.0) and NL4-3_{C5 gp120} (3.8) (Fig. 4A). Finally, compatibility between these divergent gp120 and gp41 subunits of *env* is supported by the appearance of stable inter-subtype HIV-1 recombinants in the epidemic that harbor high frequencies of recombination breakpoints at the gp120/gp41 junction (39). Nevertheless, we cannot exclude that these slight differences in envelope glycoprotein levels on the viral surface could play a role in replication efficiency since there are only 7 to 14 Env trimers per virus particle (13).

To determine whether differential gp120 expression ac-

FIG. 4. Comparing relative fitness of subtype B and C HIV-1 isolates to gp120 expression on the virus particles. Pairwise competition experiments were performed with the primary B4, B5, B6, C3, C5, C8, and C9 HIV-1 isolates and the *env* chimeric viruses, NL4-3_{B5 gp120} and NL4-3_{C5 gp120}. The actual competitions in the pairwise experiment are described in Materials and Methods and Results. (A) Total relative fitness values are plotted as the top gray bar and with the scale on the upper *x* axis. (B and C) Western blot analyses were performed on various dilutions (undiluted and 1:5, 1:25, and 1:125 dilutions of equal infectious virus units) of the primary B5, B6, C5, and C9 HIV-1, as well as the NL4-3_{B5 gp120} and NL4-3_{C5 gp120}. Blots were probed with the B13 antibody specific for a conserved linear epitope in gp120. The intensities of the gp120 band on the Western blots were plotted relative to the intensity of B5 gp120 (black bars and scale on the bottom *x* axis in panel A). Blots of NL4-3_{B5 gp120} and NL4-3_{C5 gp120} were also stripped and probed with anti-gp41 and anti-p24 antibodies in panel B. In panel C, gp120, gp41, and p24 content was also measured in pelleted and sucrose cushion purified viruses of equal titer (NL4-3_{B5 gp120} and NL4-3_{C5 gp120}).

counted for any fitness difference among primary HIV-1 isolates, Western blots were also performed on dilutions of the B5, B6, C5, and C9 viruses. Again, monoclonal antibody B13 used in the present study recognizes a highly conserved, linear epitope in the C2 region of gp120. In contrast, the antibodies to gp41 and p24 did show significant variations in the recognition of HIV-1 isolates from different subtypes (36). For example, the antibody Chessie 8 recognizes the gp41 epitope PDRPEG (1), which is found in most subtype B isolates but not in subtype C. Similar levels of gp120 expression were observed on viruses (B5, B6, C5, and C9) of equal titer (values in Fig. 4A are plotted relative to B6 gp120 intensity). All quan-

titrations were derived from the virus sample of equivalent titers and undiluted. We have recently shown that equal titers of different viruses do not equate with relative replication efficiency or fitness (36). There did appear to be a weak relationship between gp120 content and the total relative fitness value of the viruses, but the differences in fitness were several orders of magnitude greater than gp120 expression. Total relative fitness was calculated as the sum of the relative fitness values for a given HIV-1 isolate competed against all others in this set. It is important to note that the equal amount of infectious titer based on TCID₅₀ values has an accuracy of ± 0.1 log. This translates into a 25% coefficient of variance for each value of infectious titer. Thus, any differences in gp120 expression of <1.25 -fold are probably not significant. In addition, Western blots are a crude quantitative assay at best. Nonetheless, when all Western blots were performed on the same day with the same antibody dilution, this B13 antibody detected similar levels of gp120 on the surface of these very diverse HIV-1 isolates.

Ability of B5 and C5 gp120 to mediate cell-to-cell fusion. A common surrogate assay of HIV-1 entry into a host cell is a cell-to-cell fusion assay where the effector cell expresses HIV-1 *env* gp120/gp41 and the target cell expresses CD4 and CCR5 receptors, as well as a reporter construct (2, 18, 22). We have used two cell fusion assays to determine whether the B5 gp120/HXB2 gp41 compared to the C5 gp120/HXB2 gp41 can mediate more rapid and efficient cell fusion. The kinetic fusion efficiencies of the B5 and C5 gp120s were first determined in a β -lactamase reporter cell-cell fusion assay (Fig. 5) (32, 46). QT6 effector cells expressing T7 polymerase from vTF1.1 and transiently cotransfected with pEXP-B5 gp120/HXB2 gp41 (or C5 version) and β -lactamase expression constructs containing T7 promoters and infected with a recombinant vaccinia virus encoding T7 polymerase (vTF1.1) to drive expression. These effector QT6 cells were then added to CD4⁺/CCR5⁺ HeLa (JC53) target cells labeled with the green fluorescent dye CCF2-AM. Cell-cell fusion was detected by assaying for a shift from green to blue fluorescence, indicating β -lactamase cleavage of CCF2. The QT6 cells expressing B5 compared to C5 gp120 mediated more efficient fusion, as indicated by a greater accumulation of blue fluorescence starting at 40 to 60 min (Fig. 5B).

The kinetic fusion assay relies on transient expression of B5 and C5 gp120. Thus, we performed a second assay in which 293T cells were stably transformed with the same pEXP-*env* constructs used in the kinetic assay. Zeocin-resistant, stably transformed cells were shown to express equal amounts of both unspliced RNA encoding the B5 or C5 *env* gp120 and multiply spliced mRNA encoding for the Tat and Rev proteins, both of which are transcribed from the pEXP-*env* constructs (data not shown). The B13, B12, and 2F8 antibodies are cross-reactive for divergent *env* proteins of primary HIV-1 isolates but did not demonstrate an affinity sufficient for analyses by fluorescence-activated cell sorting even though these antibodies have been used successfully in the past to measure the surface expression of subtype B laboratory strains (data not shown). A feasible measure of the *env* glycoproteins in the cell or on the cell surface was staining permeabilized cells for HXB2 gp41 by using the anti-gp41 Chessie 8 antibody, followed by incubation with a secondary antibody and flow cytometry (Fig. 6A). Using this method, we found that both the 293T+B5 gp120/HXB2 gp41 cells (293T/B5) and 293T+C5 gp120/HXB2 gp41 cells (293T/C5) cells expressed similar levels of *env* gp41 glycopro-

FIG. 5. Kinetics of cell fusion mediated by transiently expressed B5 gp120/HXB2 gp41 or C5 gp120/HXB2 gp41. QT6 effector cells were cotransfected with pEXP-B5 gp120/HXB2 gp41 or pEXP-C5 gp120/HXB2 gp41 vectors and a β -lactamase expression construct containing T7 promoters and infected with a recombinant vaccinia virus encoding T7 polymerase (vTF1.1) to drive expression. These effector QT6 cells were then added to CD4⁺/CCR5⁺ HeLa (JC53) target cells labeled with CCF2-AM as previously described (32, 46). (A) Cell-cell fusion was detected by assaying for a shift from green to blue fluorescence, indicating β -lactamase cleavage of CCF2. Subpanels I, III, and V show the green fluorescence (405-nm excitation and 460-nm emission) of JC53 cells labeled CCF2-AM and not fused to effector cells, whereas panels II, IV, and VI show the blue fluorescence (405-nm excitation and 520-nm emission) resulting from the target cells fusing to the QT6 cells transfected with pcDNA3.1, pEXP-B5 gp120/HXB2 gp41 (B5), or pEXP-C5 gp120/HXB2 gp41 (C5), respectively. Fluorescent images were captured at 240 min. (B) After coinubation of the effector and target cells, the change in blue to green fluorescence was also monitored over a 240-min time course.

teins (Fig. 6A). As described below, the slight increase in cells expressing gp41 in 293T/B5 compared to 293T/C5 cells (46.9 versus 44.2% in a gated population and 10% increase in geometric mean) was unlikely to be the sole determinant of differential fusion efficiency (250% difference). We cannot rule out that 293T/B5 cultures may have had cells with a higher density of B5 gp120/gp41 and that these cells were more efficient at fusion than cells with lower gp120/gp41 density in the culture. However, the high-gp120/gp41-density cells ($>1,000$ FITC; Fig. 6A) were $<5\%$ of the gp120/gp41-positive 293T cells. As described below, the addition of more 293T/C5 cells than 293T/B5 or visa versa to the fusion assay did not alter the increase of B5- over C5-mediated cell fusion (Fig. 6E).

The 293T and zeocin-resistant 293T/B5 and 293T/C5 cells were incubated alone or in combination with U87.CD4.CCR5 cells (puromycin and G418 resistant) in the presence or absence of a selection cocktail (puromycin, G418, and zeocin)

FIG. 6. Competitive fusion assays using cells stably expressing B5 gp120/HXB2 gp41 or C5 gp120/HXB2 gp41. Stable expression of B5 gp120/HXB2 gp41 or C5 gp120/HXB2 gp41 was measured in 293T cells transfected with the pEXP vectors and then passaged under zeocin selection. (A) Expression of the *env* glycoproteins in the 293T cells was measured by intracellular gp41 staining with anti-gp41 antibody (Chessie 8) and flow cytometry (see Materials and Methods). 293T cells expressing equal amounts of B5 gp120/HXB2 gp41 or C5 gp120/HXB2 gp41 (zeocin resistant) were then mixed with the target CD4⁺/CCR5⁺ U87 cells (puromycin/G418 resistant) in the presence or absence of selection media. (B) Relative B5 and C5 gp120-mediated fusion was monitored. (C) Effects of zeocin, puromycin, and G418 selection of 293T/B5 cells at 48 h and after a PBS wash. The U87.CD4.CCR5, 293T/B5, and 293T/C5 cells added alone or together to the selection media was then removed from the plates at 48 h and after the wash. Extracted DNA was also PCR amplified with mtDNA primers (see Materials and Methods). The relative intensities of the HIV-1 and mitochondrial PCR-amplified products are plotted in panel D. Panel E shows the relative B5 to C5 gp120-mediated 293T cell fusion to CD4⁺/CCR5⁺ U87 cells when different ratios of the two effector cells were added to each experiment. The relative amounts of the B5 to C5 *env* DNA were determined by using HTA and W_D determination as described in Materials and Methods.

(Fig. 6B). DNA was extracted from cells harvested at 48 h and subjected to PCR amplification of the HIV-1 *env* DNA to be used in HTA. In the absence of the U87 target cells, the 293T, 293T/B5 gp120, and 293T/C5 gp120 cells did not survive a 48-h incubation with the selection cocktail due in part to the rapid cell killing by puromycin (37) (Fig. 6C). It is important to note that G418 and zeomycin do not have significant effects for several days. Under selection, addition of the stably *env* transformed 293T effector cells with the U87 target cells (1:10 or 1:100 effector/target ratios) only eliminated those effector cells

that did not fuse with the target cells. Killing by the selection media was verified by performing quantitative PCR for mitochondrial DNA (mtDNA) and HIV-1 DNA from cell extracts from various conditions (Fig. 6D). In the absence of selection (data not shown), both mtDNA and HIV-1 DNA was easily PCR amplified from 293T/B5 or/C5 cells. However, in the presence of the selection media (Fig. 6B), neither HIV-1 nor mtDNA could be amplified from the cells that remained on the plate after a PBS wash (Fig. 6C and D). As described earlier, U87.CD4.CCR5 cells were resistant to puromycin, whereas

zeocin had minimal or no effect on these zeocin-sensitive cells after 48 h as indicated by the efficient amplification of mtDNA (Fig. 6D). The addition of the 293T/B5 or/C5 effector to excess of U87.CD4.CCR5 did not result in a detectable increase in mtDNA (Fig. 6D). However, cell fusion was evident considering HIV-1 DNA was now readily amplified due to the survival of the 293T/B5 or/C5 cells that fused with the U87 cells (Fig. 6D). Quantitative PCR revealed a 10- to 100-fold decrease in *env* HIV-1 DNA in the 293T+U87 cell mixtures incubated in the presence compared to the absence of the selection cocktail (data not shown).

When the effector cells were mixed (1:10, 10:1, and 1:1 of 293T B5 gp120:293T C5 gp120) and added to the U87.CD4.CCR5 target cells (1:100 effector/target ratio), there was a 3.0-fold (± 1.2) increase in B5 gp120-mediated fusion over C5 gp120-mediated fusion in this competitive assay ($P < 0.0001$, Student *t* test) (Fig. 6E). The relative fusion efficiency was measured by first PCR amplifying the HIV-1 *env* DNA that survives selection and then subjecting this DNA to an HTA (as outlined in Fig. 6B). The B5 and C5 heteroduplexes, derived from these cell fusion competitions assays, migrated to the same positions on a nondenaturing gel as did the heteroduplexes derived from B5 versus C5 virus competitions. It is important to note that there was a slight increase in B5 over C5 gp120/HXB2 gp41 expression in the 293T cells, but it is unlikely that this difference could account for a threefold fusion increase in this single cycle assay. The addition of the B5 and C5 293T cells separately to U87 target cells resulted in a slight but not significant increase in B5- over C5-mediated fusion (data not shown).

Measuring the binding avidity of the parental and chimera viruses to target cells. In the cell fusion assays described above, we were unable to separate the steps of receptor binding and actual membrane fusion, both of which are mediated by the envelope glycoproteins. It is quite possible that the B5 and C5 gp120 differ in affinity for CD4 and CCR5 or in ability to mediate the gp41 conformational changes occurring prior to membrane fusion (46). Enhanced CD4/CCR5 binding could result in a fitness difference when considering (i) the difference in primary B5 and C5 HIV-1 fitness appears to map to the *env* gene (6) and (ii) that just the gp120 cloned into the NL4-3 backbone appears to encode for this fitness difference.

All viruses used in the binding assays were pelleted and purified on sucrose cushions to limit the presence of free or shed gp120. As shown in Fig. 4C, this purification did not alter the ratios of various proteins (gp120, gp41, and p24 capsid) to infectious titers between the NL4-3_{B5 gp120} and NL4-3_{C5 gp120} (Fig. 4C) or between the B5 and C5 isolates (data not shown). To test whether CD4/CCR5 binding on the cell surface plays a role in differential entry/fitness, we first incubated the parental, chimeric, and competitor (NSI/R5 B2 and SI/X4 D1) viruses with CEM-NKR-CCR5 suspension cells (CD4⁺/CXCR4⁺/CCR5⁺) or CEM-SS cells (CD4⁺/CXCR4⁺) at 15°C to allow virus to bind to cell surface receptors but prevent virus-cell fusion (Fig. 7A). We found that all viruses except the SI/X4 D1 isolate bound at least 10- to 100-fold more efficiently to the CEM-NKR-CCR5 cells than to the CEM-SS cells (Fig. 7B). In the present study, we describe the relative binding efficiency of virus to cells as "avidity" due to multiple contact sites rather than using the term "affinity," which generally describes a dis-

tinct interaction site. To measure binding avidity, CEM-NKR-CCR5 cells were preincubated with each of the parental or chimeric viruses (MOI of 0.0001) and then incubated with various concentrations of competitor viruses (NSI/R5 B2 or SI/X4 D1). Rather than performing direct but limited binding competitions between primary B5 versus C5 viruses or the chimeric B5 *env* versus C5 *env* viruses, we could assess and compare the relative binding avidity of all parental and chimeric viruses to cells when competed against a reference NSI/R5 competitor virus. After the addition of the competitor viruses (B2 or D1) at MOIs of 0.00001 to 0.0625 and rigorous washes, RNA was extracted from both lysed cells and attached virions and then subjected to RT-PCR. The amplified HIV-1 cDNA was then used in an HTA as described earlier and shown in Fig. 7C. Plotting the amount of the parental or chimeric viruses that remain bound to cells after the addition of increasing concentrations of the B2 competitor reveals that the parental B5 and NL4-3_{B5 gp120} have an avidity for the CEM-NKR-CCR5 cells that is 3.15- to 12-fold higher than that of the parental C5 and NL4-3_{C5 gp120} (Fig. 7D and E). In contrast, the SI/X4 D1 virus could not efficiently compete off either the C5 or B5 viruses from the CEM-NKR-CCR5 cells (Fig. 7C).

Differential inhibition of receptor binding and cell fusion. The same 293T effector cells stably expressing B5 or C5 gp120 were added to Ghost cells (CD4⁺/CCR5⁺ HOS cells harboring an HIV-2 LTR-GFP reporter construct) in the presence or absence of two entry inhibitors: T-20 (enfuvirtide) and PSC-RANTES. Upon cell fusion, the Tat from the 293T cells can be transported to the nucleus of the GHOST cells and drive GFP expression through the HIV-2 LTR. Due to autofluorescence, the maximal inhibition of cell fusion was 0.2 (i.e., the ratio of GFP fluorescence with maximal drug inhibition to no drug control) (Fig. 8). A low concentration of 1 nM PSC-RANTES did not inhibit cell fusion mediated by the B5 gp120/HXB2 gp41 envelope, whereas this concentration completely inhibited C5 gp120/HXB2 gp41-mediated fusion (Fig. 8B). Similar results were observed with 1 nM T-20, which blocks the formation of the gp41 six- α -helix bundle necessary for membrane fusion (Fig. 8B). Both B5 gp120- and C5 gp120-mediated fusion were almost completely blocked by the presence of 100 nM T-20. Although 100 nM PSC-RANTES completely blocked B5-mediated cell fusion (Fig. 8A), the amount of C5-mediated fusion actually increased from 0.2 to 0.46 with the addition of 1 to 100 nM of PSC-RANTES, respectively (Fig. 8B). This rebound in cell fusion due to the presence of high PSC-RANTES concentrations has been previously reported (56) and may be related to PSC-RANTES multimerization on the cell surface and an actual enhancement of viral entry, i.e., uncoupled from the inhibition effects. With the exception of the 100 nM PSC-RANTES treatment, PSC-RANTES and T-20 blocks cell fusion mediated by B5 gp120/HXB2 gp41 less efficiently than that mediated by C5 gp120/HXB2 gp41. This differential sensitivity to entry inhibitors is similar to the differences in fitness, overall entry and, more specifically, the increased avidity of the B5 viruses (parental and chimeric) over the C5 viruses for the surface of CEM-NKR-CCR5 cells (Fig. 7D).

DISCUSSION

The process of HIV-1 entry into host cells has received a renewed focus in HIV research due in part to the development of entry inhibitors, a new target for antiretroviral drugs. The benefit of these entry inhibitors is now being tested in several clinical trials, but preliminary findings already suggest that diversity in the viral envelope gene likely results in variable sensitivity to entry inhibition (25, 31, 45, 55). In the present study we examined the impact of viral entry on replication efficiency of "wild type" HIV-1 isolates. Time course competition experiments had previously suggested that the fitness of wild-type HIV-1 isolates is dominated by the efficiency of host cell entry and not by reverse transcription, integration, or transcription from the LTR. Variable entry efficiency may be related to the high diversity in the envelope gene, which is generated due to strong host immune selective pressure (47, 59). However, this is not to suggest that other steps do not vary in activity or efficiency between "wild-type" primary HIV-1 isolates. Recent studies on the HIV-1 LTRs derived from different subtypes indicate that these LTRs not only function at different efficiencies but are also influenced by the cellular environment (e.g., levels of active transcription factors) (57). However, when these LTRs of different subtypes (including B and C) were introduced into a neutral HIV-1 backbone (LAI), the different LTRs were not sufficient to result in fitness differences (57). This observation was somewhat surprising considering that substantial difference in fitness do exist among primary HIV-1 isolates. For example, primary subtype B isolates can easily outcompete primary subtype C isolates and are typically 100-fold more fit than C (6). We and others have attributed this fitness difference to the efficiency of host cell entry (6, 44) and, in the present study, we confirmed that a subtype C *env* was significantly less fit than a subtype B *env* when both were placed into a neutral NL4-3 backbone.

Creation of viral chimeras, by placing the gp120 coding region into a neutral HIV-1 backbone, indicates the contribution of different HIV-1 *env* genes to viral fitness. For this purpose, we developed a relatively rapid yeast recombination/cloning technique that is not dependent on presence of conserved restriction enzyme sites to clone HIV-1 *env*, i.e., genes that often share <75% sequence identity (Fig. 1A). Using these *env* chimeric and original parental viruses in direct time course competitions, it was clear that the gp120 coding region of the HIV-1 *env* gene was the dominant factor controlling the fitness of these viruses. Even parallel competitions involving the parental or chimeric viruses with a set of primary HIV-1 isolates

FIG. 8. Inhibition of B5 or C5 gp120-mediated cell-cell fusion by using PSC-RANTES or T-20. The 293T cells stably transformed with either pEXP-B5 gp120/HXB2 gp41 or pEXP-C5 gp120/HXB2 gp41 were added to CD4⁺/CCR5⁺ GHOST cells along with PSC-RANTES (1 or 100 nM), T-20 (1 or 100 nM), or no drug. HIV-1 Tat from the transfected 293T cells induces LTR-driven GFP expression in the Ghost cells upon cell fusion. (A) Decrease in GFP fluorescent/fused cells upon the addition of 1 or 100 nM PSC-RANTES. (B) Plot of relative GFP fluorescence in the presence compared to the absence of entry inhibitors.

indicated that the parental and *env* chimeric counterparts competed with nearly equal efficiencies. Interestingly, the small fitness differences that were observed with competitions involving the parental and respective *env* chimeric virus may be due to the relatively minor contributions of other regions of the HIV-1 genome, e.g., LTR (57). These results would further confirm the notion that HIV-1 entry efficiency may be dominant in controlling fitness but not the only factor that would have an effect.

These initial experiments may have established the role of entry in fitness but have not defined the mechanism(s) involved in differential entry efficiencies. Increased cell fusion mediated by NL4-3B5 gp120 over that mediated by the NL4-3C5 gp120 provides again direct evidence for the importance of the entry/fusion processes in viral fitness. To prevent cell fusion and examine only the virus binding avidity to host cells, we performed competitive binding assays with whole virus particles. This novel technique revealed that the differences in entry efficiency were likely due to greater binding avidity of CD4/

FIG. 7. Using CD4⁺/CCR5⁺ cells to measure the binding avidity of parental and *env* chimeric viruses. A competitive binding assay was performed by using the B5 and C5 parental and *env* chimeric viruses purified on sucrose cushions (Fig. 4C). Each of these viruses (MOI of 0.0001) were preincubated with CEM-NKR-CCR5 (CD4⁺/CCR5⁺/CXCR4⁺) or CEM-SS (CD4⁺/CXCR4⁺) cells and then competed off with either an NSI/R5 HIV-1 isolate (B2-92BR017) or an SI/X4 HIV-1 isolate (D1-92UG021) at increasing concentrations (MOI of 0.0001 to 0.0625). (A) Binding assay schematic diagram. (B) Binding of the NSI/R5 HIV-1 isolates or the SI/X4 strain (D1) to both CEM-NKR-CCR5 or CEM-SS cells at 15°C in the absence of competitor was measured by a quantitative reverse transcription-radioactive PCR assay. The viral RNA from each virus (sample virus and competitor) remaining on cells after washing was RT-PCR amplified and used in an HTA (see Materials and Methods). (C) HTAs performed on competitions involving B5, C5, NL4-3B5 gp120, and NL4-3C5 gp120 bound to CEM-NKR-CCR5 cells and then competed off with B2 virus. The lower HTA in panel C displays the inability of the SI/X4 D1 virus to compete off the NL4-3B5 gp120 virus from CEM-NKR-CCR5 cells. The amounts of each parental or chimeric virus relative to the increased competitor virus (x axis) were plotted, and examples of NL4-3B5 gp120 and NL4-3C5 gp120 binding curves are shown in panel D. The binding constants (K_d), derived from each of these binding competitions curves (e.g., panel D) are shown in panel E.

CCR5 by the B5 viruses. These differences in binding avidity were also directly related to the sensitivity of a receptor binding/cell fusion event to an R5 entry inhibitor (PSC-RANTES) and fusion inhibitor (T-20) (Fig. 8). We and others have previously shown that sensitivity to R5 entry inhibitors may be closely related to HIV-1 fitness, entry efficiency, and more specifically, to CCR5 binding (34, 45, 46, 55). The gp120 region, and more specifically, the V3 loop has been implicated in determining sensitivity to entry inhibitors (19, 29). It is important to stress that only the gp120 coding regions of the B5 and C5 viruses were introduced into these chimeric viruses, suggesting that differential T-20 sensitivity was not related to the HXB2 gp41 sequences. Thus, T-20 sensitivity appears to be partially linked to gp120 affinity for the CCR5 receptor, suggesting that CCR5 binding may be the rate-limiting step in the entry process (45).

The impact of host cell entry on HIV-1 fitness (or replication efficiency) has significant implication for a variety of HIV-related research areas. It should be stressed that a 100-fold increase in relative replication efficiency between primary wild-type HIV-1 isolates may also translate to intrinsic resistance to these entry inhibitors (42, 55). Aside from this relationship with exogenous drug pressures, the fitness of the *env* gene in the context of the entire HIV-1 isolate may also be a key to HIV-1 evolution during disease progression and global expansion (42). Increasing HIV-1 fitness (e.g., ex vivo replication efficiency) during HIV-1 disease appears to correlate with markers of progression, e.g., decreasing CD4 cell counts and increasing viral loads (7, 9, 10, 43; R. Troyer and E. J. Arts, unpublished data). Phylogenetic studies revealed that this increasing fitness during disease was related to specific changes in the V3 loop sequence (51) (Troyer and Arts, unpublished). These results predict that in the absence of therapy we may be able to eventually identify specific *env* sequence arrangements that are associated with both differential fitness and rates of disease progression. In the presence of antiretroviral therapy, the emergence of drug resistance due to mutations in these coding regions comes at a cost to the virus (8, 24, 38, 62). However, the fitness of drug resistance mutations in PR-RT are typically not measured in the context of the entire primary HIV-1 isolates (42). Thus, the role of entry on the fitness of HIV-1 isolates harboring drug resistance mutations in PR-RT is still poorly understood.

HIV-1 entry and fitness may also play a role in HIV-1 transmission, spread in the human population, and global evolution. Regardless of the human ethnicity of the host cell, subtype C HIV-1 isolates are significantly less fit in terms of relative replication efficiency than any other group M isolates (e.g., subtypes A, B, D, and E) (6), and yet subtype C now dominates the worldwide epidemic. We are now examining the possibility that subtype C attenuation may be driving this global expansion. The poor relative fitness of subtype C HIV-1 isolates appears to be related to the efficiency of host cell entry and maps to the HIV-1 *env* gene (6). Why a dominating subtype would harbor an *env* gene that mediates poor entry efficiency is not clear but, interestingly, the subtype C *env* gene is even retained in almost all circulating intersubtype recombinant HIV-1 forms (CRF) isolated in different parts of the world (39). For example, subtype C *env* genes are found in CRF07_BC and CRF08_BC from China and in CRF10_CD

from Tanzania (27, 28, 40, 52). In the case of CRF10, the gp120 coding region is derived from subtype C and gp41 from subtype D, suggesting a functional complementation between these divergent subunits similar to that observed in our NL4-3_{B5} (or C5) gp120 viruses.

In conclusion, we have found that the fitness difference among primary wild-type HIV-1 isolates is directly related to efficiency in host cell entry. By producing chimeric *env* viruses with common HIV-1 genetic backbones, we have confirmed that only the gp120 region of the HIV-1 *env* gene was necessary to recapitulate the fitness of the entire parental primary HIV-1 strain. Furthermore, it appears that entry efficiency among wild-type HIV-1 strains may be distilled down to the relative binding avidity of the virus to the host cell. In other words, the ability of primary HIV-1 isolates to bind to CD4/CCR5 may predict its competitive fitness or replication efficiency in comparison to other wild-type strains.

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