The HIV-1 Env envelope glycoprotein (Env) complex comprises a trimer of surface-exposed gp120 subunits that are noncovalently associated with a trimer of gp41 transmembrane subunits (1–3). The gp120-gp41 complex is derived from a precursor, gp160, following cleavage by host cell subtilisins in medial-golgi compartments (4, 5). The mature gp120-gp41 complex is incorporated into virions as they bud from the plasma membrane. The binding of gp120 to cell surface CD4 leads to the creation of a binding site for a chemokine receptor, usually CCR5 and/or CXCR4 (6–14). The interaction between gp120 and these cellular receptors initiates conformational changes in the gp120-gp41 complex, triggering the refolding of gp41 into a fusion-active state (15–23). HIV-1 isolates have been classified into 3 phylogenetically related groups, M, N, and O. The M group represents the majority of known viral sequences, comprising multiple subtypes and circulating recombinant forms and exhibiting up to 35% amino acid sequence divergence in Env. Envelope glycoprotein sequence variation is concentrated in 5 hypervariable regions (V1–V5) of gp120 that alternate with conserved (C1–C5) regions (24). The three-dimensional structure of the core domain of monomeric gp120 in complex with domains 1 and 2 of CD4 and the Fab domain of a neutralizing monoclonal antibody (mAb) has been revealed (25, 26). The gp120 core comprises residues from C1–C5 forming a conserved inner domain and a less conserved highly glycosylated outer domain. The inner and outer domains are linked through 4 antiparallel β-strands referred to as the bridging sheet (Fig. 1A). The V1–V4 hypervariable regions are excluded from the core domain by disulfide bridges, forming mobile solvent-exposed loops (25–27). The gp41 ectodomain comprises the conserved N-terminal fusion peptide, coiled-coil forming sequence and disulfide-bonded loop, and a C-terminal helical segment that exhibits a higher degree of sequence variation. The ectodomain is linked to the transmembrane domain and an −150-residue C-terminal cytoplasmic domain.

Structural, molecular genetic, and biochemical studies indicate that the CD4 and chemokine receptor binding sites are juxtaposed and in close proximity to the bridging sheet, and the V1V2 and V3 loops (Fig. 1A) (25, 26, 28–32). Current binding models propose that attachment of CD4 to gp120 leads to a re-organization of the V1V2 and V3 loops and the formation of the chemokine receptor binding site (see Ref. 25). The chemokine receptor specificity of an HIV-1 isolate determines its cellular preference or tropism. HIV-1 strains that utilize CCR5 (“R5” strains) preferentially infect CD4+ RO+ memory T cells. However, in −50% of infected individuals, the onset of symptomatic disease is preceded by the emergence of strains able to utilize CXCR4 (“X4” and “R5X4” dual tropic strains) that broadens viral tropism to both CD4+ RO+ memory and CD4+ RA+ naive T cells (33, 34). The chemokine receptor preference of HIV-1 isolates is determined largely by V3 sequences, with the CCR5 to CXCR4 switch being generally associated with increased positive charge in V3, often in conjunction with sequence changes in V1V2 and C4 (see Ref. 35).

The fusion activation of gp41 is considered to involve the propagation of a conformational signal from CD4/chemokine
FIG. 1. A, hypothetical location of gp120-gp41 contact residues in relation to the crystal structure of the gp120 core domain in complex with domains 1 and 2 of CD4 (25). Gp120 inner domain, blue; outer domain, red; bridging sheet, green; CD4-binding residues, cyan; chemokine receptor-binding region (28, 31), yellow; Ca trace of CD4 domains 1 and 2, pink. The locations of the V1V2 and V3 loops are shown in black; the N- and C-terminal extremities of C1 and C5 are shown as blue lines. Gp41 is shown diagrammatically with the oligomerization helix (51, 98) as a purple barrel. The gp120-gp41 contact residues Leu593, Trp596, Gly597, Lys601, and Trp610 identified previously for the TCLA strain, BH8, that were targeted with mutations in this study are indicated by annotated spheres. B, alignment of HIV-1, HIV-2, and SIV gp41 disulfide-bonded and flanking region sequences. The gp120 contact residues Leu300, Trp306, Gly307, Lys301, and Trp310 identified previously for the TCLA strain, BH8, that were targeted with mutations in this study.
receptor-bound gp120 through the sites of gp120-gp41 contact, culminating in the insertion of the fusion peptide into the target membrane and the refolding of the gp41 ectodomain into a six-helix bundle, promoting membrane fusion (15, 19, 22, 23). The sites of gp120-gp41 contact have been mapped by in vitro mutagenesis to conserved residues within the disulfide-bonded region of gp41 and in the extremities of C1 and C5 of gp120 (Fig. 1B, 36, 37). The introduction of cysteines to the gp41 disulfide-bonded region and to gp120 C1- or C5-generated disulfide-linked gp120-gp41, confirming that these regions are in close contact (38). The reduction of one such covalently associated gp120-gp41 complex, linked through cysteines introduced at residues 501 of gp120 and 605 of gp41, led to the restoration of cell-cell fusion and entry function indicating that the engineered intersubunit disulfide had formed in a functional Env context (39, 40).

Protein-protein interaction surfaces can exhibit structural plasticity, a mechanism whereby an interface adapts to mutations as binding partners coevolve (41). Gp120-gp41 represents an extreme example of a coevolving protein-protein complex with V1, V2, and V3 amino acid sequences diverging by 77, 36, and 46%, respectively, over 7 years in an infected individual, whereas up to 35% sequence divergence has been observed in Env among HIV-1 pandemic strains (24, 42). In this study, we examined the functional contribution of 5 conserved gp120 contact residues of the gp41 disulfide-bonded region (37), in the context of Env sequence evolution. Whereas the overall gp120-gp41 association function of the disulfide-bonded region was conserved in Env from diverse HIV-1 strains, the relative contribution of the individual residues to gp120-anchoring and membrane fusion varied among the strains tested and depended on V1V2 and V3 sequences. These results suggest that the coevolution of gp120 and gp41 is associated with changes to the functional contribution of conserved amino acids in the gp120-gp41 association site.

EXPERIMENTAL PROCEDURES

HIV-1 Env Expression Vectors—The following HIV-1 molecular clones were used for derivation of pCDNA3.1/myc-HisA (Invitrogen)-based env expression vectors: pAD8 (43) from K. W. Peden, CBER, Food and Drug Administration, Bethesda, MD; p89.6 (44) from R. G. Collman, University of Pennsylvania School of Medicine, Philadelphia, PA; pSIV89.6-KpnI024.2 and pSIV89.6-KpnI037.8 (45) from B. Hahn through the National Institutes of Health AIDS Reference and Reagent Program, QH0515.1, QH1549.13, and QH1521.34 (46) from M. L. Greenberg, Department of Surgery, Duke University Medical Center, Durham, NC; and pTMenv.2, which contains the BBS env open reading frame (37). The env region was excised from pAD8 with EcoRI and BspI and this fragment was cloned into the EcoRI-EcoRV sites of pCDNA3.1 to give pCDNA3.1-AD8env. The env region of p89.6 was excised using HindIII and EcoRV and then cloned into the corresponding sites of pCDNA3.1 to give pCDNA3.1–89.env. The KpnI env gene fragments of pSIV89.6-KpnI024.2 and pSIV89.6-KpnI037.8 were cloned into the KpnI sites of pCDNA3.1–89.env. QH0515.1, QH1549.13, and QH1521.34 KpnI-XhoI env gene fragments were cloned into the corresponding sites in pCDNA3.1–89.env. Finally, a BBS env gene fragment was excised from pTMenv.2 using KpnI and SalI and ligated into the KpnI-XhoI sites of pCDNA3.1–89.env. In vitro mutagenesis was carried out by standard overlapped extension PCR techniques. For the sequential isolates, QH0515.1 and QH1549.13, the L593V, W596L, G597A, K601E, and W610F mutations were first introduced into QH1549.13 Env and the wild-type and mutated gene fragments encompassing gp41 residues Ser36 to Thr43 were used to replace the corresponding region of QH0515.1. BBS-AD8 Env chimeras were made by replacing the VIV2-C2-V3 (DraIII-Bsu36I fragment, AD836), V12 (DraIII-StuI, AD8StuI), or V3 (AaeI-Bna36I, AD8BnaI) regions of BBS with those of AD8 in pCDNA3.1-BBSenv. The AD8StuI chimera contains AD8 gp120 linked to BBS gp41 through the common subtilisin cleavage sequence Arg-Glu-Lys-Arg and was constructed using overlap extension PCR. The sequences of mutated Env regions were confirmed by automated DNA sequencing using the ABI prism method.

Cells—293T and BHK-21 cells were maintained in Dulbecco’s modification of minimal essential medium, 10% fetal calf serum (complete medium). The cells (250,000 cells per 4.5-mm2 well of Linbro 12-well dishes, ICN Biomedicals Inc, Aurora, OH) were transfected by the FuGENE 6 procedure (Roche Applied Science). U87 astroglioma cells stably expressing CD4 and CCR5 (U87.CD4.CCR5; obtained from the AIDS Research and Reference Reagent Program from H. Deng and D. Littman (47)) were maintained in complete medium supplemented with 1 μg/ml puromycin and 300 μg/ml G418.

Antibodies—Immunoglobulin G14 (IgG14) was purified from the plasma of an HIV-1-positive individual using protein A-Sepharose (Amersham Biosciences). The anti-gp41 monoclonal antibody C8 (48) was obtained from G. Lewis through the AIDS Research and Reference Reagent Program, NIAID National Institutes for Health, whereas mAbs 2F5 (49) was obtained from Polysymton Scientific (Vienna, Austria). The polyclonal anti-gp120 antibody DO-12 was obtained from the AIDS Research and Reference Reagent Program, NIAID, from M. Phelan (50).

Western Blotting—At 36 h post-transfection, 293T cells were lysed for 10 min on ice in phosphate-buffered saline (PBS) containing 1% Triton X-100, 0.02% sodium azide, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM dithiothreitol. Lysates were clarified by centrifugation at 10,000 × g at 4°C prior to 10% polyacrylamide gel electrophoresis in the presence of SDS under nonreducing conditions. Protein samples were transferred to nitrocellulose prior to Western blotting with mAbs C8 or mAb 2F5 using the enhanced chemiluminescence procedure (Roche Applied Science).

Bioisytthetic Labeling and Immunoprecipitation—At 16 h post-transfection, 293T cells were incubated for 30 min in cysteine- and methionine-deficient medium (ICN, Costa Mesa, CA), and then labeled for 45 min with 150 μCi of Trans35S-label (ICN). The cells were then washed and chased in complete medium for 4.5 h prior to lysis. In most experiments, incubations were performed at 37°C. However, for 292U037, QH15213#34, QH05151#1, and QH1549#3, the pulse and chase were performed at 35°C for enhanced gp160 processing. Cell lysates and clarified culture supernatants were immunoprecipitated as described previously (51) using IgG14.

Surface Biotinylation—At 24 h post-transfection, 293T cells were chilled on ice, washed twice with ice-cold PBS and then incubated with 1.25 mM sulfo-NHS long chain biotin (Pierce) in PBS for 30 min on ice. The biotylated cells were washed once with ice-cold PBS and then quenched with 50 mM glycine/PBS, pH 8.0 (500 μl) for 30 min on ice. Biotinylated cells were lysed by the addition of 170 μl of buffer A containing X-100, 2.4 mM KCl, 0.2 mM Tris-HCl, pH 7.4, clarified by centrifugation at 10,000 × g for 10 min at 4°C, and precleared with bovine serum albumin-Sepharose CL-4B for 16 h at 4°C. Envelope proteins were immunoprecipitated from the precleared cell lysates with IgG14 and protein G-Sepharose (Amersham Biosciences). Biotinylated Env proteins were visualized following SDS-PAGE on 4–12% polyacrylamide gradient gels under reducing conditions, transfer to nitrocellulose and blotting with neâ€” neutravidin-horseradish peroxidase (Pierce) and chemiluminescence (Roche Applied Science).

Luciferase Reporter Assay for Cell to Cell Fusion—Luciferase reporter assays for cell to cell fusion were performed as described with minor modifications (37). 293T effector cells were transfected with Env-expression constructs and then infected with the recombinant vaccinia virus vTF7.3 at 5 h post-transfection for expression of T7 RNA polymerase. 100X-21 or 293T target cells were cotransfected with pt4uc (a dual expression vector for cytomegalovirus promoter-driven CD4 expression and bacteriophage T7 promoter-driven firefly luciferase expression (37)) and a chemokine receptor expression construct: pc.FUSIN, pc.CCR5, or pc.CCR3 (9) obtained from Dr. N. Landau through the National Institutes of Health AIDS Research and Reference Reagent Program. At 24 h post-transfection, effectors and targets were resuspended in 1 ml of complete medium containing 1 μg/ml actinomycin D and 40 μg/ml cytosine arabinoside and cocultured for a further 3 h (BHK-21) or 16 h (293T) at 37°C. The cells were then lysed and assayed for luciferase activity using the Promega luciferase assay system.

Single Cycle Infectivity Assays—Env-pseudotyped luciferase reporter are indicated with diamonds. The strains analyzed in this study are highlighted in bold text and their chemokine receptor preferences indicated. HIV-1 reference strains were selected from Kuiken et al. (24). The HXB2R numbering system is used throughout this report. Cylinder indicates the N-terminal α-helix of the gp41 6-helix bundle core domain.
viruses were produced by cotransfecting 293T cells (350,000 cells per well of a 6-well Linbro culture plate) with 1 μg of Env expression vector and 1 μg of luciferase reporter virus vector pNL4.3.Luc.R'E' (obtained from the AIDS Research and Reference Reagent Program from N. Landau (53)). Supernatants containing pseudotyped virions were harvested at 72 h post-transfection and filtered through 0.45-μm filters. The ability of wild-type and mutated Env to mediate infection via CCR5 was assessed in U87.CD4.CCR5 cells. The cells (60,000 cells per well of a 48-well plate, Greiner Labtek) were infected with 200 μl of virus-containing supernatant for 18 h, after which the inoculum was replaced with 500 μl of complete medium. At 52 h postinfection, the cells were assayed with the Promega luciferase assay system using a Berthold Microlumat LB luminometer. The ability of wild-type and mutated Env to mediate infection via CXCR4 was assessed in 293T cells (250,000 cells per well of a 12 well plate) that had been transiently transfected with 1 μg of pcFUSIN and 1 μg of pCDNA.T4 24 h previously (37). The cells infected with 800 μl of pseudotyped virus-containing supernatant for 18 h after which the virus inoculum was replaced with 2 ml of complete medium. The cells were assayed for luciferase activity at 50 h postinfection. The protein composition of pseudotyped virions was assessed by first pelleting the virions from 9 ml of culture supernatant through a 1.5-ml 25% (v/v) sucrose/PBS cushion (Beckman SW41Ti rotor, 25,000 rpm, 2.5 h, 4 °C) followed by reducing SDS-PAGE in 7.5–15% polyacrylamide gradient gels and Western blotting with IgG14 and DV-012.

RESULTS

Previously, we identified five conserved residues, Leu503, Trp560, Gly566, Lyr576, and Trp580, in the disulfide-bonded region of gp41 that are important for association with gp120 for a T cell line-adapted (TCLA) HIV-1 isolate BH8. We reasoned that these residues cluster in the gp120 association site and are likely to play a role in receptor-induced conformational changes in gp120-gp41, leading to gp41 fusion activation (Fig. 1A) (37). The functional role of these residues may differ in primary strains. Indeed, the gp120-gp41 complex of such strains exhibit greater stability and resistance to neutralization with soluble CD4 (sCD4) when compared with TCLA HIV-1 (54). Subtle functional differences in gp120-gp41 of primary strains relating to chemokine receptor specificity have also been observed. For example, suboptimal sCD4 concentrations lead to enhanced entry by R5 viruses, whereas an R5X4 strain, 89.6, is neutralized by sCD4 when compared with TCLA HIV-1 (55). Furthermore, primary R5 viruses are neutralized more effectively than X4 viruses by the gp41 inhibitory peptide T-20/DP178 (56), which acts at the level of a gp41 fusion intermediate induced by gp120-CD4 (16). We reasoned that these differences may reflect evolutionary changes in the mode whereby gp120 and gp41 interact during fusion. To investigate this idea, the L593V (M593V for 89.6), W596L, G597A, K601E, and W610F mutations, shown previously to disrupt BH8 gp120-gp41 association and fusion function (37), were introduced into Env of selected HIV-1 primary strains. Fig. 1B shows a comparison of the disulfide-bonded region and flanking sequences of isolates analyzed in this study (bold type) and those of group M reference strains. In addition to Env-derived strains from clade A (92UG037 (R5), B (89.6 (R5X4), AD8 (R5), QH1521.34 (R5X4)), and D (92UG024 (X4)) isolates, we included two sequential isolates (QH0515.1 (R5) and QH1549.13 (R5X4)) to examine the function of the gp41 disulfide-bonded region of closely related Env sequences exhibiting different chemokine receptor preferences. We assessed the effects of the mutations on gp120-gp41 association, gp41 synthesis and stability, surface expression, and membrane fusion activity.

Functional Analysis of the Disulfide-bonded Region from Clade B Env: AD8 (R5) and 89.6 (R5X4)—We first compared the functional characteristics of gp41 disulfide-bonded region mutants derived from the macrophage-adapted R5 strain, AD8, and the primary R5X4 strain, 89.6. The gp120 anchoring ability of gp41 mutants was assessed by SDS-PAGE after immunoprecipitation of pulse-chase-labeled glycoproteins from transfected cell lysates and culture supernatants. In agreement with previous studies (for example, see Ref. 36), gp160 and gp41-associated gp120 were immunoprecipitated from wild-type Env-transfected cell lysates (Fig. 2A, lanes un-t), whereas gp120 that had been spontaneously shed from the envelope glycoprotein complex was obtained from culture supernatants (Fig. 2A, lanes un-s). The AD8 mutant, W596L, exhibited a gp120 anchoring ability that was similar to wild type. However, decreased cell-associated gp120 and increased shedding of gp120 were observed for AD8-L593V and -K601E, and for the 89.6 mutants M593V, W596L, and K601E. The W610F mutation resulted in loss of gp120-gp41 association in both contexts. Whereas AD8-G597A was able to associate with low but detectable amounts of gp120, its 89.6 counterpart lacked gp120 association ability. These results contrast those obtained previously for the TCLA X4 strain, BH8, where only W596L and K601E mutants retained low levels of gp120 anchoring ability (see Ref. 37 and Fig. 6B).

The gp120 anchoring ability of gp41 mutants was reflected in a surface biotinylation assay (Fig. 2B). Cell surface-biotinylated gp120 was detected for 89.6-M593V and W596L and K601E of both strains, but not for G597A and W610F. Whereas AD8-L593V was found to anchor significant amounts of gp120 (Fig. 2A), biotinylated gp120 was not detected for this mutant consistent with a labile gp120-gp41 complex (Fig. 2B). The level of surface-biotinylated gp120 relative to gp160 was diminished for 89.6 when compared with AD8 (Fig. 2B) and is consistent with an ~2-fold lower efficiency of gp160 cleavage to gp120 and ~3-fold weaker gp120-gp41 association for 89.6 relative to AD8 in pulse-chase immunoprecipitation experiments (Fig. 2A).

Western blot assays employing the anti-gp41 mAbs, C8 and 2F5, were used to examine whether differences in gp120-gp41 association correlated with differences in the synthesis and/or stability of gp41 mutants. Fig. 2C shows similar levels of gp160 and processed gp41 for wild-type and 9 of 10 mutants, consistent with the formation of translocation-competent glycoproteins that are cleaved in the Golgi complex, yielding stable gp41 molecules. However, for AD8-W610F (but not 89.6-W610F), we observed decreased amounts of gp41 relative to precursor gp160 suggesting that this mutation may be associated with a folding defect.

Ability of AD8 and 89.6 Mutants to Mediate Cell-Cell Fusion and Viral Entry—We assessed the fusogenicity of AD8 and 89.6 mutants using a luciferase reporter assay of cell-cell fusion. The membrane fusion activity of AD8 mutants correlated with their ability to associate with gp120 (Figs. 2A and 3A). Thus, AD8-W596L and -K601E mutants exhibited wild-type fusion and gp120 association activities. L593V exhibited an intermediate phenotype, whereas G597A retained low but significant levels of gp120 association and fusion activity (p < 0.005, G597A fusion activity relative to mock transfections). The W610F mutant lacked both functions, however, we cannot rule out the possibility that this phenotype is because of a compromised gp41 structure (see Fig. 2C).

We next investigated whether wild-type and mutated AD8 Env-pseudotyped luciferase reporter viruses could establish a single cycle of replication in U87.CD4.CCR5 target cells. Fig. 3, A and B, indicates that the entry competence of AD8-L593V, -W596L, and -K601E mutants related to wild-type was reduced when compared with their relative cell-cell fusion activities. Thus, L593V exhibited an ~20% reduction in fusogenicity and a 2.4 log10 reduction in entry competence, whereas the fully fusogenic mutants, W596L and K601E, exhibited ~0.8 log10 reductions in entry competence. The G597A and W610F mutants lacked the ability to mediate viral entry.
The protein content of AD8-Env pseudotypes was assessed by first pelleting the virions through a sucrose cushion and then subjecting the viral pellets to SDS-PAGE and Western blot (Fig. 3C). The probing of filters with IgG414 (IgG414 does not detect denatured gp160, gp120, and gp41 in Western blot assay) revealed similar amounts of CA, MA, RT, and IN for all constructs. The anti-gp120 antibody DV-012 indicated the presence of both gp120 and gp160 in wild-type AD8 Env pseudotypes and is consistent with previous analyses of pseudotyped particles (e.g., Ref. 57). Reduced levels of gp120 relative to p24 CA were observed for the W596L and K601E mutants thus providing an explanation for the associated decreases in entry competence. By contrast, only trace levels of virion-associated gp120 were detected for L593V, G597A, and W610F mutants, consistent with decreased or absent gp120 anchoring ability and entry competence (Figs. 2A and 3B).

The cell-cell fusion activity of 89.6 mutants was assessed using cellular targets transfected with CD4 and CCR5, CCR3, or CXCR4, alternative chemokine receptors utilized by this strain for entry in vitro. The fusogenicity of 89.6 mutants with CXCR4 targets correlated generally with their capacity to anchor gp120, with wild-type activities observed for M593V, W596L, and K601E compared with abolished activities for G597A and W610F (Figs. 2A and 3A). However, when CCR5 and CCR3 targets were employed in the fusion assays, chemokine receptor-dependent differences in fusogenicity were observed for some 89.6 mutants. For example, 89.6-W596L retained gp120-gp41 association and wild-type levels of CXCR4-dependent fusion function but exhibited 70 and 90% reductions in fusogenicity with CCR5 and CCR3 targets, respectively (p < 0.005, CCR3 fusion activity relative to CCR5). In addition, M593V and K601E mutants exhibited small but significant reductions in fusogenicity with CCR3 targets relative to CCR5 and CXCR4 targets.

The infectivity of 89.6 Env pseudotyped virions was assessed using U87.CD4.CCR5 and 293T.CD4.CXCR4 target cells. Fig. 3B indicates that of the five 89.6 Env mutants, only M593V retained the ability to mediate viral entry via either chemokine receptor but at reduced levels relative to wild type (CCR5, 1.6 log10 reduction; CXCR4, 1.1 log10 reduction). Western blot analysis of the pseudotyped particles with IgG414 indicated similar amounts of CA, MA, RT, and IN for all constructs (Fig. 3C). The probing of filters with DV-012 indicated that high levels of wild-type, M593V and K601E gp160 were incorporated into virions relative to gp120 and is consistent with the proportion of gp160 and gp120 detected at the cell surface (Fig. 2B) where HIV-1 assembly occurs. Particles containing gp120 derived from the fusogenic mutant, K601E, were not infectious suggesting that this mutant cannot complete fusion in a viral context or that entry may be blocked at a post-fusion stage. In contrast, gp120 derived from W596L was not incorporated into particles thereby explaining their lack of entry competence. This lack of incorporation may be because of labile gp120-gp41 association for W596L in this context. Thus, conservative substitutions in the disulfide-bonded region of BH8, AD8, and 89.6 are sufficient to disrupt gp120-gp41 association, indicating that the overall role of the disulfide-bonded region in anchoring gp120 is conserved for these strains. However, marked differences in the contribution of individual targeted residues to gp120-gp41 anchoring ability and entry competence (Figs. 2A and 3B).

Fig. 2. Biochemical analysis of AD8 and 89.6 gp41 mutants. A, gp120 anchoring ability of gp41 mutants. At 24 h post-transfection, cells were labeled with Tran35S-label for 45 min and chased in complete medium for 4.5 h before lysis. Cell lysates (C) and clarified culture supernatants (S) were immunoprecipitated with IgG14 and protein G-Sepharose. Gp160 and gp120 bands were visualized following SDS-PAGE in 10% polyacrylamide gels under reducing conditions and scanning in a PhosphorImager SF. Mutant gp120-gp41 association relative to wild type was determined by the method of Helseth et al. (36) (mutant gp120)cell × [wild-type gp120]supernatant/[mutant gp120]supernatant × [wild-type gp120]cell]. +, >0.7; +, 0.4–0.7; +/−, <0.1; −, no association. Representative of two independent experiments. B, cell surface expression of Env mutants. At 16 h post-transfection, cells were washed twice with PBS, incubated with 1.25 μM sulfo-NHS long chain biotin for 30 min, and then quenched with 50 mM glycine/PBS. Envelope proteins were immunoprecipitated from pre-cleared cell lysates with IgG14 and protein G-Sepharose. Biotinylated Env proteins were visualized following SDS-PAGE in 4–12% polyacrylamide gradient gels under reducing conditions, transfer to nitrocellulose, and blotting with neutravidin-horseradish peroxidase. C, synthesis and processing of HIV-1 Env glycoprotein mutants in 293T cells. At 36 h post-transfection, the cells were lysed and subjected to reducing SDS-PAGE in 10% polyacrylamide gels. Proteins were transferred to nitrocellulose prior to Western blotting with mAb C8 (AD8) or 2F5 (89.6) and chemiluminescent detection. Arrowhead, ~80-kDa species corresponding in molecular mass to a biosynthetic intermediate or degradation product of gp160 (99). Representative of three independent experiments.
FIG. 3. A, cell-cell fusion activities of Env glycoprotein mutants using a luciferase reporter assay. 293T effector cells were transfected with wild-type and mutated Env expression vectors and then infected with vT7.3 while target BHK-21 cells were cotransfected with pT4luc and pc.CKR5, pc.CCR3, or pc.FUSIN. At 16 h postinfection, effectors and targets were mixed and cocultured for 3 h prior to lysis and assay for luciferase activity. The relative fusion activities of Env proteins are expressed as (ratio of luciferase activity induced by mutant Env to luciferase activity induced by wild-type Env)/100. The mean ± S.E. from four independent transfections are shown. Statistical analysis was performed by using a two-sample t test assuming unequal variances: *, p < 0.004 relative to wild type; †, p < 0.0001 relative to wild type; ‡, p < 0.005 relative to mock- and W610F-transfected cells. Inset, mutant gp120-gp41 association relative to wild type: ++, >0.7; +, 0.4–0.7; +/−, <0.1; −, no association. B, ability of AD8 and 89.6 Env glycoproteins to mediate virus entry in a single cycle infectivity assay. Env-pseudotyped luciferase reporter viruses were prepared by cotransfecting 293T cells with 1 μg of Env expression construct and 1 μg of pNL4.3.Luc.R’−E−. At 72 h post-transfection, the virus-containing supernatants were filtered and used to infect U87.CD4.CCR5 (gray bars) or 293T.CD4.CXCR4 (black bars) monolayers for 18 h. The cells were assayed for luciferase activity at 52 h postinfection. The mean ± S.E. from triplicate transfections are shown. *, p < 0.01 relative to wild type; †, p < 0.03 relative to no Env. Statistical analysis was performed by using a two-sample t test assuming unequal variances. Representative of three independent experiments. C, analysis of viral proteins. Env-pseudotyped virions were pelleted from a 9-ml transfection supernatant through a 25% sucrose cushion. The viral pellets were boiled in sample buffer containing 1% β-mercaptoethanol and subjected to SDS-PAGE in 7.5–15% polyacrylamide gradient gels. The proteins were visualized by Western blotting with IgG#14 (upper panel) and DV-012 (lower panel) antibodies followed by enhanced chemiluminescence. RLU, relative light units.
**Functional Analysis of the Disulfide-bonded Region from Primary HIV-1 Isolates**—To obtain a broader understanding of the functional evolution of the gp41 disulfide-bonded region, we extended our analysis to EnvVs derived from a panel of primary isolates exhibiting various chemokine receptor specificities. We first examined the biosynthesis and stability of mutated gp41 glycoproteins in a Western blot assay using the anti-gp41 mAb 2F5. Normal processing and/or stability was observed for the majority of gp41 mutants (Fig. 4A). However, reproducible decreases in gp41 relative to gp160 were observed for 92UG037-L593V, -G597A, -K601E, and -W610F, 92UG024-G597A and -W610F, and QH1521.34-L593V, consistent with compromised gp41 maturation and/or stability.

A variety of gp120-gp41 association and fusion profiles were observed for these primary strains. L593V, G597A, and W610F mutations resulted in the loss of both gp120 association and fusion functions irrespective of whether these mutations were associated with compromised gp41 maturation and/or stability (Fig. 4, B and C). By contrast, the functional effects of W596L and K601E mutations were strain-specific. For example, W596L mutated 92UG037 and 92UG024 gp41 mediated normal gp120 association and cell-cell fusion, whereas QH1521.34-W596L exhibited little gp120 association. The K601E mutation resulted in weak (92UG037, QH1521.34) or absent gp120 anchoring (92UG024) with only QH1521.34 retaining CCR5- and CXCR4-dependent fusion activity at low but significant levels (p < 0.01, CCR5-dependent fusion; p < 0.05, CXCR4-dependent fusion for K601E relative to mock transfections). The gp120 association and fusion profiles observed for AD8 (R5), 89.6 (R5X4), and BH8 (X4) Env mutants are therefore not reflected in these primary strains, indicating that the functional contribution of the disulfide-bonded region amino acids is strain-specific.

**Functional Analysis of the Disulfide-bonded Region from Sequential HIV-1 Isolates**—The variety of mutational effects observed for the primary isolates is likely to be related to amino acid sequence variation in the Env ectodomain (14–26% sequence divergence in residues 31–677). It was therefore of interest to determine whether these mutations had more uniform effects in sequential isolates exhibiting a closer evolutionary relationship. QH0515.1 (R5) and QH1549.13 (R5X4) were isolated by Hu and co-workers (46) at 0 and 1,023 days post-serum conversion, respectively, from a Trinidadian male. QH0515.1 (R5) and QH1549.13 (R5X4) exhibit only 7.5% amino acid sequence divergence in the Env ectodomain (residues 31–677). All QH1549.13 mutants exhibited comparable gp160: gp41 ratios in Western blotting assays, indicating apparently normal synthesis and/or stability, whereas only QH0515.1-L593V gp41 was diminished relative to gp160 (Fig. 5A). Unexpectedly, the five mutations had markedly different effects on gp120-gp41 association, the five QH1549.13 mutants retaining wild-type amounts of gp120, whereas only QH0515.1-W596L and -K601E retained trace levels of gp120 (Fig. 5B). Fusion function was ablated by the mutations in both strains (Fig. 5C). These data indicate that the five target residues make variable contributions to gp120-gp41 association and membrane fusion function in closely related sequential HIV-1 isolates.

The **V1V2 and V3 Regions of Gp120 Modulate the Functional Role of Gp120-contact Residues**—To identify Env determinants that modulate the functional contribution of Leu597, Trp598, Gly607, Lys610, and Trp614, we analyzed a panel of chimeric Env proteins comprising variable and conserved domains of AD8 gp120 (macrophage-adapted, R5) on a BH8 Env background (TCLA, X4) (Fig. 6A). These two strains respond differently to the L593V, W596L, G597A, K601E, and W610F mutations: the five BH8 mutants exhibit ablated or markedly decreased gp120-gp41 association and fusion function, whereas three of five AD8 mutants (L593V, W596L, and K601E) retain these activities (Fig. 6B and Ref. 37). We first examined if V1V2 and V3 sequences, shown by others to be important for defining chemokine receptor specificity and tropism (35, 46, 58–60), contribute to the different phenotypes exhibited by corresponding AD8 and BH8 mutants. Replacement of the V1V2-C2-V3 region of BH8 with the corresponding region of AD8 (Fig. 6A, chimera AD8-V1–3) resulted in the utilization of CCR5 instead of CXCR4 as a fusion co-receptor (Fig. 6C), consistent with the chemokine receptor specificity of AD8. Near wild-type levels of gp120-gp41 association and CCR5-dependent fusion function were conferred to the AD8-V1–3-W596L and -K601E mutants (p > 0.15 relative to AD8-V1–3 transfections) (Fig. 6, B and C). Similar results were obtained for W596L and K601E mutants in chimeras that contained AD8 V1V2 and V3 but not C2 sequences (data not shown) indicating that the AD8-like phenotype of W596L and K601E mutants depends on the AD8 hypervariable loops. By contrast, AD8-V1–3-L593V and -G597A mutants were fusion-defective and had weak gp120-gp41 association (Fig. 6, B and C). The fusogenicity of L593V was not restored even after the entire gp120 domain of BH8 was replaced with that of AD8 in chimera AD8 (Fig. 6D) indicating that determinants outside AD8 gp120 may also be required for a functional chimeric L593V mutant.

We next asked if the AD8 V1V2 and V3 hypervariable regions could independently confer AD8-like characteristics to BH8 mutants by replacing these BH8 sequences with those of AD8 in chimeras AD8-V1V2 and AD8-V3 (Fig. 6A). The “wild-type” AD8-V1V2 chimera exhibited reduced gp120-gp41 association and an ~50% reduction in CCR5-dependent fusion function with respect to BH8 (Fig. 6, B and C). These results are consistent with previous observations that the functionality of Env chimeras containing heterologous V1V2 sequences is dependent on the Env background sequence context (61–65). AD8-V1V2, L593V, -W596L, and -K601E mutants lacked both gp120 association and cell-cell fusion function (Fig. 6, B and C), indicating that V1V2 of AD8 cannot complement the functional defects associated with these mutations in a BH8 background. Replacement of the V3 loop of BH8 with that of AD8 in AD8-V3 rendered BH8 with the associated utilization of CCR5 as a fusion cofactor instead of CXCR4 (Fig. 6C), consistent with the role of V3 in determining chemokine receptor specificity. However, AD8 V3 did not transfer an AD8-like phenotype to BH8 mutants. The ability of the AD8-V2 mutant to anchor gp120 resembled that of the BH8 mutants and 4 of 5 mutants were fusion-incompetent, with only the AD8-V3-K601E capable of CCR5-dependent fusion. These effects were not because of defects in Env biosynthesis as all chimeras were synthesized and processed to gp41 at normal levels (data not shown). Our findings indicate that V1V2 and V3 sequences act cooperatively to influence the functional role played by Trp607 and Lys610 within the gp41 disulfide-bonded region, whereas these determinants were not sufficient to functionally complement the chimeric L593V mutant. The variable nature of V1V2 and V3 may therefore account, in part, for phenotypic differences exhibited by corresponding mutants in diverse HIV-1 EnvVs. As the primary site of gp41 contact involves residues within gp120 C1 and C5 (36, 38) and deletion of V1V2 and V3 does not ablate gp120-gp41 association (66), we expect that V1V2 and V3 indirectly modulate the functional contribution of gp41 disulfide-bonded loop residues.

**DISCUSSION**

Protein-protein interaction surfaces exhibit a high degree of shape and charge complementarity, burying more than 600 Å² of protein surface (67, 68). A subset of interfacial residues
A typical example of conformational flexibility in Env glycoproteins involves the disulfide-bonded region of gp41, which is critical for Env function. This region contributes dominantly to the binding energy and is referred to as a "hotspot" (69, 70). The available evidence indicates that the disulfide-bonded region of gp41 and the C1 and C5 regions of gp120 interact to form an interface in the HIV-1 glycoprotein complex (36–38). Previously, we found that subtle mutagenesis of conserved residues (L593V, W596L, G597A, and W610F) within the disulfide-bonded region of the TCLA isolate HIV-1BH8 was sufficient to disrupt gp120-gp41 association and fusion function but not other indicators of glycoprotein folding such as precursor processing and gp41 stability. Env surface expression, and antigenic conformation (37). These observations are consistent with these gp41 residues contributing to a binding hotspot for gp120. In this study, we re-examined the functional role of Leu593, Trp596, Gly597, Trp610, and also Lys601, in Env derived from divergent primary HIV-1 strains, observing strain-specific contributions of individual residues to gp41 folding and/or stability, gp120-gp41 association, and membrane fusion function. For example, the five residues were important for efficient gp120-gp41 association and membrane fusion for BH8, QH1521.34, and QH0515.1, whereas Trp596 and Lys601 were dispensable for AD8, 89.6, 92UG037, and 92UG024 Env function. These data are consistent with a conserved role for the gp41 disulfide-bonded region in gp120 association. However, individual conserved residues within this region make strain-specific contributions to Env function, consistent with an adaptable gp120-gp41 association site.

The strain-specific functional effects of the mutations did not correlate with the chemokine receptor specificity of the isolate nor with its relatedness to other Env sequences. For example, the W596L and K601E mutations had distinct effects in dual tropic Envys, with 89.6 mutants retaining gp120 association and membrane fusion for BH8, QH1521.34, and QH0515.1, whereas Trp596 and Lys601 were dispensable for AD8, 89.6, 92UG037, and 92UG024 Env function. These data are consistent with a conserved role for the gp41 disulfide-bonded region in gp120 association. The AD8 and 89.6 mutant panels exhibited similar phenotypic profiles with L/M593V, W596L, and K601E mutants able to associate with gp120 and to mediate cell-cell fusion. However, marked differences in Env function were observed for Env-expressing 293T cells were labeled with Tran35S-label for 45 min and chased in complete medium for 4.5 h before lysis. Cell lysates (C) and clarified culture supernatants (S) were immunoprecipitated with IgG14 and protein G-Sepharose. Gp160 and gp120 bands were visualized following SDS-PAGE in 4–12% gradient gels under reducing conditions and scanning in a PhosphorImager SF. Mutant gp120-gp41 association relative to wild type: +, p < 0.05; ++, p < 0.01; ++++, p < 0.002 relative to wild type; –, no association. Representative of two independent experiments. C, cell-cell fusion activities of Env glycoprotein mutants. Assays were performed as described in the legend to Fig. 3 except for QH1521.34, where effectors were cocultured for 16 h with 293T targets transfected with pT4lac and pc.FUSIN prior to assay. The relative fusion activities of Env proteins were calculated as described in the legend to Fig. 3. The mean ± S.E. from three independent transfections are shown. Statistical analysis was performed as described in the legend to Fig. 3: 224, p < 0.05 relative to wild type; *, p < 0.002 relative to wild type; †, p < 0.00001 relative to wild type; ‡, p < 0.01 relative to mock-transfected cells.
These two strains in a viral context. The three AD8 mutants were competent to mediate viral entry, the level of infection correlating with the amount of gp120 incorporated into virions. However, of the three fusion-competent 89.6 Env mutants, M593V, W596L, and K601E, only M593V retained the ability to mediate viral entry via either chemokine receptor but at reduced levels relative to wild-type (CCR5, 1.6 log10 reduction; CXCR4, 1.1 log10 reduction). Whereas the entry defect associated with 89.6-W596L can be explained by an absence of virion-associated gp120, M593V and K601E were able to incorporate gp120. The M593V and K601E mutants may be capable of mediating cell-cell fusion through a functional cooperation between high concentrations of gp120-gp41 at the cell surface. By contrast, the limited number (7–14) of glycoprotein spikes on a virion may not be sufficient to overcome a kinetic or other defect associated with the M593V and K601E substitutions (71, 72).

An unusual cell-cell fusion phenotype was observed for 89.6-W596L. Whereas this mutant retained gp120 association function, its fusion competence depended on the chemokine receptor utilized as a fusion cofactor, with wild-type levels of fusion achieved with CXCR4, ~30% fusion activity with CCR5, and background fusion levels with CCR3. As the gp120-CD4 complex can bind to chemokine receptors independently of gp41 (13, 14, 28), it is unlikely that the fusion defects associated with W596L have resulted from a direct effect on receptor binding. Distinct regions of 89.6 Env have been shown to be important for the utilization of CXCR4, CCR5, and CCR3 in membrane fusion and viral entry (73, 74). Different modes of binding by 89.6 gp120 to these chemokine receptors may evoke distinct fusion-activation signals involving the site of gp120-gp41 association. For example, Trp596 may play a critical role in CCR3-triggered fusion, whereas this residue is dispensable when CXCR4 initiates the activation signal. Thus, residues within the disulfide-bonded region can play a direct role in fusion independent of an apparent role in gp120 association, perhaps by mediating the fusion-activation signal from receptor-bound gp120 to gp41. It will be of interest to determine whether 89.6-W596L and the QH1549.13 gp41 mutants are defective in CD4 and chemokine receptor-induced conformational changes that accompany Env fusion competence (16–18, 21).

Immune surveillance and preferential tropism for cellular targets are considered to represent significant forces driving Env sequence evolution (24, 46, 75–77). Amino acid changes in gp120 V3 alone, or in conjunction with changes in V1 and V2, have a profound influence on Env function with respect to CD4-induced conformational changes (55, 78–80), chemokine receptor specificity and tropism (35, 46, 58–60), CD4-independent entry (81), chemokine receptor affinity and kinetics of membrane fusion (82), viral replication competence (83, 84), and sensitivity to neutralization by antibody (85–87), sCD4 (55, 78, 88), and T-20/DP178 antiviral peptide (56, 82). We found that the transfer of V1V2 plus V3 sequences, but not V1V2 or V3 alone, from the R5 strain, AD8, to an X4 BH8 Env background resulted in the transfer of some phenotypic characteristics of AD8, namely, full gp120-gp41 association and CCR5-dependent fusion competence to the BH8-W596L and -K601E mutants. These results indicate that V1V2 and V3 sequences impact on the gp120-gp41 association site and on its role in Env fusion activity, potentially accounting for some of the different phenotypes associated with particular mutations.

**Fig. 5. Functional consequences of disulfide-bonded loop mutations in Env of sequential HIV-1 isolates.** A, synthesis and processing of HIV-1 Env glycoprotein mutants in 293T cells. At 36 h post-transfection, the cells were lysed and subjected to reducing SDS-PAGE in 10% polyacrylamide gels. Proteins were transferred to nitrocellulose prior to Western blotting with mAb 2F5 and chemiluminescent detection. *, reproducible decreases in gp41. Arrowhead, biosynthetic intermediate or degradation product of gp120. Representative of two independent experiments. B, gp120 anchoring ability of gp41 mutants. Env-expressing 293T cells were labeled with Tran35S-label for 45 min and chased in complete medium for 4.5 h before lysis. Cell lysates (C) and clarified culture supernatants (S) were immunoprecipitated with IgG#14 and protein G-Sepharose. Mutant gp120-gp41 association relative to wild type: ++, >0.7; +, 0.4–0.7; +/−, <0.1; −, no association. Representative of two independent experiments. C, cell-cell fusion activities of Env glycoprotein mutants. Assays were performed as described in the legend to Fig. 3. The mean ± S.E. from three independent transfections are shown.
Fig. 6. Functional analysis of disulfide-bonded region mutations in AD8-BH8 chimeric glycoproteins. A, schematic representation of AD8-BH8 Env chimeras. B, gp120 anchoring ability. Cell lysates (C) and clarified culture supernatants (S) were immunoprecipitated with IgG#14 and protein G-Sepharose. Gp160 and gp120 bands were visualized as described above. Representative of three independent experiments. Mutant gp120-gp41 association relative to wild type: ++, >0.7; +, 0.4–0.7; +/−, <0.4; −, no association. C, cell-cell fusion activity. 293T effector cells were transfected with wild-type and mutated Env expression vectors and then infected with vTF7.3 while target 293T cells were cotransfected with pT4-luc plus either pc.CKR5 or pc.FUSIN. At 24 h postinfection, effectors and targets were mixed and cocultured for 16 h prior to lysis and assay for luciferase activity. D, gp120 anchoring ability and cell-cell fusion activity AD8120 chimeras. Cell-cell fusion data for BH8 were obtained from Ref. 37. The mean ± S.E. from three independent transfections are shown. nd, not determined.
in diverse Env contexts. That the three hypervariable regions were required for the transfer of AD8-like properties to W596L and K601E BH8 gp41 mutants is consistent with the idea that these regions act cooperatively in receptor-induced structural transitions leading to the membrane fusion activity of Env (65, 89, 90). However, determinants outside V1V2 and V3 are also likely to influence the function of the disulfide-bonded region as chimerization of AD8 gp120 to BH8 gp41 did not functionally complement the defect associated with L593V.

Our findings with AD8-BH8 Env chimeras that the phenotype of particular mutants is dependent on V1V2 and V3 sequences is consistent with the results of other investigations of hypervariable loop deletion mutants. Whereas deletion of V1V2 plus V3 does not cause dissociation of gp120 from gp41 (66), this deletion (but not individual V1V2 or V3 deletions), neverthless, impacts on the gp120-gp41 association site because an 89, 90). However, determinants outside V1V2 and V3 are also likely to influence the function of the disulfide-bonded region as chimerization of AD8 gp120 to BH8 gp41 did not functionally complement the defect associated with L593V.

Functional Evolution of the HIV-1 Gp120-Gp41 Association Site
Functional Evolution of the HIV-1 Envelope Glycoprotein 120 Association Site of Glycoprotein 41
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J. Biol. Chem. 2003, 278:42149-42160.
doi: 10.1074/jbc.M305223200 originally published online August 15, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M305223200

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