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The Selection of Low Envelope Glycoprotein Reactivity to Soluble CD4 and Cold during Simian-Human Immunodeficiency Virus Infection of Rhesus Macaques

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Envelope glycoprotein (Env) reactivity (ER) describes the propensity of human immunodeficiency virus type 1 (HIV-1) Env to change conformation from the metastable unliganded state in response to the binding of ligands (antibodies and soluble CD4 [sCD4]) or incubation in the cold. To investigate Env properties that favor *in vivo* persistence, we inoculated rhesus macaques with three closely related CCR5-tropic simian-human immunodeficiency viruses (SHIVs) that differ in ER to cold (ER_{cold}) and ER to sCD4 (ER_{sCD4}); these SHIVs were neutralized by antibodies equivalently and thus were similar in $ER_{antibody}$. All three SHIVs achieved high levels of acute viremia in the monkeys without alteration of their Env sequences, indicating that neither ER_{cold} nor ER_{sCD4} significantly influences the establishment of infection. Between 14 and 100 days following infection, viruses with high ER_{cold} and ER_{sCD4} were counterselected. Remarkably, the virus variant with low ER_{cold} and low ER_{sCD4} did not elicit a neutralizing antibody response against the infecting virus, despite the generation of high levels of anti-Env antibodies in the infected monkeys. All viruses that achieved persistent viremia escaped from any autologous neutralizing antibodies and exhibited low ER_{cold} and low ER_{sCD4}. One set of gp120 changes determined the decrease in ER_{cold} and ER_{sCD4}, and a different set of gp120 changes determined resistance to autologous neutralizing antibodies. Each set of changes contributed to a reduction in Env-mediated entry. During infection of monkeys, any Env replication fitness costs associated with decreases in ER_{cold} and ER_{sCD4} may be offset by minimizing the elicitation of autologous neutralizing antibodies.

Human immunodeficiency virus type 1 (HIV-1) is the cause of AIDS, which results from the depletion of CD4-positive T lymphocytes (1, 2). Infection of HIV-1 target cells is mediated by the trimeric viral envelope glycoprotein (Env) spike. The Env spike is composed of three protomers consisting of two subunits (gp120 and gp41) that are generated by cleavage of a gp160 precursor glycoprotein (3–5). HIV-1 Env mediates entry into $CD4^+$ T lymphocytes by first engaging the receptor CD4, which initiates a conformational change in gp120 that allows the binding to chemokine receptors, typically CCR5 or CXCR4 (6–16). The gp120 interactions with CD4 and CCR5/CXCR4 also trigger conformational changes in gp41 that promote membrane fusion and virus entry (5, 17). This two-receptor system allows HIV-1 to bury vulnerable Env elements until engagement with the target cell, thus providing a steric mechanism to escape immune recognition by potentially neutralizing antibodies (18).

Neutralizing antibodies are elicited during natural HIV-1 infection. Most early-arising neutralizing antibodies are strain restricted and drive the evolution of Env changes that allow virus escape (19). More broadly neutralizing antibodies are elicited in only a minority of HIV-1-infected people, usually arising several years after the initiation of infection (20–23). Although passive administration of broadly neutralizing antibodies can prevent immunodeficiency virus infections in animal models (24–28), the contribution of neutralizing antibodies to the control of viremia in an established infection and their impact on disease progression are unknown. HIV-1 variants clearly escape early autologous neutralizing antibodies, but the impact of the accumulating escape-associated Env changes on viral fitness is still uncertain. Evidence has been presented that donor neutralization escape phenotypes revert in recipients upon transmission, suggesting that potential fitness costs associated with autologous escape are selected against in the absence of neutralization pressure in the new hosts (29–32). Likewise, virus escape mutants that arose in HIV-1-infected individuals passively treated with broadly neutralizing antibodies reverted after antibody levels diminished (33). On the other hand, another study found little evidence that replication rates of neutralization-resistant viruses consistently differ from those of early or transmitted/founder viruses from the same individual (34). Thus, the contribution of the neutralizing antibody response to the control of an already established HIV-1 infection remains unclear.

Chimeric simian-human immunodeficiency viruses (SHIVs) have been used to investigate the role of HIV-1-specific proteins in virus evolution, interaction with the host immune system, and pathogenesis (35–41). Although simian immunodeficiency virus SIVmac infection of rhesus macaques exhibits many parallels with HIV-1 infection of humans, the HIV-1 and SIVmac Envs differ in the degree of dependence on the CD4 receptor for entry, in the use

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of alternative coreceptors (besides CCR5), and in the composition of most neutralizing antibody epitopes (42). Therefore, SHIVs with both CCR5-using (R5), CXCR4-using (X4), and dual-tropic (R5X4) HIV-1 Envs have been created and studied (40, 43–48). The vast majority of transmitted/founder and early isolates of HIV-1 are R5 and infect the activated subset of CD4-positive T lymphocytes; in some HIV-1 subtypes, R5X4 or X4 variants that can infect naive T lymphocytes arise later in the course of infection (49–53). Some R5X4 and X4 SHIVs are efficient pathogens, causing rapid CD4-positive T-lymphocyte depletion and AIDS-like illness in nearly all infected monkeys (43, 54, 55); however, the rapidity with which the host immune system is compromised in these animals often results in the lack of generation of neutralizing antibodies (36, 39, 56). Therefore, infection of monkeys with R5 SHIVs is thought to mimic early HIV-1 infection more closely with respect to target cells, the pattern and timing of antiviral immune responses, and immunopathogenesis. However, R5 SHIVs are inconsistent with respect to establishing persistent infections that lead to immunodeficiency and AIDS-like illness. An understanding of the HIV-1 Env properties that determine *in vivo* consistency would expedite the development of R5 SHIVs with diverse transmitted/founder HIV-1 Envs for vaccine and other prevention studies.

HIV-1 Env has evolved several features (variable surface loops, carbohydrates, and structural lability) that minimize the generation and impact of potentially neutralizing antibodies. Both the trimer apex, where the gp120 V1/V2 variable regions are thought to reside, and the gp120 outer domain can be targeted by neutralizing antibodies with various degrees of breadth, and both regions are shielded by glycans (57, 58). The Env trimer apex "opens" upon CD4 engagement, consistent with the V1/V2 stem changing conformation to form the gp120 bridging sheet that, along with the V3 loop, engages the coreceptor (59, 60). The propensity ("intrinsic Env reactivity" [ER]) of HIV-1 Env variants to change their conformation from the unliganded state globally influences viral sensitivity to inactivation by neutralizing antibodies, soluble CD4 (sCD4), CD4-mimetic compounds, and prolonged exposure to cold (61–63). ER is inversely related to the magnitude of the activation energy barrier between the unliganded state of Env and lower-energy downstream states.

Although HIV-1 Env properties related to ER have been studied in tissue culture assays (61–63), their contribution to the establishment and persistence of infections *in vivo* is not understood. Here, we study the infection of rhesus macaques inoculated with three closely related R5 SHIVs. Two of the R5 SHIVs (SHIV-KA and SHIV-KY) were created by grafting the tropism-determining gp120 V3 loops from the R5 HIV-1_{ADA} and HIV-1_{YU2} strains, respectively, into the dual-tropic, pathogenic SHIV-KB9 (44). The third R5 SHIV, SHIV-KY62, was derived from a rhesus macaque that had been inoculated 62 weeks earlier with SHIV-KY. These three SHIVs were found to be equally sensitive to neutralization by antibodies. However, the infectivity of SHIV-KA and SHIV-KY was inhibited more effectively by sCD4 binding and incubation in the cold than that of SHIV-KY62. These results corroborate previous observations (64, 65) suggesting that some HIV-1 Env variants exhibit global sensitivity to cold, sCD4, and antibodies, whereas other Env variants exhibit specific sensitivity to one or two of these agents. Here, we use ER to refer to global Env reactivity to multiple agents and introduce the terms ER_{cold}, ER_{sCD4}, and $ER_{antibody}$ to indicate Env reactivity to each of these specific agents. A high level of global ER implies that a virus variant exhibits high levels of ER_{cold}, ER_{sCD4}, and $ER_{antibody}$. Thus, relative to SHIV-KY62, SHIV-KY and SHIV-KA exhibit increased ER_{cold} and ER_{sCD4} but are comparable in $ER_{antibody}$ and ER. This provides an opportunity to investigate the specific contribution of ER_{cold} and ER_{sCD4} to the efficiency of SHIV infection in monkeys. We examined the evolution in monkeys of Env phenotypes such as *in vitro* fitness and sensitivity to neutralizing antibodies, sCD4, and cold. The *env* genetic determinants of these properties were mapped. The results provide insights into the properties of Env that allow the establishment of persistent infection in monkeys and reveal differences between these *in vivo* fitness requirements and Env properties that dictate replication efficiency in tissue culture systems.

MATERIALS AND METHODS

Cells and virus. Rhesus monkey peripheral blood mononuclear cells (PBMC) were isolated by Ficoll enrichment from 10 ml of blood-EDTA. Cells were stimulated for 72 h with 1 to 2 μg/ml of phytohemagglutinin (PHA) (Sigma-Aldrich) and cultured in the presence of 10% interleukin-2 (IL-2) (Hemagen Diagnostics) in RPMI plus 10% fetal bovine serum (FBS). $CCR5^+$ CEMx174 5.25 M7 cells were a kind gift from Nathaniel Landau and were cultured in RPMI plus 10% FBS. $CD4^+$ $CCR5^+$ and $CD4^+$ $CXCR4^+$ Cf2Th cells were cultured with Dulbecco's modified Eagle's medium (DMEM) plus 10% FBS in the presence of 400 μg/ml G418 and 150 μg/ml hygromycin (66). Ghost cell lines expressing CD4 and several chemokine receptors were cultured in DMEM plus 10% FBS supplemented with 500 μg/ml G418, 100 μg/ml hygromycin, and 1 μg/ml puromycin (from Vineet N. KewalRamani and Dan R. Littman, NIH AIDS Research and Reference Reagent Program) (67). 293T, COS-1, and HOS cells were cultured in DMEM plus 10% FBS. Penicillin-streptomycin was added to all culture media. Cells were grown at 37°C with 5% CO_2.

SHIV-KA and SHIV-KY were constructed by introducing the *env* sequences encoding the gp120 V3 loop from HIV-1_{ADA} and HIV-1_{YU2}, respectively, into the *env* sequence in the 3′ KB9 proviral clone (44). Viruses were generated by ligation of 5′ and 3′ proviral halves, as previously described for SHIV-KB9 (68). Briefly, 10 μg of 5′ KB9 and either 3′ KA or 3′ KY was digested with SphI (New England BioLabs) for 3 h before ligation with 2,000 U of high-concentration T4 DNA ligase (New England BioLabs) for 5 min at room temperature. Ligation products were immediately transfected by using Lipofectamine 2000 (Invitrogen) into HEK293T cells. Viral supernatants were harvested after 24 h, passed through a 0.45-μm filter, and transferred to CEMx174 5.25 M7 cell cultures for 24 h. Virus was removed from cultures after 24 h, and the medium was replaced with fresh RPMI plus 10% FBS. Viral infection was monitored by observing green fluorescent protein (GFP) expression in the cells under a fluorescence microscope and by assaying reverse transcriptase (RT) activity in cell supernatants for several days. Peak RT activity was typically detected 10 to 14 days after infection, and virus was harvested at this time. Cells were removed by centrifugation and 0.45-μm filtration of the virus-containing supernatant.

Single-round reporter viruses were produced by transfection of HEK293T cells seeded onto 10-cm^2 plates with 2 μg of a packaging plasmid (pCMVΔP1ΔenvpA), 6 μg of an HIV-1 long terminal repeat (LTR)-driven luciferase reporter plasmid (pHIvec2.luc), and 2 μg of an Env expression plasmid (pSVIIIenv), using Lipofectamine 2000. Viral supernatants were harvested after 48 h, passed through a 0.45-μm filter, frozen in a dry ice-ethanol bath, and stored at −80°C. Virus stocks were normalized for RT activity prior to use.

Animals. PBMC from Mamu A01-negative rhesus macaques were prescreened for susceptibility to infection by SHIV-KA, SHIV-KY, and SHIV-KY62. Approximately 1 million Ficoll-enriched PBMC were infected with 50,000 RT units of virus following PHA stimulation. Virus was removed after 24 h, and RT activity in the culture supernatants was mon-

itored for 17 days. The monkeys whose PBMC supported the highest level of *in vitro* replication were chosen for inoculation. Two animals were intravenously inoculated with approximately 5,000,000 RT units of each virus. Blood samples were drawn from each animal twice weekly for the first 2 weeks, weekly for weeks 2 to 4, biweekly for weeks 4 to 10, and monthly after week 10.

Antibodies and soluble CD4. The IgG1 b12 and b6 antibodies, which recognize the CD4-binding site of gp120; the PG9 antibody, which recognizes a quaternary structure-dependent epitope; and PGT128, which recognizes a carbohydrate-dependent gp120 epitope, were kind gifts from Dennis Burton (57, 58, 69, 70). The F105 and VRC01 antibodies, which also recognize the gp120 CD4-binding site, were kindly provided by Marshall Posner and John Mascola, respectively (71, 72). James Robinson provided the 17b antibody, which recognizes a CD4-induced gp120 epitope, and the 39F antibody, which binds to the gp120 V3 region (73). The 2G12 antibody, which targets a carbohydrate-dependent gp120 epitope, and the 4E10 antibody, which recognizes the membrane-proximal external region (MPER) of gp41, were provided by Hermann Katinger (74, 75). The anti-CD4 antibody OKT4 was purchased from eBioscience.

sCD4 was purified from transfected HEK293F supernatants, as previously described (76), and quantified by Bradford analysis. The anti-HIV-1 activity of each sCD4 preparation was normalized to that of earlier stocks by comparative sCD4 sensitivity assays.

Plasmids and site-directed mutagenesis. All envelope glycoprotein expression vectors were derived from pSVIIIenv-KB9, which contains the entire KB9 *env* and *rev* genes flanked by HIV-1_{HXB2} LTR elements (68). The Asp718-BamHI fragments of some single-genome amplicons of SHIV-KA and SHIV-KY from days 70 to 98 after inoculation of monkeys (see below) were subcloned into pSVIIIenv-KB9. Other amplicons from days 154 to 434 after infection exhibited changes in regions outside the Asp718-BamHI fragment and required complete *env* sequence cloning by introducing SphI and BclI sites at the 5′ and 3′ extremities, respectively, of *env*. Digested fragments were ligated with 2,000 U of high-concentration T4 DNA ligase (New England BioLabs) for 5 min at room temperature.

Mutations specifying single-amino-acid changes were introduced into the pSVIIIenv-KY or relevant plasmids according to the QuikChange II XL site-directed mutagenesis protocol (Stratagene). All plasmids were transformed into Top10 cells (Invitrogen), cultured at 37°C, and completely sequenced.

Viremia measurements. The numbers of viral RNA (vRNA) copies per ml of plasma were quantified by using an ultrasensitive branched DNA detection assay with a detection limit of 125 RNA copies per ml (Siemens). For monkeys 331-08 and 401-08, viral loads were determined by using a Qiagen QIAsymphony Virus/Bacteria Midi kit and QIAgility Applied Biosystems StepOne quantitative real-time PCR (CHAVI Viral Core Laboratory, Duke Human Vaccine Institute, Durham, NC); viral loads in these two monkeys were always well above the detection limit of the assay (250 to 500 copies/ml).

Single-genome analysis. Amplification of single *env* segments was modified slightly from a previously described method (77). Briefly, viral RNA was isolated from infected plasma by using a QiaAmp kit (Qiagen) and eluted with AVE buffer diluted 1:5 with nuclease-free water. cDNA was generated from vRNA templates by using 0.24 μM reverse primer 1 (SHIV U3/R [CAGAGCGACTGAATACAGAGCG]) and Superscript III (Invitrogen), as recommended by the polymerase manufacturer. Limiting dilutions were applied to cDNA templates for the first round of amplification to provide an average of a single DNA copy per reaction in a 96-well plate. Each 20-μl PCR mixture included 0.2 mM deoxynucleoside triphosphate (dNTP), 2 mM $MgSO_4$, and 0.5 U of High Fidelity Platinum *Taq* polymerase (Invitrogen). Primers used for the first round of amplification were 0.25 μM primer 2F (GTAGCATTAGTAGTAGCAAT) and SHIV U3/R, which anneal to positions 6203 and 9673 of the SHIV-KB9 genome, respectively. Templates were amplified through 35 cycles of 94°C for 15 s, 55°C for 30 s, and 68°C for 4 min. Second-round PCR products were amplified from 2 μl of first-round PCR products using 0.5 μM primers 4F (GACAGGTTAATTGATAGACTA) and 1R (TGGGTTTCTC CATGGAGT), which anneal to positions 6293 and 9000, respectively, through 45 cycles of 94°C for 15 s, 58°C for 30 s, and 68°C for 3 min. A positive ratio of 1:3 or less decreases the likelihood of multiple template amplification, thereby eliminating founder bias and recombination via primer-template switching during PCR (77). Each amplicon was purified by using the Qiagen PCR purification kit before sequencing.

Single-round infectivity assays. Cf2Th or GHOST cells expressing CD4 and the coreceptor were plated at a density of 6,000 cells/well in a 96-well flat-bottom luminometer plate (PerkinElmer) 24 h prior to infection with HIV-*luc* pseudovirus. Viruses were prepared at 50,000 or 150,000 RT units/ml and serially diluted 1:2 with media to deliver 1,250 to 10,000 or 3,750 to 30,000 RT units/well in a total volume of 200 μl/well. Each infection was performed twice in triplicate. Cells were lysed with passive lysis buffer (Promega) after 48 h, and luciferase activity was measured in a luminometer (Berthold), as previously described (61, 64).

Assays to measure virus inhibition by autologous plasma, monoclonal antibodies, sCD4, and cold. $CD4^+$ $CCR5^+$ Cf2Th cells were preplated for infection, as described above. The HIV-*luc* pseudovirus, normalized by RT, and heat-inactivated plasma, monoclonal antibody, or soluble CD4 were preincubated for 1 h at 37°C prior to infection. For autologous neutralization assays, 5,000 RT units/well of virus was preincubated with a 1:50 dilution of heat-inactivated plasma. Neutralization by a single dose of broadly acting monoclonal antibodies was performed with 10 μg/ml of 2G12, b12, VRC01, 4E10, 17b, or 39F and 5 μg/ml of PGT128. Virus was exposed to 0 to 20 μg/ml of sCD4 prior to infection for 1 h at 37°C. Virus was tested for cold sensitivity by incubation on ice for either 0, 24, 48, and 96 h or 0, 2, 4, 8, 16, and 24 h before infection. All infected cells were incubated at 37°C for 48 h and assayed for luciferase activity, as described above.

Env expression, processing, and recognition by autologous and heterologous plasma antibodies. Env variants were expressed in HEK293T cells by transfection of pSVIIIenv and pTat at a ratio of 2:1 using Lipofectamine 2000 (Invitrogen). After 24 h, cells were radiolabeled with 100 μCi/ml of ^{35}S protein-labeling mix (PerkinElmer) following depletion with cysteine- and methionine-free DMEM supplemented with 10% dialyzed FBS (Gibco Invitrogen). Cell-associated Env was harvested by NP-40 lysis of cells 24 h after labeling. Env was immunoprecipitated from 100 μl of cleared lysates with 3 μl of a mixture of polyclonal sera from HIV-1-infected individuals or inactivated plasma from the infected monkeys along with protein A-Sepharose beads (Roche) and washed three times with NP-40 buffer. Steady-state levels of expressed Env were determined by resolving gp160 and gp120 on a 10% Bis-Tris denaturing polyacrylamide gel (Invitrogen) run with morpholinepropanesulfonic acid (MOPS) buffer. Gels were visualized by PhosphorImager scanning (GE Healthcare Life Sciences).

Assays measuring sCD4 binding to the Env trimer. Assays to measure soluble CD4 binding to trimeric Env complexes on cell surfaces were performed as previously described (61, 64). Briefly, plasmid pSVIIIenv was cotransfected with the HIV-1 Tat-expressing plasmid pTat into COS-1 or HOS cells. After 3 days, cells were washed with blocking buffer before incubation with sCD4 or the 2G12 anti-Env antibody (for normalization of Env expression) for 45 min at room temperature and then washed again with blocking buffer. Cells were incubated with an anti-human IgG or anti-CD4 (OKT4) antibody conjugated with horseradish peroxidase (HRP) for 45 min at room temperature and washed several times with buffer. HRP activity was measured in a luminometer after addition of Western Lightning oxidizing and luminol reagents (PerkinElmer Life Sciences).

The sCD4 reactivity of Env (ER_{sCD4}) was calculated by using the formula $\text{sCD4 reactivity} = \dfrac{1}{IC50_{sCD4} \times (\text{sCD4 binding to Env trimer})}$, where $IC50_{sCD4}$ is the 50% inhibitory concentration of sCD4.

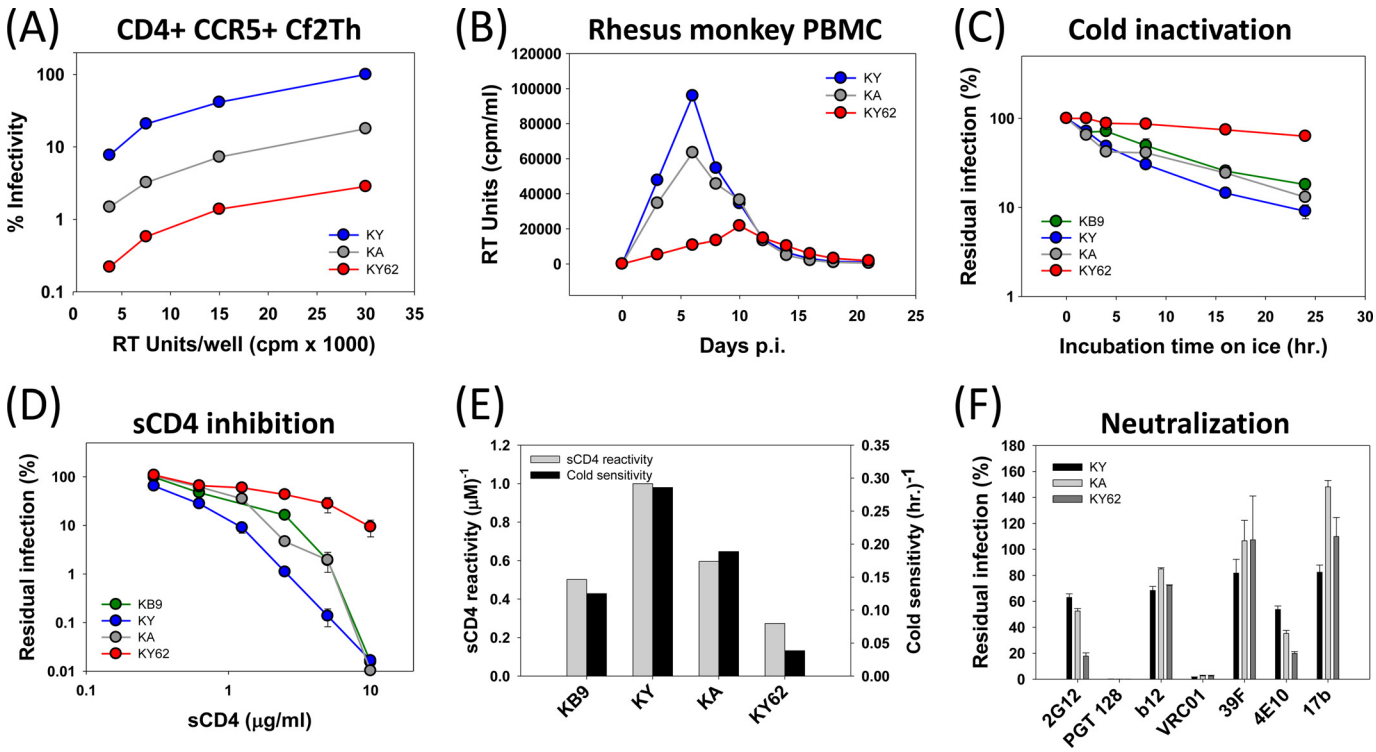

FIG 1 Infectivity and ER-related properties of SHIV Envs. All virus preparations were normalized by reverse transcriptase (RT) activity and used to test infectivity and sensitivity to inhibition. (A) Infectivity of KY, KA, and KY62 Env-pseudotyped reporter viruses was tested in $CD4^+$ $CCR5^+$ Cf2Th cells. The Cf2Th $CD4^+$ $CCR5^+$ cells were lysed 48 h after infection and assayed for luciferase activity. All values are expressed as percent infection at a given virus level compared to the value observed for the maximum input of the KY virus. (B) The infectivity of full-length SHIVs was tested in PBMC pooled from three rhesus macaques. Cell supernatants were collected at the times indicated and assayed for RT activity. (C to F) Cold inactivation (C and E), sCD4 inhibition (D and E), and neutralization sensitivity (F) experiments were performed by using reporter viruses pseudotyped with the KB9, KY, KA, or KY62 Envs. (C) Single-round reporter viruses were incubated on ice for 0 to 24 h before infection. (D and F) Viruses were preincubated at 37°C with 0 to 20 µg/ml of sCD4 (D) or with 10 µg/ml of 2G12, b12, 39F, 17b, 4E10, or VRC01 or 5 µg/ml of PGT128 (F) 1 h before infection. All infections were performed in triplicate with Cf2Th $CD4^+$ $CCR5^+$ target cells, using RT-normalized levels of input virus. Cells were lysed after 48 h and evaluated for luciferase activity. Error bars represent standard errors of the means from two separate experiments. (E) sCD4 reactivity and cold sensitivity of the indicated SHIV Envs are shown. sCD4 reactivity was calculated as described in Materials and Methods and normalized to the ER_{sCD4} of viruses pseudotyped with KY Env, which was set at a value of 1. Cold sensitivity was calculated as the reciprocal of the time (IT_{50}) on ice required to inactivate 50% of the viral infectivity.

RESULTS

Tropism and replicative abilities of the SHIVs. The SHIVs used in this study were derived from the dual-tropic, pathogenic SHIV-KB9 (44). The tropism-determining gp120 V3 regions from the R5 primary viruses HIV-1_{ADA} and HIV-1_{YU2} replace the SHIV-KB9 gp120 V3 region in SHIV-KA and SHIV-KY, respectively. The *env* gene of SHIV-KY62 was derived from a virus isolated 62 weeks after the infection of a monkey with SHIV-KY. The Envs of SHIV-KA, SHIV-KY, and SHIV-KY62 were tested for the ability to infect $CD4^+$ GHOST cell lines expressing different chemokine receptors, including CXCR4, Bob/Gpr15, Bonzo/Strl33, CCR1, CCR2b, CCR3, CCR4, CCR5, CCR8, and CX3CR1. Only the $CD4^+$ $CCR5^+$ cells were infected by viruses bearing the SHIV-KA, SHIV-KY, and SHIV-KY62 Envs (data not shown). Thus, the substitution of the SHIV-KB9 gp120 V3 region with the V3 region of the R5 HIV-1 isolates resulted in SHIVs that utilize CCR5 but not CXCR4 as a coreceptor.

The ability of the SHIV Envs to mediate virus infection was tested. In single-round infections, recombinant HIV-1 with the KY Envs infected cells more efficiently than viruses with the KA Envs, and these were more efficient than viruses with the KY62 Envs (Fig. 1A). Multiple-round replications of SHIV-KA, SHIV-KY, and SHIV-KY62 in $CD4^+$ $CCR5^+$ CEMx174 5.25 M7 cells and in rhesus macaque PBMC were compared. In these cells, SHIV-KY replicated equivalently to or slightly better than SHIV-KA; both SHIV-KY and SHIV-KA replicated more efficiently than SHIV-KY62 (Fig. 1B and data not shown). Thus, in multiple assays, the Envs from passaged SHIV-KY62 supported virus replication less efficiently than the Envs of either SHIV-KY or SHIV-KA.

Properties related to ER of SHIVs. Global changes in intrinsic Env reactivity (ER) of HIV-1 Env influence HIV-1 sensitivity to cold, soluble CD4, and antibodies (61). Thus, a high-ER Env will, by definition, exhibit high levels of ER_{cold}, ER_{sCD4}, and $ER_{antibody}$. Specific changes in ER_{cold}, ER_{sCD4}, and $ER_{antibody}$ are also possible for individual HIV-1 Env variants that are not globally reactive (64, 65). To evaluate properties of the parental SHIV-KB9, SHIV-KA, SHIV-KY, and SHIV-KY62 related to ER, recombinant viruses pseudotyped with the Envs were tested for infectivity following incubation on ice and following incubation with sCD4 and neutralizing antibodies. The susceptibility to inactivation in the cold (ER_{cold}) exhibited the rank order KY > KA > KB9 ≫ KY62 (Fig. 1C).

Env reactivity to sCD4 (ER_{sCD4}) reflects the inhibition of HIV-1 infectivity by sCD4 relative to the binding of sCD4 to the

trimeric Env complex (61). Recombinant viruses with the KY and KA Envs as well as parental KB9 Env were more sensitive to sCD4 than were viruses with KY62 Env (Fig. 1D). When sCD4 binding to the Env complex was measured, the sCD4 reactivity of each Env could be calculated and exhibited the rank order KY > KA > KB9 > KY62 (Fig. 1E). As has been previously observed (61), cold sensitivity and sCD4 reactivity correlated (Fig. 1E).

Increases in ER result in globally increased HIV-1 sensitivity to neutralizing antibodies (64). Viruses pseudotyped with the KB9, KA, KY, and KY62 Envs were neutralized comparably by a number of monoclonal antibodies directed against gp120 or gp41 (Fig. 1F). Viruses with KY62 Env were slightly more sensitive than the other viruses to the glycan-directed anti-gp120 antibody 2G12 and to the MPER-directed anti-gp41 antibody 4E10. All four viruses were resistant to the weakly neutralizing anti-CD4BS antibody F105 or b6 (70, 71) and to the quaternary structure-dependent antibody PG9/PG16 (due to a lack of a critical glycosylation site at asparagine 160 [data not shown]) (58, 78). Thus, these viruses exhibited comparable sensitivity to neutralizing monoclonal antibodies, suggesting that the KB9, KA, KY, and KY62 Envs have similar levels of global intrinsic reactivity (ER) and $ER_{antibody}$.

The parental virus for these studies, SHIV-KB9, is highly pathogenic and was derived by serial passage of the less virulent SHIV-89.6 in rhesus macaques (44). The Env ectodomains of SHIV-KB9 and SHIV-89.6 differ by only 12 amino acid residues. We compared 89.6 and KB9 Env properties related to ER. Sensitivity to sCD4 and CD4-binding affinity, and therefore ER_{sCD4}, are similar for KB9 and 89.6 (64). Viruses with the KB9 and 89.6 Envs exhibited comparable sensitivity to most neutralizing antibodies, with the exception of IgG1 b12 and AG1121 (38). Thus, the KB9 and 89.6 Envs exhibit similar global ER and $ER_{antibody}$. However, the cold sensitivities of viruses with these Envs differed significantly, with the average half-life on ice being >10-fold higher for KB9 than for 89.6 (Fig. 2A and B). The Env residues responsible for this difference in ER_{cold} mapped to residues 151 (V1), 225 (C2), and 305 (V3) (Fig. 2A and B). Of note, residues 225 and 305 also contribute to the increased virulence and syncytium-forming ability of KB9 relative to those of 89.6 (79). As was observed for SHIV-KY and SHIV-KY62, passage of SHIV-89.6 in monkeys produced a virus, SHIV-KB9, with a significant reduction in ER_{cold}.

The Env properties of the SHIVs are summarized in Fig. 2C. The KY and KA Envs exhibited higher ER_{sCD4} and ER_{cold} than did parental KB9 Env. Chimerism in the V3 loop of SHIV-KY and SHIV-KA presumably contributed to the observed increases in ER_{sCD4} and ER_{cold}. Infection of a monkey with SHIV-KY resulted in a virus, SHIV-KY62, with decreases in both ER_{sCD4} and ER_{cold}. Likewise, after monkey passage, SHIV-89.6 with a high ER_{cold} gave rise to SHIV-KB9, with a significantly reduced ER_{cold}. The ER_{sCD4} of 89.6 is low and did not change upon *in vivo* passage in monkeys. Thus, high levels of ER_{cold} and ER_{sCD4} are apparently counterselected *in vivo*. This implies that low ER_{cold} and ER_{sCD4} are advantageous in the host, even when global neutralization sensitivity and ER are similar (as is the case for all five SHIVs in this study).

Infection of rhesus monkeys with SHIV variants. To test the hypothesis that low ER_{cold} and ER_{sCD4} values are advantageous *in vivo* and to investigate the coevolution of the virus and host immune responses, SHIV-KY62 was inoculated intravenously into each of two rhesus macaques. We retrospectively studied the infection of four monkeys, two inoculated intravenously with the same dose of SHIV-KY and two inoculated with SHIV-KA. All six

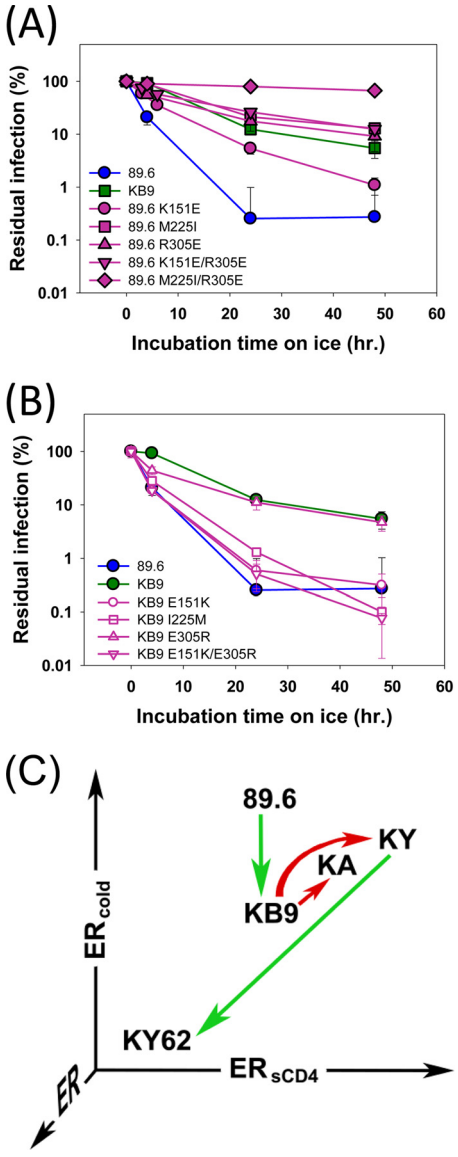

FIG 2 Env determinants of differences in cold sensitivity between SHIV-89.6 and SHIV-KB9. (A and B) Residues in 89.6 Env that differ from those in KB9 Env were altered, alone or in combination, to those found in KB9 Env and vice versa. Recombinant luciferase-expressing HIV-1 pseudotyped with the designated Envs was incubated on ice for the indicated times, and infectivity was determined following incubation with Cf2Th $CD4^+$ $CCR5^+$ cells. Experiments were performed as indicated in the Fig. 1 legend. (C) The approximate ER_{cold} and ER_{sCD4} values of the SHIV Envs used in this study are plotted. The red arrows indicate phenotypic changes that resulted from modifications of Env introduced in the laboratory. The green arrows indicate phenotypic changes that arose during passage of the virus in monkeys. Note that the $ER_{antibody}$ of all the studied Envs is low.

monkeys exhibited high levels (1×10^7 to 5×10^7 RNA copies/ml plasma) of viremia at day 10 postinoculation (p.i.) (Fig. 3A and B). Thus, no significant differences in the abilities of these SHIVs to replicate during the earliest, ramp-up phase of infection were apparent (Fig. 3A and B). Subsequently, the level of viremia decreased in all the animals, with the rate of decrease being dependent upon the particular virus. Plasma viral RNA levels diminished in both SHIV-KA-infected monkeys to near or below

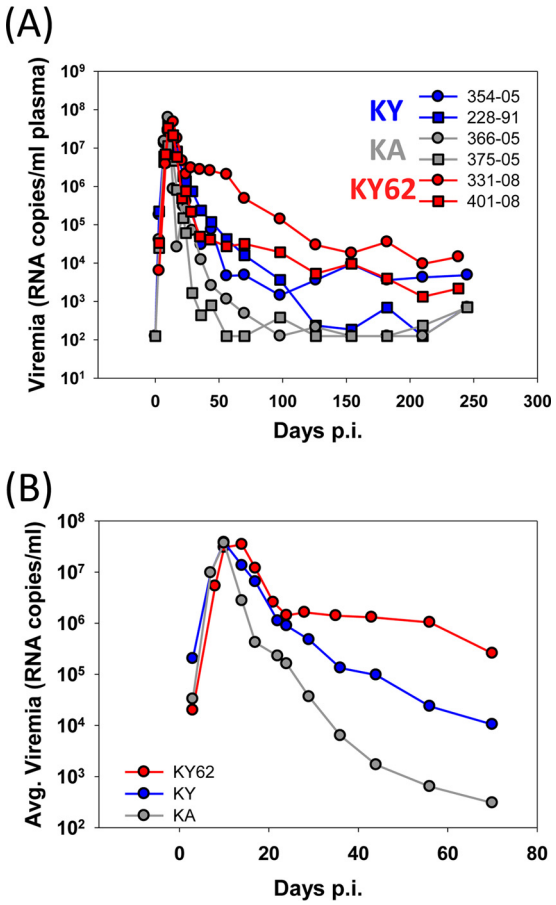

FIG 3 Viremia in SHIV-infected monkeys. (A) Plasma virus levels in rhesus macaques intravenously inoculated with SHIV-KA, SHIV-KY, and SHIV-KY62. (B) Average viremia levels in SHIV-infected monkeys.

the limit of detection (125 RNA copies/ml plasma) by 100 days p.i. (Fig. 3A). Of the two animals inoculated with SHIV-KY, one monkey (monkey 354-05) maintained a level of viremia of between 10^3 and 10^4 RNA copies/ml plasma for at least 440 days p.i. (Fig. 3A and data not shown). Viral loads in the other SHIV-KY-infected monkey (monkey 228-91) decreased to between 100 and 1,000 RNA copies/ml plasma by 150 days p.i. The highest levels of chronic viremia (between 10^3 and 10^5 RNA copies/ml plasma) at between 100 and 200 days p.i. were seen for the two monkeys inoculated with SHIV-KY62. Thus, differences in ER_{cold} and ER_{sCD4} among the SHIV variants did not apparently affect the ability of the virus to establish an acute infection. However, differences among the KA, KY, and KY62 Envs resulted in different rates of decline of viremia after the peak. The area under the curve (AUC) of viremia versus time was calculated for days 14 to 98 p.i. The average AUC was lowest for the SHIV-KA-infected monkeys and highest for the SHIV-KY62-infected animals (Table 1). Thus, SHIV-KY62, although containing an Env with lower fitness in tissue culture and lower ER_{cold} and ER_{sCD4} than the KY and KA Envs, successfully persisted in the infected monkeys.

Host antibody response in SHIV-infected monkeys. Early antibody responses to the infecting SHIVs in the monkeys were examined. All of the SHIV-infected monkeys generated detectable antibodies against Env by 28 days p.i. (Fig. 4A). By 70 days p.i., the anti-Env antibody titer was slightly higher in SHIV-KY62-infected monkeys than in monkeys infected with SHIV-KA or SHIV-KY (Fig. 4A).

To evaluate the initial neutralizing antibody response in the SHIV-infected monkeys, single-round infection assays were performed in the presence of plasma from the infected animals. Recombinant HIV-1 carrying the KA, KY, or KY62 Envs was incubated with autologous and heterologous plasma from several time points during the first 70 days of infection and then incubated with $CD4^+$ $CCR5^+$ Cf2Th cells. Antibodies that neutralized viruses with the KA and KY Envs were generated by day 28 p.i. in both SHIV-KA-infected monkeys and further increased in potency by days 42 to 70 p.i. (Fig. 4B, D, and E). Neutralizing activity against viruses with KY and KA Envs appeared in the plasma of the SHIV-KY-infected monkeys by day 42 p.i. but was less potent than that seen in the SHIV-KA-infected animals. The plasma from the SHIV-KA- and SHIV-KY-infected monkeys did not neutralize viruses with the KY62 Envs (Fig. 4F). Remarkably, despite the generation of antibodies against Env in SHIV-KY62-infected monkeys, no neutralizing activity against viruses with KA, KY, or KY62 Envs was detected in the plasma of these monkeys (Fig. 4B and F). Thus, differences in Env apparently influence the generation of and/or susceptibility to the early neutralizing antibody response in the infected monkeys. Moreover, the potency of the early neutralizing antibody response against the infecting virus correlated inversely with the average level of chronic viremia at between 98 and 210 days p.i. (Spearman = 0.948; $P < 0.05$) (Table 1). These results are consistent

TABLE 1 Viremia and autologous neutralization in infected monkeys

Monkey	SHIV strain	Postpeak viremia (AUC) (10^6 RNA copies/ml)[a]	Avg postpeak viremia (AUC) (10^6 RNA copies/ml)[b]	Chronic viremia (RNA copies/ml)[c]	Autologous neutralization onset (days)[d]
366-05	KA	6.43	8.7	6,020	28
375-05	KA	10.96		8,624	32
354-05	KY	49.24	58.42	544,698	42
228-91	KY	67.6		82,404	59
331-08	KY62	262	161.2	4,433,000	≫70
401-08	KY62	60.41		816,718	≫70

[a] Area under the viral load-time curve (AUC) from days 14 to 98 p.i. Viral loads were measured in RNA copies/ml of plasma.
[b] Average AUC for each virus group.
[c] AUC from days 98 to 210 p.i.
[d] Days p.i. when autologous neutralization potency reached 99% inhibition of input virus at a plasma dilution of 1:50.

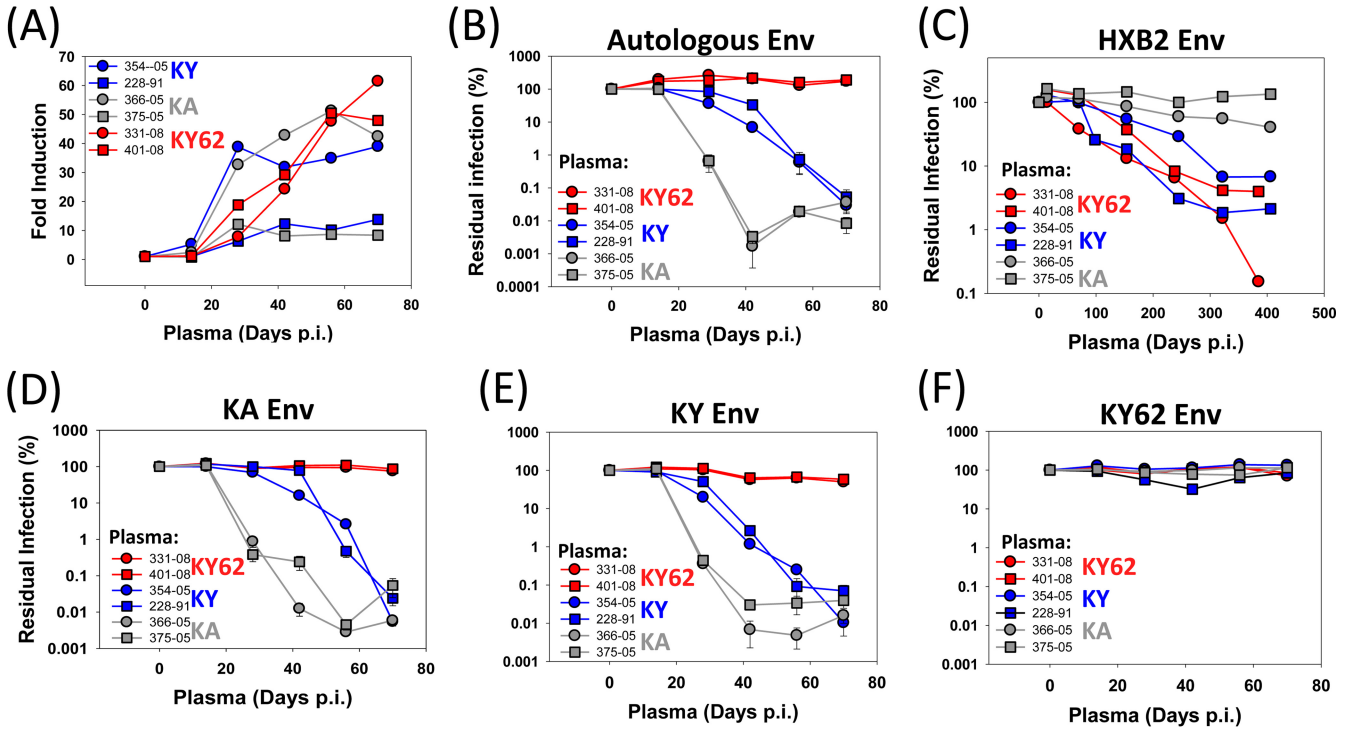

FIG 4 Generation of anti-Env antibodies in SHIV-infected monkeys. (A) Levels of antibodies in the plasma of SHIV-infected monkeys that recognize Env of the inoculated SHIV. (B to F) Reporter viruses pseudotyped with the indicated Env were preincubated for 1 h at 37°C with a single dilution of autologous or heterologous plasma (1:50) from the indicated times postinfection. After 1 h at 37°C, the virus-plasma mixtures were incubated with $CD4^+$ $CCR5^+$ Cf2Th cells. Luciferase activity was measured in the target cells. Residual infection is shown as the percentage of reporter activity observed with day 0 (uninfected) plasma. Error bars represent the standard errors of the means.

with a contribution of neutralizing antibodies to the control of viremia at between 40 and 200 days p.i. in this model system.

Antibodies that neutralize heterologous strains of HIV-1 typically arise later than autologous neutralizing antibodies in the course of SHIV infection of monkeys or HIV-1 infection of humans (21, 37, 57, 80, 81). To examine whether any heterologous neutralizing antibodies were generated during infection with the SHIV-KA, SHIV-KY, and SHIV-KY62 variants, the plasma of the infected monkeys was tested for the ability to neutralize HIV-1 vectors pseudotyped with HXB2 Env. HXB2 Env is derived from a laboratory-adapted HIV-1 strain, so viruses with HXB2 Env are generally sensitive to neutralization (82). In this sensitive assay, antibodies neutralizing the viruses with HXB2 Env were detected in the plasma of monkeys infected with SHIV-KY and SHIV-KY62 by days 70 to 406 p.i. (Fig. 4C). In contrast, the plasma from monkeys infected with SHIV-KA, which did not achieve a detectable level of chronic viremia, did not efficiently neutralize viruses with HXB2 Env. We also examined the Env-directed antibody response in infected monkeys from the late chronic stage (day 322 p.i.) by immunoprecipitating HXB2 Env with various concentrations of plasma; a trend was observed between the titers of anti-Env antibodies at this stage and the level of chronic viremia, although this association did not reach statistical significance (Spearman $r_S = 0.83$; $P = 0.058$). However, the level of cross-neutralization of viruses with HXB2 Env seen at this time point strongly correlated with the level of postacute viremia (Spearman $r_S = 0.94$; $P = 0.017$); this suggests a possible relationship between the broadening of the neutralization response to the HXB2 virus and the level of early infection. Higher levels of anti-Env antibodies in the plasma, however, did not correlate with more potent HXB2 cross-neutralization (Spearman $r_S = 0.54$; $P = 0.30$).

We also examined the ability of the monkey plasma from 70 to 406 days p.i. to inhibit infection mediated by Envs from heterologous primary HIV-1 isolates (YU2, ADA, and JRFL), which are generally more resistant to neutralization than the HXB2 isolate. None of the sera efficiently neutralized these viruses (data not shown). Thus, the HXB2-neutralizing activity observed in the plasma of SHIV-KY- and SHIV-KY62-infected monkeys at 70 to 406 days p.i. was not sufficiently broad or potent to inhibit the entry of neutralization-resistant primary viruses.

Timing of *in vivo* phenotypic changes related to ER. The hypothesis that low values of ER_{sCD4} and ER_{cold} are advantageous *in vivo* is supported by our observation that SHIVs with high ER_{sCD4} and/or ER_{cold} were apparently counterselected in the infected monkeys and replaced by SHIV variants with lower ER_{sCD4} and ER_{cold}. To investigate this selection further, we examined the ER_{sCD4} and ER_{cold} of viruses containing Env variants PCR amplified from SHIV-KY- and SHIV-KY62-infected monkeys at multiple time points of infection. As the Env sequences of the viruses at the time of peak viremia (14 days p.i.) did not differ from those of the inoculated SHIVs (see below), ER_{sCD4} and ER_{cold} did not change during the first 2 weeks of infection. Notably, by 70 days p.i., the circulating SHIV-KY viruses in monkeys 228-91 and 354-05 had Envs that were cold and sCD4 resistant, relative to KY Env in the inoculated SHIV (Fig. 5A and B). In the monkey in which infection persisted (monkey 354-05), the Envs from viruses at

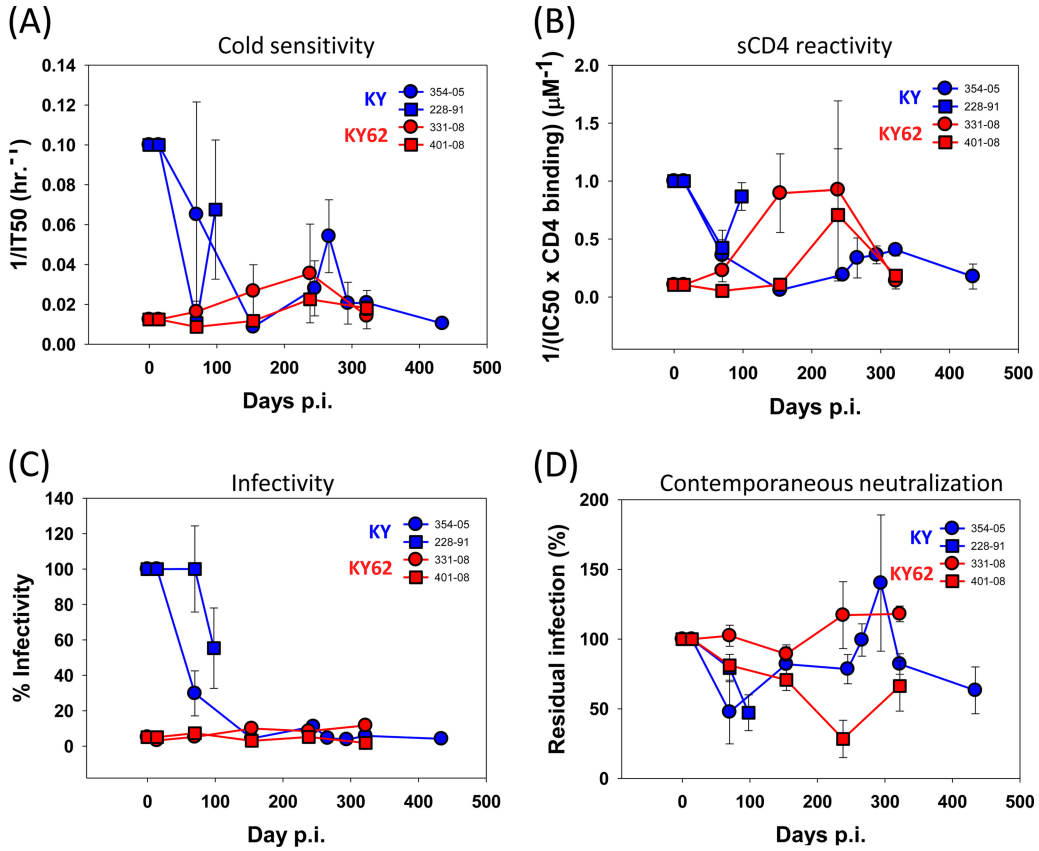

FIG 5 ER_{cold}, ER_{sCD4}, infectivity, and autologous neutralization of envelope glycoprotein isolates. Env variants derived from SHIV-KY- or -KY62-infected monkeys at the indicated times after inoculation were tested for infectivity and sensitivity to inhibition by incubation of pseudotyped reporter viruses on ice or with inhibitors (sCD4 and contemporaneous plasma). The viruses were then added to Cf2Th $CD4^+$ $CCR5^+$ cells, and luciferase activity was measured 48 h later. (A) Cold sensitivity is shown as the reciprocal of the average time on ice required for a 50% reduction in virus infectivity (IT_{50}). (B) Average soluble CD4 reactivity was calculated as described in Materials and Methods. (C) Average infectivity of variants was calculated from infectivity of maximum virus input levels relative to KY virus infectivity observed with the same RT-normalized virus input. Each experiment was performed with four virus dilutions to ensure that subsaturating infection concentrations were compared. (D) Average autologous neutralization of variants was performed by measuring inhibition of infectivity in the presence of 2% contemporaneous plasma compared to preinfection plasma from the same monkey. All experiments were performed as described in the Fig. 1 legend.

later time points remained relatively resistant to cold and sCD4. Transient increases in ER_{sCD4} and ER_{cold} were observed in Envs from monkey 354-05 at around 250 to 300 days p.i.; however, by day 434 p.i., the Envs from the circulating viruses returned to a more resistant state. In contrast, in monkey 228-91, which controlled SHIV-KY infection, the Envs from circulating viruses became more sensitive to cold and sCD4 at 98 days p.i. compared with the day 70 Envs (Fig. 5A and B). These increases in ER_{cold} and ER_{sCD4} coincided with a decrease of viremia in this monkey to undetectable levels. Envs from the monkeys infected with SHIV-KY62, a virus with low ER_{cold} and low ER_{sCD4}, exhibited slight increases in ER_{cold} and more substantial increases in ER_{sCD4} between days 150 and 250 p.i. However, both ER_{cold} and ER_{sCD4} returned to low levels by 322 days p.i.

Our results indicate that SHIVs with low or high ER_{cold} and ER_{sCD4} can efficiently establish infection. However, between days 14 and 100 p.i., as the monkeys seroconverted, SHIVs with high ER_{cold} and high ER_{sCD4} were counterselected. Transient increases in ER_{cold} and ER_{sCD4} were apparently tolerated, but long-term-persistent SHIVs generally exhibited low values of these parameters.

Viral fitness and neutralization resistance. The ability of recombinant HIV-1 pseudotyped with the Envs derived from SHIV-infected monkeys to infect target cells was examined. Compared with single-round viruses pseudotyped with the KY Envs, viruses pseudotyped with Envs derived from SHIV-KY-infected monkeys beyond 70 days p.i. were less infectious for $CD4^+$ $CCR5^+$ Cf2Th cells (Fig. 5C). Viruses pseudotyped with the parental SHIV-KY62 Envs, as well as Envs derived from the SHIV-KY62-infected monkeys (monkeys 331-08 and 401-08), were also significantly impaired for the ability to infect $CD4^+$ $CCR5^+$ Cf2Th cells (Fig. 5C). The proteolytic maturation and subunit association of the Envs were comparable (data not shown), indicating that the basis for the poor replication of the Envs that evolved in the monkeys did not involve a global disruption of the Env trimer. Thus, shortly after the peak of acute viremia, the SHIV-KY Envs exhibited decreases in ER_{cold}, ER_{sCD4}, and the ability to support virus entry into cultured $CD4^+$ $CCR5^+$ cells.

To examine whether the observed evolution of Env sequences in the SHIV-KY-infected monkeys might have resulted from pressure exerted by neutralizing antibodies, recombinant HIV-1 viruses pseudotyped with the Envs obtained at different times of infection were tested for sensitivity to neutralization by plasma from infected monkeys. Compared with viruses bearing the pa-

rental KY Envs (Fig. 4D), viruses with Envs from day 70 p.i. were resistant to neutralization by contemporaneous plasma from the SHIV-KY-infected monkeys (Fig. 5D). The ability of plasma from the SHIV-KY- and SHIV-KY62-infected monkeys to neutralize contemporaneous viruses was limited (Fig. 5D). These results suggest that SHIVs that establish chronic infections escape from neutralizing antibodies present in the plasma.

As the viruses in SHIV-KY-infected monkey 354-05 evolved resistance to autologous neutralizing antibodies, the viruses from days 70 to 245 p.i. became more sensitive to neutralization by the b12 CD4-binding-site antibody (data not shown). This increased sensitivity was specific, as the sensitivity of the viruses to the gp41-directed 4E10 antibody did not change during the course of infection (data not shown). Following the appearance of antibodies in the plasma of monkey 354-05 that neutralized viruses with the heterologous HXB2 Envs (beginning at days 245 to 434 p.i.), the SHIV-KY viruses in this animal reacquired resistance to the b12 antibody. These observations suggest that antibodies that drive this viral resistance may arise in this monkey only after 250 days p.i.

Evolution of SHIV Env sequences in infected monkeys. The HIV-1 *env* genes described above were derived by a single-genome-amplification (SGA) procedure from SHIV-infected monkeys, allowing an assessment of the diversification of the viruses over time. As mentioned above, *env* sequences analyzed near the time of peak viremia (day 10 p.i.) exhibited no nonsynonymous changes compared with the *env* sequences of the parental SHIVs (data not shown). This result implies that SHIV-KA, SHIV-KY, and SHIV-KY62 were all efficient in establishing infection in the inoculated monkeys and that no Env adaptation was required to achieve high levels of viremia.

The *env* sequences of the SHIVs circulating in plasma during later phases of infection (70 to 434 days p.i.) were analyzed by SGA. The *env* sequences were amplified from the plasma of both SHIV-KY62-infected monkeys at multiple time points. Although *env* sequences were obtained from the plasma of both SHIV-KY-infected macaques at 70 days p.i. and from one of the monkeys (monkey 228-91) at 98 days p.i., *env* sequences could not be detected in monkey 228-91 at any subsequent time. In contrast, in the SHIV-KY-infected monkey (monkey 354-05) with higher levels of chronic viremia, *env* sequences were amplified from plasma on days 154 to 434 p.i. Between 8 and 40 amplicons were sequenced at each time point. No *env* sequences could be detected in the plasma of the SHIV-KA-infected monkeys on day 70 p.i., and no sequence divergence from the inoculate was found in amplicons from day 24 or 36 p.i., the last time point of amplifiable levels of vRNA in monkey 375-05 or 366-05, respectively. Thus, as might be expected, the success rate of SGA reflects the abundance of viruses in plasma.

Amplicons from SHIV-KY-infected monkey 354-05 on days 28, 42, and 56 p.i. demonstrated minimal but diverse changes, particularly in the gp120 V2 and β20-β21 regions (data not shown). This phylogenetic "rake" of diverse viruses is "purified" by days 70 and 154 p.i., so that only three and two clones, respectively, were identified at each time point (Fig. 6A). This pattern is similar to the Env amplicon assortment from monkey 228-91 on day 70 p.i., which demonstrates charge changes and glycosylation rearrangements in V2 (Fig. 6B).

From days 70 to 434 p.i., specific Env clonal lineages emerged in the circulating SHIV-KY viruses; a partial summary of the analyzed sequences is presented in Fig. 6B. The SHIV-KY viruses in both monkey 228-91 and monkey 354-05 exhibited similar changes in the gp120 V2 variable region involving an acidic substitution (N167D), a shift of a glycan from Asn186 to Asn187 (S187N), and additional basic-to-acidic substitutions near the altered glycosylation site (K185E and K190E). These changes contribute to a general increase in the acidity of the V2 region as infection proceeds, a trend previously noted for monkeys infected with SHIV-89.6P (83). The SHIV-KY Envs in monkey 354-05 also gained an N-linked site of glycosylation in the V1 variable region (S142N). Thus, the SHIV-KY Envs in both monkeys exhibited alterations of an N-linked glycosylation site and the appearance of acidic residues in the gp120 V2 variable region. In addition, a late-arising gp120 V3 change (L317W) became fixed in the SHIV-KY viruses in monkey 354-05 by day 294 p.i. In both SHIV-KY-infected animals, sporadic changes in β19 to β21 (I420M, V430A, and T440A) were also observed. In monkey 354-05, *env* diversity increased after 154 days p.i. by both point mutations and recombination.

Single genomes from SHIV-KY62-infected monkeys were amplified on days 10, 70, 154, 238, and 322 p.i. The animal with the highest sustained viremia, monkey 331-08, contained Envs with the most sequence diversity and the highest degree of change from parental SHIV-KY62; this may reflect the requirement to escape the stronger neutralizing antibody response generated in this animal than that in monkey 401-08 (data not shown). As was observed for SHIV-KY infection, most early substitutions in KY62 Env involved alterations in the gp120 V2 and β21 regions (Fig. 6C). Both SHIV-KY62-infected monkeys exhibited β21 A433T changes early in infection, with the K432R change emerging later in monkey 331-08. Some common acidic-to-basic changes in the V2 region were observed for both SHIV-KY62-infected animals. Viruses from monkey 331-08 also acquired changes in the gp120 V3 region not associated with known tropism determinants, suggesting that these changes arise in response to other selective pressures. Other noteworthy changes include a deletion in V1 and a few hydrophobic residue changes in the V2 stem as well as the addition of a potential N-linked glycosylation site at asparagine 160. The glycan at this position is important for the integrity of the quaternary structure-dependent neutralizing antibodies PG9 and PG16 (58).

Env determinants of resistance to autologous neutralizing antibodies, soluble CD4, and cold. As mentioned above, Env sequences in SHIV-KY-infected monkey 354-05 exhibited greater sequence diversity between days 28 and 56 than at subsequent time points (days 70 and 154), suggesting the existence of a genetic bottleneck at this stage of the infection. This is consistent with the changes in fitness and resistance to autologous neutralizing antibodies, sCD4, and cold associated with the Envs derived from this animal. Of the three sequences recovered from monkey 354-05 on day 70, isolate C showed the highest phylogenetic relatedness to later isolates, including SHIV-KY62 (Fig. 6A). To investigate the Env determinants of the observed viral phenotypes, we studied the day 70 Env sequences because the relevant phenotypes were already established by this time, yet relatively few Env changes had occurred compared with the Env from the inoculated SHIV-KY. The amino acid residues in d70C Env that had changed during the course of infection were reverted to those found in KY Env, either individually or as a group. Conversely, the d70C Env changes were introduced into the KY Envs. The infectivity and sensitivity to autologous neutralizing antibodies, sCD4, and cold of the Env

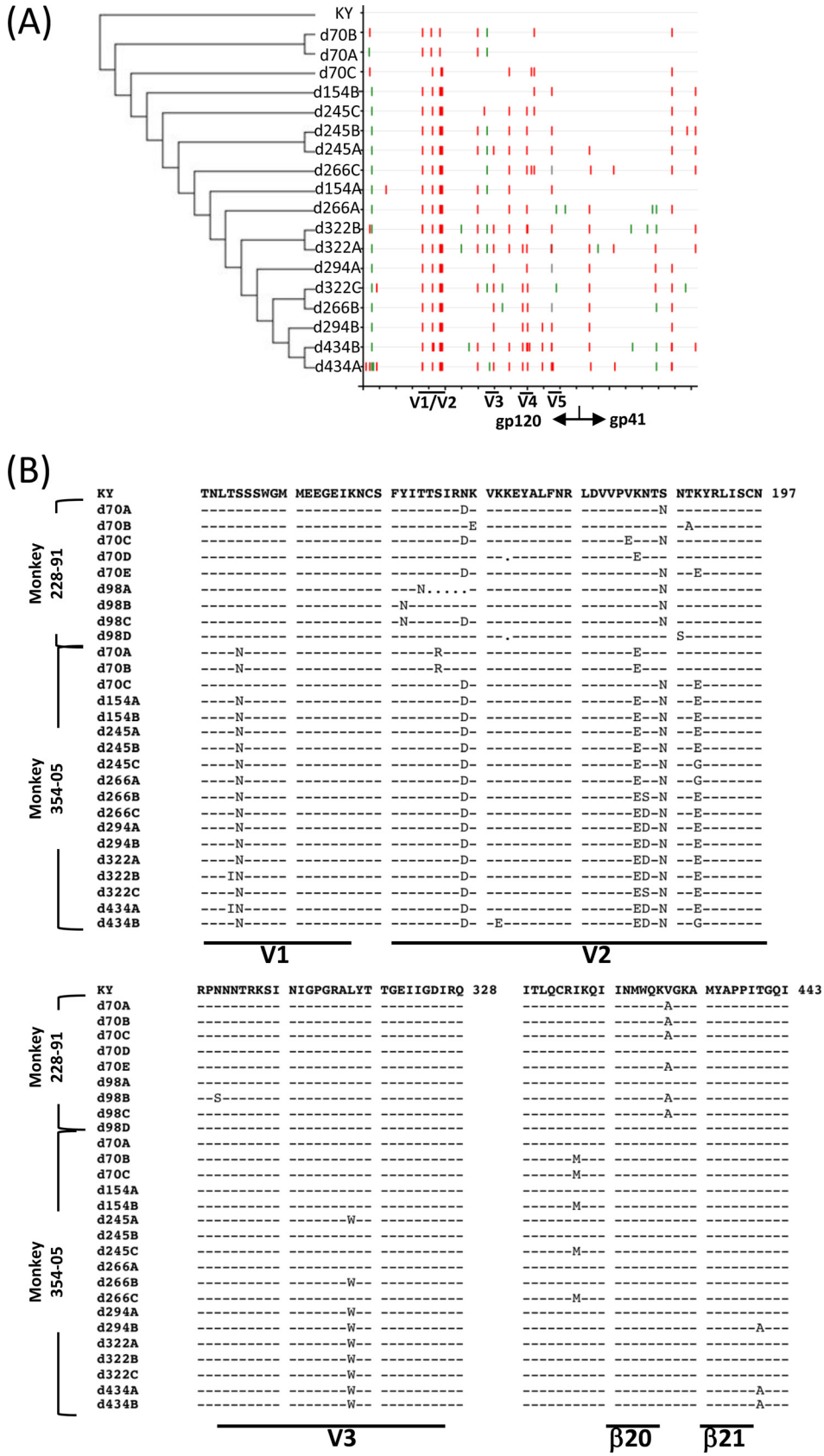

FIG 6 Phylogeny and alignment of SHIV-KY and -KY62 Env sequences. (A) Cladogram (left) and Highlighter analysis (right) of *env* sequences from animal 354-05 showing synonymous (green) and nonsynonymous (red) mutations. Relative positions of gp120 variable regions are shown, and the gp120/gp41 cleavage point is delineated. (B and C) Alignment of Env sequences spanning the V1/V2, V3, and C4 gp120 regions from SHIV-KY-infected animals 228-91 (days 70 and 98) and 354-05 (days 70 to 434) (B) or SHIV-KY62-infected animals 331-08 and 401-08 (days 70 to 322) (C). Phylogenic tree construction was calculated with PhyML 3.0 (ATCG) (102). Alignments were generated by using Highlighter and SeqPublish (Los Alamos National Laboratory). All amino acid positions are numbered according to the HXB2 HIV-1 prototype.

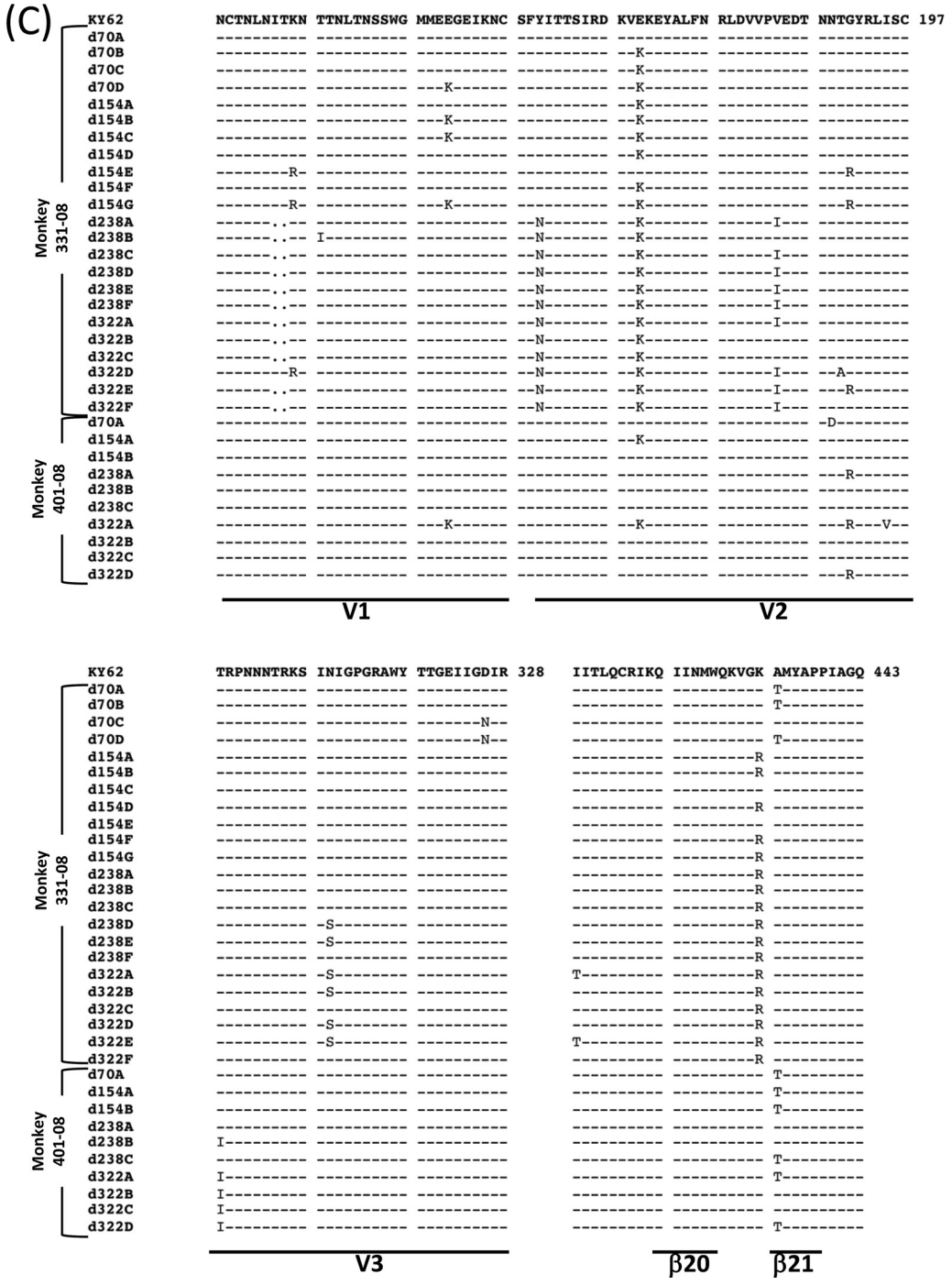

FIG 6 continued

variants are shown in Fig. 7 and Tables 2 and 3. Collectively, the changes in the gp120 V2 variable region were necessary and sufficient to explain the resistance to autologous neutralizing antibodies of viruses with the d70C Envs (Fig. 7A and B). The shift in the V2 N-linked glycosylation site and nearby acidic substitution (S187N/K190E) were the major determinants of resistance to neutralizing antibodies. Other V2 region changes, S164R and K185E, were also found to contribute to the resistance to autologous neutralizing antibodies of the d70A and d70B Envs relative to that of the KY Env (Fig. 8B and D). Thus, gp120 V2 region changes determine resistance to autologous neutralizing antibodies in the SHIV-KY-infected monkey.

Determinants of resistance to cold and sCD4 in the d70C Envs, relative to the Envs of the inoculated SHIV-KY, were investigated. The I420M change in the β19-β20 turn of gp120 made a major contribution to these phenotypes (Fig. 7C and D). Likewise, the I420M change was the major determinant of the relative cold and sCD4 resistance of the d70B Envs (Fig. 8E and F). Changes in

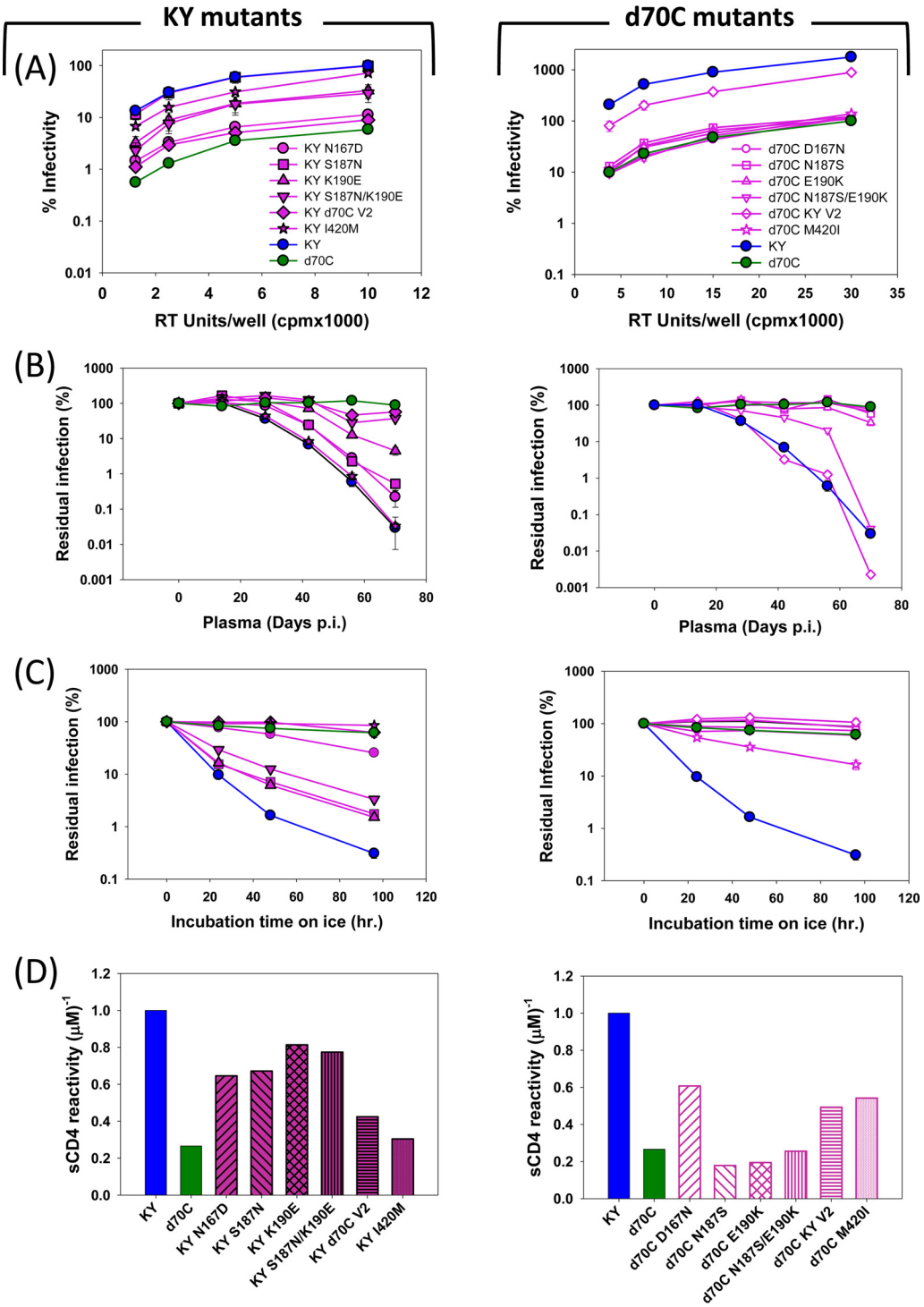

FIG 7 Env mutant infectivity, autologous neutralization, ER_{cold}, and ER_{sCD4}. Viruses pseudotyped with KY (left) or d70C (right) Env recombinants were evaluated for infectivity and inhibition by plasma antibodies, cold, and sCD4 relative to the values observed for viruses pseudotyped with wild-type KY or d70C Envs, respectively. (A) Relative infectivity of the viruses pseudotyped with the indicated Env variants. (B) Neutralization of the recombinant viruses by monkey 354-05 plasma from days 0 to 70 p.i. (C and D) Cold sensitivity (C) and sCD4 reactivity (D) of the recombinants are shown. A summary of the KY and d70C Env phenotypes can be found in Tables 2 and 3, respectively. All experiments were performed as described in the Fig. 1 legend. The error bars indicate the standard errors of the means.

residue 420 also made small contributions to the decrease in Env entry efficiency (Fig. 7A). Independently of the I420M change, the V2 region contributed to the resistance of the d70C Envs to cold and sCD4 relative to the KY Envs (Fig. 7C and D). The N167D change in V2 alone made a significant contribution to these phenotypes. Thus, changes in gp120 residue 420 made major contributions to resistance to cold and sCD4, and in some contexts, V2 changes could independently determine these phenotypes. Of

TABLE 2 Infectivity, autologous neutralization, and sensitivity to cold and sCD4 inactivation of viruses pseudotyped with KY Env variants[e]

Mutant	Location of gp120 change	Infectivity (%)[a]	Cold IT_{50} (h)[b]	sCD4 reactivity[c]	Neutralization (%)[d]
KY	Wild type	100	7.1	1.0	99.9
KY N167D	V2 region	**12**	**55**	0.65	99.8
KY S187N	V2 region	99	9.4	0.67	99.5
KY K190E	V2 region	**33**	9.5	0.82	99.5
KY S187N/K190E	V2 region	**29**	14	0.77	**62.8**
KY d70C V2	V2 region	**11**	**>96**	**0.42**	**42.6**
KY I420M	β19	79	**>96**	**0.30**	99.9

[a] Infectivity levels represent the percentage of mutant reporter virus readouts at maximum virus input compared with equivalent RT levels of reporter virus pseudotyped with KY Env.
[b] Cold IT_{50} is calculated as the half-life of virus on ice, in hours.
[c] sCD4 reactivity (ER_{sCD4}) was calculated as described in Materials and Methods and is reported relative to the ER_{sCD4} of the KY Env, which is set at a value of 1.
[d] Percent neutralization of virus with autologous contemporaneous plasma from monkey 354-05 at a 1:50 dilution.
[e] Values in boldface type indicate phenotypes differing significantly from the wild type.

TABLE 3 Infectivity, autologous neutralization, and sensitivity to cold and sCD4 inactivation of viruses pseudotyped with KY d70C Env variants[e]

Mutant	Location of gp120 change	Infectivity (%)[a]	Cold IT_{50} (h)[b]	sCD4 reactivity[c]	Neutralization (%)[d]
d70C	Wild type	100	>96	0.27	11
d70C D167N	V2 region	117	>96	**0.61**	33
d70C N187S	V2 region	121	>96	0.18	42
d70C E190K	V2 region	110	>96	0.19	67
d70C N187S/E190K	V2 region	119	80	0.26	**99.9**
d70C KY V2	V2 region	**890**	>96	**0.49**	**99.9**
d70C G355R	LE	100	>96	0.15	41
d70C T413I	V4 region	78	>96	0.23	10
d70C M420I	β19	137	**25**	**0.54**	21

[a] Infectivity levels represent the percentage of mutant reporter virus readout at maximum virus input compared with equivalent RT levels of reporter virus pseudotyped with d70C Env.
[b] Cold IT_{50} is calculated as the half-life of virus on ice, in hours.
[c] sCD4 reactivity (ER_{sCD4}) was calculated as described in Materials and Methods and is reported relative to the ER_{sCD4} of KY Env, which is set at a value of 1.
[d] Percent neutralization of virus with autologous contemporaneous plasma from monkey 354-05 at a 1:50 dilution.
[e] Values in boldface type indicate phenotypes differing significantly from the wild type.

note, the V2 changes that determined lower ER_{cold} and ER_{sCD4} (the N167D change) and neutralizing antibody resistance (the S187N/K190E changes) differed.

The decreased infectivity of viruses with the d70C Envs relative to that of viruses with the KY Envs was investigated (Fig. 7A). Together, the changes in the gp120 V2 region were necessary and sufficient to explain the decreased infectivity of the Envs from day 70 p.i. The V2 change associated with decreased ER_{cold} and ER_{sCD4} (N167D) and the V2 changes associated with resistance to neutralizing antibodies (S187N/K190E and K190E) individually resulted in decreased infectivity when introduced into the KY Env.

In summary, between peak viremia and day 70 p.i., the SHIV-KY Envs in monkey 354-05 exhibited a number of changes. The S187N/K190E changes in the gp120 V2 region, which involved a glycosylation shift and acidic substitution, conferred escape from autologous neutralizing antibodies. Another change in V2, N167D, which does not involve a change in glycosylation, decreased ER_{cold} and ER_{sCD4}. Changes in gp120 residue 420 in the β19-β20 turn independently decreased ER_{cold} and ER_{sCD4}. Changes associated with neutralization resistance and with reductions in ER_{cold} and ER_{sCD4}, particularly the V2 changes, contributed to the decreased ability of Env to mediate infection in tissue-cultured target cells. This fitness cost was apparently offset by the benefits of escaping neutralization by antibodies and other *in vivo* advantages associated with low ER_{cold} and ER_{sCD4}.

Late determinants of Env reactivity to cold and sCD4. Between days 245 and 322 p.i., transient increases in ER_{cold} and ER_{sCD4} were observed in the Envs of circulating SHIV-KY viruses in persistently infected monkey 354-05 (see above). These phenotypic changes correlated temporally with the emergence of Envs in the circulating viruses with two gp120 changes that became fixed in the circulating SHIVs: L317W near the tip of the V3 loop and T440A in the LF loop. These changes were introduced into d266A Envs that had neither change; leucine 317 and alanine 440 in d266B Envs were altered to tryptophan and threonine residues, respectively. Conversely, the tryptophan at residue 317 and alanine at residue 440 were reverted to leucine and threonine residues, respectively, in the d434A and d434B Envs. The most cold- and sCD4-resistant Envs in this mutant panel contained tryptophan 317 and alanine 440 (Table 4 and Fig. 9). Envs with leucine 317 and threonine 440 were most sensitive to cold and sCD4. Envs with only one of the resistance-associated residues exhibited intermediate phenotypes. Thus, the observed L317W and T440A changes contributed to the reemergence of SHIV-KY variants with low ER_{cold} and ER_{sCD4} in monkey 354-05 beyond 322 days p.i.

DISCUSSION

The infection of rhesus macaques with SHIVs represents an opportunity to study the properties of the HIV-1 Envs that contribute to the burst of acute viremia, establishment of persistent infection, evasion of the host immune response, and pathogenesis. Intrinsic envelope reactivity (ER) is a property of the HIV-1 Envs that reflects their propensity to negotiate transitions from the metastable unliganded state to other conformations (64). ER is inversely related to the activation barriers that separate the metastable, unliganded state of Env from lower-energy conformations (Fig. 10). ER can influence the consequences of the binding of ligands (e.g., antibodies) to the HIV-1 Env trimer and thereby exert an impact on virus sensitivity to neutralization (61). HIV-1 Envs with high ER are invariably sensitive to bound sCD4 and to cold, two properties that often correlate (64). By modulating the free energy of the metastable, unliganded state (Fig. 10, asterisk), Env reactivity to the binding of different ligands and to cold exposure could be globally altered. Not all cold-sensitive Envs are intrinsically reactive, however, and recent studies suggest that the global intrinsic envelope reactivity (ER), sensitivity to cold (ER_{cold}), and reactivity to bound sCD4 (ER_{sCD4}) can be independently regulated (64, 65). The viral phenotypes characterized in this study support this hypothesis and suggest that the heights of the activation barriers for virus inactivation by cold, sCD4, and antibodies can be individually modulated (Fig. 10). Viruses with high global ER or high $ER_{antibody}$ are expected to be counterselected by neutralizing antibodies in the host. However, as HIV-1 does not generally encounter either low temperature or sCD4 in human hosts, the importance of cold resistance and sCD4 reactivity for HIV-1 replication and persistence *in vivo* is unknown. Here, we inoculated rhesus monkeys with three R5 SHIV variants that exhibit similar sensitivities to neutralizing antibodies (and thus are similar in global ER and $ER_{antibody}$) but differ in ER_{cold} and ER_{sCD4}.

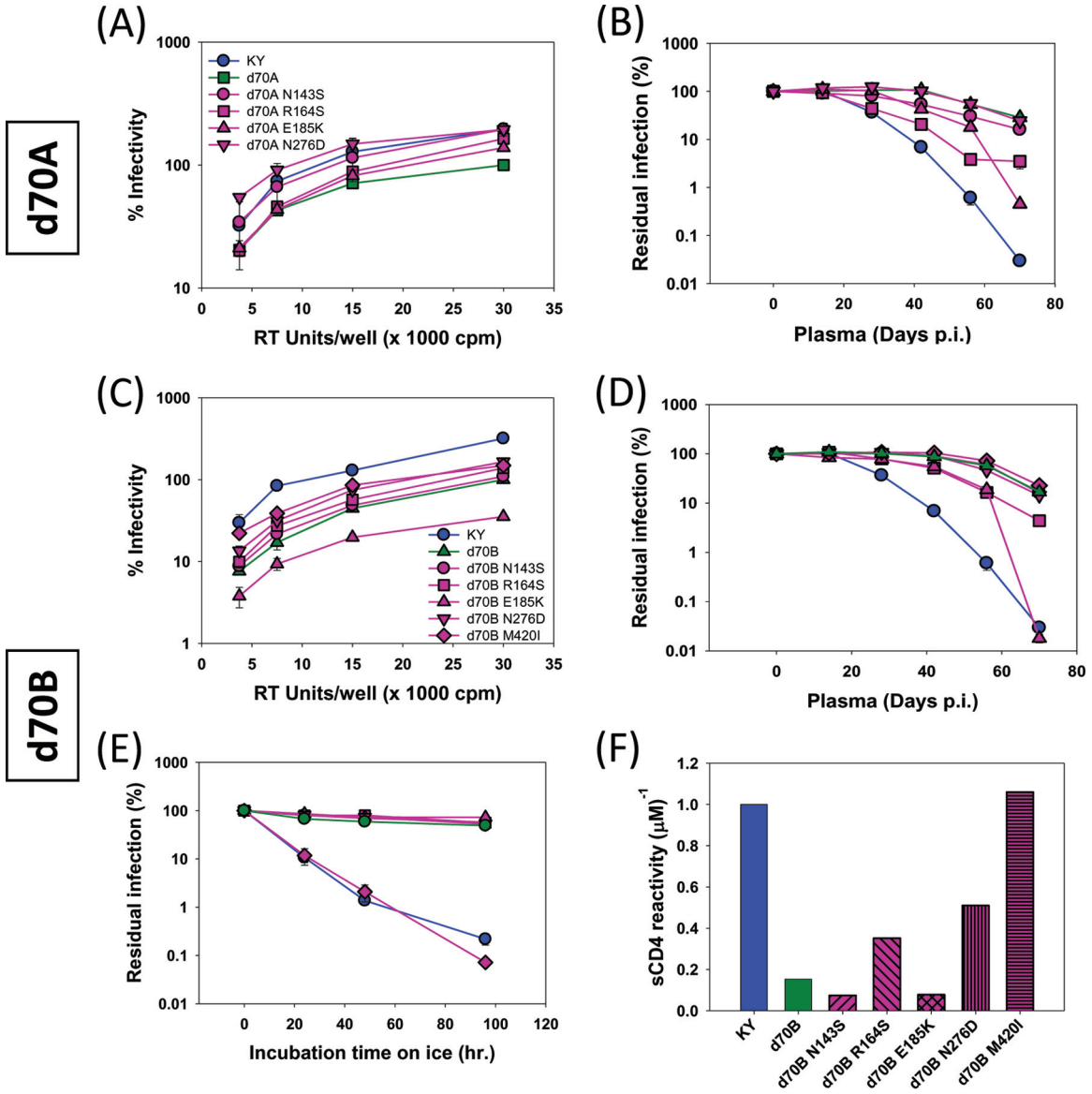

FIG 8 Early mutant infectivity, autologous neutralization, ER_{cold}, and ER_{sCD4}. (A and C) Viruses pseudotyped with d70A or d70B Env mutants were evaluated for infectivity, relative to the values observed for wild-type d70A or d70B Envs, respectively. (B and D) Sensitivity of the pseudotyped viruses to neutralization by autologous plasma from days 0 to 70 is shown. (E and F) d70B mutants were also tested for cold sensitivity (E) and sCD4 reactivity (F). All experiments were performed as described in the Fig. 1 legend. Standard errors of the means are indicated by error bars.

SHIV-KA and SHIV-KY differ only in the sequences of the gp120 V3 region, and SHIV-KY62 contains an Env that evolved in an infected monkey inoculated with SHIV-KY. Viruses with the KA, KY, and KY62 Envs were neutralized comparably by a panel of monoclonal antibodies directed against the HIV-1 Envs. However, the KA and KY Envs exhibited higher ER_{cold} and ER_{sCD4} values than those of the KY62 Envs. In tissue-cultured cells, SHIV-KA and SHIV-KY replicated more efficiently than SHIV-KY62. Upon intravenous inoculation into monkeys, however, all three SHIVs were indistinguishable in their ability to replicate and achieve a high level of acute viremia. Thus, the basic capacities of SHIV-KA, SHIV-KY, and SHIV-KY62 to replicate in monkeys prior to the generation of an efficient antiviral response appear to be similar. This implies that the differences in the ER_{cold} and ER_{sCD4} among the viruses exert negligible effects on the ability to initiate and establish an infection in monkeys. This is consistent with the observation that although transmitted/founder HIV-1 strains are all relatively resistant to neutralizing antibodies (i.e., they are "tier 2" viruses, with ER values in the low range), they exhibit a wide range of ER_{cold} and ER_{sCD4} levels (64).

Soon after the peak of viremia, host humoral immune responses emerged, and the courses of SHIV-KA, -KY, and -KY62 infection differed. By 14 to 28 days p.i., antibodies directed against Env were generated in the monkeys infected by SHIV-KA, SHIV-KY, and SHIV-KY62. By 28 to 42 days p.i., neutralizing antibodies directed against the inoculated virus were detected in the plasma of SHIV-KA- and SHIV-KY-infected monkeys. The generation of antibodies that neutralize the inoculated SHIV was dependent on the Envs of the inoculated virus. SHIV-KY62 did not elicit autologous neutralizing antibodies, despite the efficient elicitation of

TABLE 4 Infectivity and sensitivity to cold and sCD4 inactivation of viruses pseudotyped with d266 and d434 Env variants from monkey 354-05

Mutant	Residue at position: 317	440	Infectivity (%)[a]	Cold IT_{50} (h)[b]	sCD4 reactivity[c]
d266A	L	T	100	15	0.89
d266A L317W	W	T	102	43	0.15
d266A T440A	L	A	63	26	0.16
d266A L317W/T440A	W	A	255	50	0.11
d266B	W	T	100	76	0.46
d266B W317L	L	T	52	35	0.26
d266B T440A	W	A	52	84	0.14
d266B W317L/T440A	L	A	72	54	0.21
d434A	W	A	100	>96	0.09
d434A W317L	L	A	72	54	0.21
d434A A440T	W	T	86	77	0.14
d434A W317L/A440T	L	T	72	28	0.36
d434B	W	A	100	79	0.37
d434B W317L	L	A	61	47	0.26
d434B A440T	W	T	90	39	0.27
d434B W317L/A440T	L	T	41	23	0.84

[a] Infectivity levels represent the percentage of mutant reporter virus readout at maximum virus input compared with the infectivity levels of the reporter viruses pseudotyped with the corresponding wild-type Env.
[b] Cold IT_{50} represents the half-life of virus on ice, in hours.
[c] sCD4 reactivity (ER_{sCD4}) was calculated as described in Materials and Methods and is reported relative to the ER_{sCD4} of KY Env, which is set at a value of 1.

anti-Env antibodies in the infected monkeys and the comparable neutralizing antibody sensitivities of the KY62 and KA or KY Envs. Presumably, Env elements commonly targeted by strain-specific neutralizing antibodies during early infection are less accessible and/or immunogenic in the KY62 Envs. Indeed, the autologous neutralizing antibodies elicited in SHIV-KA- and SHIV-KY-infected monkeys did not neutralize viruses with the KY62 Envs. Envs with low ER_{cold} and ER_{sCD4} are predicted to have higher activation barriers that decrease the spontaneous sampling of the CD4-bound or other low-energy conformations (Fig. 10) (64) and

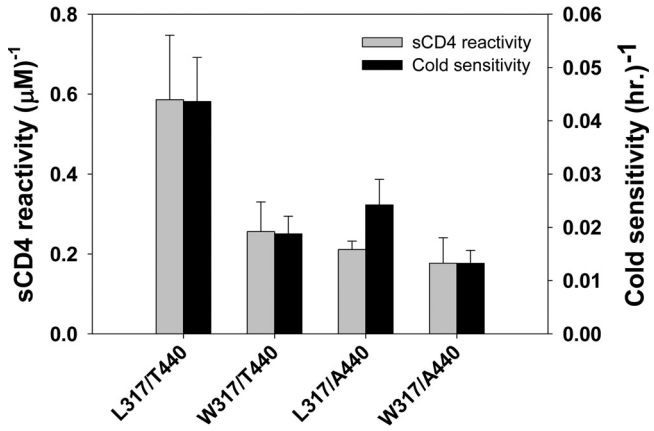

FIG 9 Changes in ER_{cold} and ER_{sCD4} in late virus variants with L317W and/or T440A Env changes. The W317 and/or A440 residue was introduced into d266 Env or was changed to L317 and/or T440 in d434 Env from monkey 354-05. Cold inactivation and sCD4 reactivity of the pseudotyped viruses were measured as described in the Fig. 1 legend. A summary of the phenotypes, including relative infectivity, cold sensitivity, and sCD4 reactivity, for each late variant is described in Table 4.

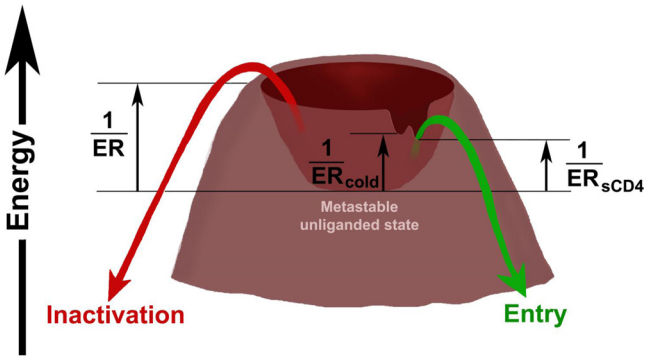

FIG 10 Energy landscape of HIV-1 Env variants. The metastable, unliganded state of the HIV-1 Env glycoproteins exists in a local energy well. The heights of the activation barriers surrounding this local energy well are inversely related to the ER, $ER_{antibody}$, ER_{sCD4}, and ER_{cold} (64). Changes in the energy of the metastable unliganded state (asterisk) result in global changes in ER, which in turn result in changes in $ER_{antibody}$, ER_{sCD4}, and ER_{cold}. Note that "notches" in the hills surrounding the energy well allow $ER_{antibody}$, ER_{sCD4}, and ER_{cold} to be independently regulated from each other and from ER. ER_{cold} and ER_{sCD4} often correlate in HIV-1 Env variants (64). The activation energy associated with the binding of target cell CD4 can influence the level of CD4 required on the cell for HIV-1 entry. At the time of seroconversion in SHIV-infected monkeys, HIV-1 Envs with more shallow notches are apparently selected, lowering ER_{cold}, ER_{sCD4}, and entry efficiency. This is predicted to result in an Env that remains longer in the metastable, unliganded state.

therefore may be less prone to elicit autologous neutralizing antibodies. Envs with low ER_{cold} and ER_{sCD4} may also be less susceptible to any neutralizing antibodies generated. Thus, the low ER_{cold} and ER_{sCD4} of the KY62 Envs may be relevant to the minimal elicitation of autologous neutralizing antibody responses by decreasing the number of Env conformations presented to the host immune system and by diminishing the impact of any elicited antibodies that can bind the Env trimer.

The generation of strain-specific neutralizing antibodies in HIV-1-infected humans is typically followed by the emergence of viruses that escape from inhibition by these antibodies (34, 84–90). The impact of neutralizing antibodies on HIV-1 fitness, viremia, and clinical course is uncertain (29, 30, 91–95). In the SHIV-infected monkeys, the decline in circulating virus after the peak of viremia was correlated directly with the potency of the neutralizing antibody response generated against the inoculated virus Envs. Furthermore, the emergence of SHIV variants resistant to the contemporaneous plasma neutralizing antibodies may contribute to the establishment of a persistent viremic state. SHIV-KA Envs did not exhibit any changes during the acute phase of infection and thus remained vulnerable to the potent neutralizing antibodies generated in the plasma of the infected monkeys. Perhaps, as a result, SHIV-KA loads in both infected macaques dropped to nearly undetectable levels by day 50 p.i. and remained there for the duration of the study. The levels of neutralizing antibodies directed against the inoculated SHIV-KY Envs were intermediate between those in SHIV-KA-infected monkeys and those in SHIV-KY62-infected animals. Although the SHIV-KY variants that emerged by 70 days p.i. in both infected monkeys were relatively resistant to the autologous neutralizing antibodies, this resistance decreased in the Envs of the viruses in monkey 228-91, which subsequently controlled viremia. In contrast, in the other SHIV-KY-infected monkey (monkey 354-05) that developed persistent viremia, viruses resistant to autologous neutraliz-

ing antibodies arose and predominated throughout infection. Finally, SHIV-KY62-infected monkeys did not develop neutralizing antibodies against the inoculated virus until much later, even though these animals efficiently generated antibodies directed against Env early in infection and subsequently made antibodies that cross-neutralized the heterologous HXB2 virus. The SHIV-KY62 viruses established a persistent viremia in these monkeys. Apparently, minimizing the elicitation of host neutralizing antibodies and/or evolving resistance to any such antibodies that are generated facilitates the persistence of these SHIVs in rhesus macaques.

Whereas the *in vivo* fitness benefit of resistance to neutralizing antibodies is intuitively obvious, the advantages of low ER_{cold} or low ER_{sCD4} are less apparent. Our observations support the proposition that an Env with low ER_{cold} and ER_{sCD4} is conducive to the ability of a SHIV to persist in monkeys. First, viruses bearing Envs with lower ER_{cold} and ER_{sCD4} were selected within the first 70 days of SHIV-KY infection of monkeys, concurrently with the generation of neutralizing antibodies. The sCD4 reactivities and cold sensitivities of the SHIV-KY Envs that were selected *in vivo* generally covaried and were determined by identical Env amino acid residues, supporting the close relationship of these two properties (64). Second, the Env changes that led to lowered ER_{cold} and ER_{sCD4} were distinct from those that resulted in resistance to autologous neutralizing antibodies. Thus, although low ER_{cold} and ER_{sCD4} on the one hand and neutralization resistance on the other hand both evolved concurrently with the generation of a neutralizing antibody response in the infected monkeys, they represent distinct and separable properties of Env. This is consistent with the observation that HIV-1 isolates with similar values of neutralization sensitivity can exhibit a wide range of ER_{cold} and ER_{sCD4} and vice versa (64, 65). Finally, the SHIV-KY progeny that successfully persisted in monkeys (including SHIV-KY62) generally maintained ER_{cold} and ER_{sCD4} levels lower than those of parental SHIV-KY. The temporal relationship between the appearance of autologous neutralizing antibodies and decreases in the ER_{cold} and ER_{sCD4} of the viral Envs is consistent with the hypothesis that low ER_{cold} and low ER_{sCD4} help the virus to evade the humoral immune response.

Of note, the initial effectiveness of the neutralizing antibody response against the inoculated virus was inversely correlated with the emergence of cross-neutralizing antibodies against the laboratory-adapted HXB2 virus. Animals inoculated with SHIV-KA developed a robust autologous neutralizing antibody response that coincided with early viral clearance, but these antibodies did not cross-neutralize the HXB2 virus. SHIV-KY Env, with higher ER_{cold} and ER_{sCD4}, induced neutralizing antibodies against the inoculated virus, but these arose more slowly and were of lower titers than those in SHIV-KA-infected monkeys. Apparently, this allowed the rapid selection of neutralization-resistant and low-ER_{cold} and low-ER_{sCD4} variants in SHIV-KY-infected animals, which may have contributed to longer persistence and the subsequent elicitation of HXB2-neutralizing antibodies. Autologous neutralizing antibodies were not detectable in SHIV-KY62-inoculated animals until very late in the infection, but these animals produced more antibodies that cross-neutralized the HXB2 virus. Envs with low ER_{cold} and ER_{sCD4} are predicted to have higher activation barriers that decrease the spontaneous sampling of the CD4-bound or other low-energy conformations (64) (Fig. 10). Therefore, these low-ER_{cold} and low-ER_{sCD4} Envs may present the unliganded Env trimer to the host immune system, rather than other Env conformers, predisposing to the generation of neutralizing antibodies with some breadth. This situation is reminiscent of the observation that Env immunogens from transmitted/founder HIV-1, which generally exhibit low ER values, elicit antibodies that can neutralize divergent tier 1 HIV-1 isolates (96).

Changes in the V2 variable region of gp120 allowed viral escape from the strain-restricted neutralizing antibodies generated in SHIV-KY-infected monkeys. Alterations in a potential N-linked glycosylation site at residue 187 and adjacent residue 190 explained most of the V2 phenotype with respect to neutralization escape. Changes in the V2 region also contributed to lowered ER_{cold} and ER_{sCD4}, but in this case, the most important residue was asparagine/aspartic acid 167. Low ER_{cold} and low ER_{sCD4} could also be achieved by changes in methionine/isoleucine 420 in the gp120 β19 strand. Thus, two redundant pathways to achieve low values of ER_{cold} and ER_{sCD4} apparently were available to the KY Envs. An understanding of how these changes affect Env neutralization sensitivity, ER_{cold}, and ER_{sCD4} will require structures of these elements in the context of the Env trimer.

The Env changes associated with neutralizing antibody escape and those associated with lowered ER_{cold} and ER_{sCD4} resulted in a decrease in the ability of Env to mediate virus entry into tissue-cultured cells. The appearance of Env variants with decreases in neutralization sensitivity, ER_{cold}, and ER_{sCD4} in SHIV-KY-infected monkeys is consistent with the positive contribution of these Env properties to virus persistence *in vivo*. Also, consistent with this proposed *in vivo* advantage of low ER_{cold}/ER_{sCD4}, SHIV-KY62 exhibited decreased elicitation/susceptibility to autologous neutralizing antibodies. Thus, SHIV-KY62 very efficiently established persistent infections in monkeys, despite the associated decrease in the ability of KY62 Env to mediate infections in PBMC and other cell types in tissue culture. The advantages of neutralizing antibody resistance and decreased ER_{cold} and ER_{sCD4} to the establishment of persistent infection apparently offset any associated decreases in the ability of the evolved Envs to mediate virus infection.

Unexpectedly, concomitant with the evolution of SHIV-KY variants that escaped autologous neutralizing antibodies, the viral Envs became more sensitive to neutralization by b12, an antibody directed against the CD4-binding site of gp120. Comparison of the sequences of Envs from monkey 228-91 that were sensitive or resistant to b12 neutralization indicates that the Env determinant is in the V2 variable region. Changes in V2 residues (Asp182 and Asn186) were previously shown to influence HIV-1 susceptibility to b12 neutralization (97–99). Also of note, SHIV-89.6 acquired resistance to b12 neutralization during the passage in monkeys that gave rise to SHIV-KB9 (38). Determinants of b12 resistance in SHIV-KB9 Env were narrowed to the V2 changes E185K and N187S, both of which reverted in d70 variants from SHIV-KY-inoculated animals 354-05 and 228-91. Thus, alterations in the V2 region likely contributed to changes in b12 sensitivity early in the course of SHIV-KY infection, but later determinants of b12 resistance remain to be characterized. As the V2 structures are distant from the b12 epitope, the mechanism whereby these V2 changes affect sensitivity to b12 inhibition also requires further studies. Of interest, when SHIV-KY-infected monkey 354-05 generated antibodies that could neutralize the heterologous HXB2 virus, the SHIV-KY variants in this animal became more resistant to b12 neutralization. This observation raises the possibility that during

the chronic stage of infection (days 250 to 430 p.i.), weakly neutralizing antibodies directed against the CD4-binding site of gp120 in the plasma of monkey 354-05 may contribute to the counterselection of SHIV-KY variants susceptible to these antibodies.

Later in the course of SHIV-KY infection of monkey 354-05, transient increases in ER_{cold} and ER_{sCD4} appeared. These increases were partial and did not return either ER_{cold} or ER_{sCD4} to preinfection values. A subsequent lowering of ER_{cold} and ER_{sCD4} was observed after day 300 p.i., with changes in the V3 tip (L317W) and LF loop (T440A) mediating this restoration. The late fluctuations in ER_{cold} and ER_{sCD4} of the SHIV-KY Envs are reminiscent of those described for a chronic R5 SHIV infection just before conversion of the virus to an X4 variant and rapid disease progression (100, 101). Such transient changes in ER_{cold} and ER_{sCD4} may arise as HIV-1 Envs adapt to altered environmental circumstances (e.g., the generation of new neutralizing antibodies or infection of different cell types) and then need to reequilibrate to optimize fitness.

An understanding of the importance of low envelope reactivity to SHIV persistence and pathogenicity should assist efforts to develop this animal model of HIV-1 infection further.

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