JOURNAL OF VIROLOGY, Sept. 1992, p. 5472–5478
0022-538X/92/095472-07$02.00/0

Vol. 66, No. 9

Identification and Characterization of Fusion and Processing Domains of the Human Immunodeficiency Virus Type 2 Envelope Glycoprotein†

ERIC O. FREED‡* AND DAWN J. MYERS

McArdle Laboratory for Cancer Research, 1400 University Avenue, University of Wisconsin at Madison, Madison, Wisconsin 53706

Received 6 December 1991/Accepted 19 June 1992

The envelope glycoprotein of the human immunodeficiency virus type 2 (HIV-2) is synthesized as a polyprotein precursor which is proteolytically processed to produce the mature surface and transmembrane envelope glycoproteins. The processed envelope glycoprotein species are responsible for the fusion between the viral envelope and the host cell membrane during the infection process. The envelope glycoprotein also induces syncytium formation between envelope-expressing cells and receptor-bearing cells. To characterize domains of the HIV-2 envelope glycoprotein involved in membrane fusion and in proteolytic processing, we introduced single amino acid mutations into the region of the HIV-2 surface glycoprotein corresponding to the principal neutralizing determinant (the V3 loop) of HIV-1, the putative HIV-2 envelope precursor-processing sequence, and the hydrophobic amino terminus of the HIV-2 transmembrane envelope glycoprotein. The effects of these mutations on syncytium formation, virus infectivity, envelope expression, envelope processing, and CD4 binding were analyzed. Our results suggest that the V3-like region of the HIV-2 surface glycoprotein and the hydrophobic amino terminus of the transmembrane glycoprotein are HIV-2 fusion domains and characterize the effects of mutations in the HIV-2 envelope glycoprotein precursor-processing sequence.

The human immunodeficiency virus type 1 (HIV-1) and the human immunodeficiency virus type 2 (HIV-2) are linked to the current worldwide epidemic of AIDS. Unlike HIV-1 infection, which has spread widely throughout the world, most HIV-2 infection is localized to regions of West Africa (7). While HIV-1 and HIV-2 share a similar genetic organization, they are considerably divergent at the amino acid sequence level. The HIV-1 and HIV-2 envelope glycoproteins, for example, share only approximately 40% amino acid sequence identity (18). HIV-2 is, however, closely related to certain simian immunodeficiency viruses (SIV). The envelope glycoproteins of HIV-2 and the SIV of the rhesus macaque (SIVmac) share approximately 75% amino acid sequence identity (8).

The envelope glycoprotein of HIV-2, like that of other retroviruses, is synthesized as a polyprotein precursor which is proteolytically processed by a host protease to generate the surface (SU) envelope glycoprotein and the transmembrane (TM) envelope glycoprotein. The SU glycoprotein binds the HIV receptor molecule CD4, while the TM glycoprotein anchors the SU/TM complex in the viral envelope or the plasma membrane of the infected cell.

Following the binding of CD4 by the HIV SU glycoprotein, the SU/TM complex triggers a membrane fusion reaction between the viral envelope and the host cell membrane. This membrane fusion reaction is an essential step in the infection process (34). Once a cell is infected, the SU/TM complex can also induce fusion between the infected cell and uninfected $CD4^+$ cells, leading to the formation of giant polynucleated syncytia (27, 33). Syncytium formation, which results in extensive cell death in culture, may contribute to the depletion of $CD4^+$ cells in infected individuals. The membrane fusion function of the HIV envelope glycoprotein therefore plays an important role in the life cycle and perhaps in the pathogenic properties of HIV.

Mutational studies have identified several domains of the HIV-1 envelope glycoprotein that are involved in the fusion process. These domains include the highly hydrophobic amino terminus of the TM glycoprotein (10, 13, 23) and the principal neutralizing determinant, or V3 loop, of the SU glycoprotein (14, 16, 19). Proper proteolytic processing of the HIV-1 envelope precursor is also required to activate the fusion function (12, 29).

Although the Cys residues that are involved in V3 loop formation in the HIV-1 SU glycoprotein (26) are conserved in HIV-2 (18), the V3 region of HIV-1 and the corresponding region of HIV-2 are poorly conserved. Especially notable is the lack of significant sequence conservation between HIV-1 and HIV-2 in the central, or tip, region of V3. This tip region, a G-P-G-R motif in particular, is highly conserved among different strains of HIV-1 (25).

Retroviral envelope glycoprotein precursors are generally cleaved by a host protease on the carboxy-terminal side of the basic pair of amino acids in Arg-X-Lys/Arg-Arg sequences (where X is any amino acid). Cleavage at Arg-X-Lys/Arg-Arg motifs is thought to be catalyzed by furin or other members of the mammalian subtilisin family of proteases (2, 20). The envelope glycoproteins of HIV-1 and HIV-2 have two sequences resembling retroviral precursor cleavage sequences: a downstream site (site 1) and an upstream site (site 2). The results of mutational analyses from several laboratories have strongly suggested that cleavage of the HIV-1 envelope glycoprotein precursor occurs primarily at the site 1 Arg (5, 12, 29). The site of cleavage of the HIV-2 envelope glycoprotein precursor has not been identified and has been the subject of debate since certain strains of HIV-2

* Corresponding author.

† We dedicate this paper to the memory of Rex Risser.

‡ Present address: Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892.

have a Thr-Arg pair rather than a basic pair of amino acids at site 1 (18, 36). A previous study indicated that mutation of the Rous sarcoma virus envelope precursor cleavage sequence from Arg-Arg-Lys-Arg to Arg-Arg-Glu-Arg almost completely blocked precursor processing (31). Cleavage of the HIV-1 envelope precursor, however, is not absolutely dependent upon the presence of a basic pair of amino acids at site 1. Mutation of the HIV-1 site 1 sequence from Arg-Glu-Lys-Arg to Arg-Glu-Asn-Arg reduced but did not block precursor processing (5), and mutation of the HIV-1 site 1 sequence to Arg-Glu-Ile-Arg did not affect precursor cleavage (12).

To characterize the domains of the HIV-2 envelope glycoprotein that are involved in processing and fusion, single amino acid substitutions were introduced in three regions of the HIV-2 SU and TM envelope glycoproteins: the region of the HIV-2 SU glycoprotein corresponding to the V3 loop of HIV-1 (the V3-like domain), the predicted site of proteolytic processing of the HIV-2 envelope precursor, and the highly hydrophobic amino terminus of the HIV-2 TM glycoprotein. The effects of these mutations on syncytium formation, envelope expression, envelope processing, CD4 binding, and infectivity were examined.

MATERIALS AND METHODS

Expression of the HIV-2 envelope glycoprotein. To study the HIV-2 envelope glycoprotein in the absence of virus replication, we constructed the HIV-2 envelope expression plasmid pCMVHIV-2env. The construction of this plasmid has been described previously (11). Briefly, the *env* region from the HIV-2_{ROD} strain of HIV-2 was removed from the plasmid pSP275 (provided by M. Emerman, Fred Hutchinson Cancer Research Center, Seattle, Wash.) and introduced into the plasmid p763 (provided by B. Sugden, University of Wisconsin, Madison). p763 contains the human cytomegalovirus immediate-early promoter/enhancer which drives HIV-2 envelope expression in pCMVHIV-2env. Since the *env* region introduced into pSP275 lacks the *rev* open reading frame, pCMVHIV-2env was provided with HIV-1 Rev in *trans* by cotransfection with pHenvKFS (11). Cotransfection of pCMVHIV-2env and pHenvKFS into the $CD4^+$ HeLa T4 cell line results in the formation of approximately 1,000 syncytia per μg of transfected pCMVHIV-2env DNA.

Mutagenesis of the HIV-2 envelope gene. The *Eco*RI-*Bam*HI fragment from pCMVHIV-2env was introduced into M13 and subjected to oligonucleotide-directed mutagenesis as described by Kunkle et al. (24). After mutagenesis, the presence of the desired mutation was detected by DNA sequencing, and the mutagenized fragment was reintroduced into pCMVHIV-2env. After recloning, DNA sequencing was performed again to confirm the presence of the mutation.

Cell culture and fusion assays. HeLa cells and HeLa T4 cells (28) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (HyClone). HeLa T4 cell medium was also periodically supplemented with G418 (900 μg/ml; GIBCO). The CD4 gene was effectively maintained by culturing the cells for several days in G418 at 2-week intervals. Transfections were performed as previously described (15). One to two days posttransfection, cells were stained and the number of syncytia were scored microscopically as described previously (13).

Radioimmunoprecipitation of the HIV-2 envelope glycoprotein. At 2 days posttransfection, HeLa T4 cells were metabolically labeled with [^{35}S]methionine for approximately 15 h, lysed, and immunoprecipitated with serum from rhesus macaques infected with SIVmac. Macaque serum was provided by K. Schultz, University of Wisconsin, Madison. The immunoprecipitated envelope glycoproteins were subjected to polyacrylamide gel electrophoresis, the gels were treated with En^{3}Hance (New England Nuclear Corp., Boston, Mass.), dried, and placed on film.

CD4-binding assay. Our method for analyzing CD4 binding has been described previously (14, 16). Briefly, labeled cell lysates were immunoprecipitated with the anti-CD4 monoclonal antibody OKT4 (Ortho Diagnostic Systems, Inc., Raritan, N.J.). Before polyacrylamide gel electrophoresis, the immunoprecipitates were reprecipitated with serum from a rhesus macaque infected with SIVmac. Envelope glycoproteins were thus precipitated in the first immunoprecipitation by their association with CD4; the envelope glycoproteins detected in this assay therefore represent CD4-bound envelope glycoproteins.

HIV-2 infectivity assays. To analyze the ability of the mutant HIV-2 envelope glycoproteins to function in the infection process, we used wild-type or mutant HIV-2 envelope glycoproteins in a *trans*-complementation assay with the replication-defective HIV-1 proviral clone pGB108 (provided by G. L. Buchschacher, Jr., and A. T. Panganiban, University of Wisconsin, Madison). pGB108 is a derivative of pNL4-3 (1) containing an *env* deletion into which was cloned the hygromycin resistance gene expressed from a simian virus 40 promoter. The use of pGB108 in *trans*-complementation assays has been described elsewhere (8a, 11). Briefly, HeLa cells (5×10^5 cells per 60-mm tissue culture dish) were cotransfected with pCMVHIV-2env (or mutant-containing derivatives) and pGB108. At 1 day posttransfection, the medium was changed, and at 2 days posttransfection, the supernatant was harvested, mixed with DEAE-dextran (to a final concentration of 8 μg/μl), and used to infect HeLa T4 cells. On the day following infection, the medium was replaced with medium containing 200 μg of hygromycin B per ml. The cells were scored for hygromycin-resistant colonies at 8 to 13 days postinfection.

RESULTS

Mutagenesis of the HIV-2 envelope glycoprotein. An amino acid sequence comparison of the V3 loop of HIV-1 and the corresponding region of HIV-2 is presented in Fig. 1A. An amino acid sequence comparison of the envelope precursor processing regions of HIV-1 and HIV-2 is presented in Fig. 1B. To determine whether the V3-like region of the HIV-2 SU glycoprotein is involved in fusion, single amino acid changes were introduced at positions throughout the predicted loop region (Fig. 2). Pro at amino acid 308 was mutated to Arg (308PR), the Ile at amino acid 316 was changed to Val (316IV), the Gly at amino acid 321 was changed to Glu (321GE), the His at amino acid 325 was mutated to Asp (325HD), and the Cys at amino acid 340, which is predicted to be involved in disulfide bridge formation (21), was changed to Ser (340CS).

Single amino acid mutations were also introduced in the HIV-2 envelope precursor processing region (Fig. 3). The Arg at amino acid 501 was mutated to Lys (501RK), Thr (501RT), His (501RH), and Gln (501RQ); the Thr at residue 510 was changed to Lys (510TK); and the Arg at residue 511 was mutated to Lys (511RK), Thr (511RT), and Gln (511RQ). Finally, a polar substitution (Val to Glu) was introduced in the hydrophobic amino terminus of the TM glycoprotein (2VE).

Effects of HIV-2 envelope glycoprotein mutations on syncy-

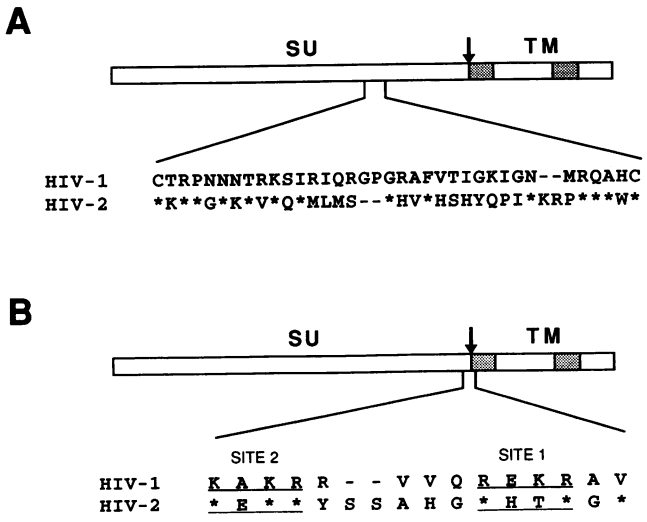

FIG. 1. HIV-1 and HIV-2 amino acid sequence comparisons. Amino acid sequence comparison of the principal neutralizing determinant (the V3 loop) of HIV-1 and the corresponding region of HIV-2 (A) and the HIV-1 and HIV-2 envelope precursor processing regions (B). HIV-1 sequence, HIV-1_{BRU} (35); HIV-2 sequence, HIV-2_{ROD} (18). The shaded regions represent hydrophobic domains; the arrows indicate the site of envelope precursor cleavage. Asterisks represent amino acid sequence identity.

tium formation. After mutagenesis, the mutant *env* genes were introduced into the HIV-2 envelope glycoprotein expression vector pCMVHIV-2env (see Materials and Methods) (11). To analyze the effects of the HIV-2 mutations on syncytium formation, the wild-type and mutant pCMVHIV-2env expression constructs were introduced via transfection into the $CD4^+$ HeLa T4 cell line (28). The number of syncytia resulting from transfection with mutant-containing pCMVHIV-2env expression vectors was compared with the number of syncytia resulting from transfection with wild-type pCMVHIV-2env (Table 1). In the V3-like region of the HIV-2 SU glycoprotein, the 308PR, 321GE, 325HD, and 340CS mutations completely abolished syncytium formation induced by the HIV-2 envelope glycoprotein. The conservative 316IV mutation, however, had no effect on syncytium formation.

In the region suspected of being the target of precursor proteolytic processing, mutation of both the site 1 and site 2

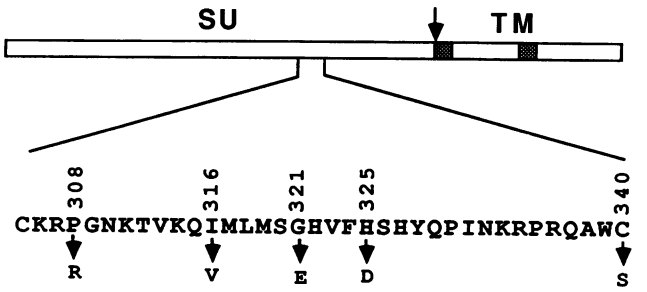

FIG. 2. Single amino acid mutations introduced into the V3-like region of the HIV-2 SU envelope glycoprotein. The positions of mutated residues are indicated. The shaded regions represent hydrophobic domains; the arrow above the bar indicates the site of envelope precursor cleavage.

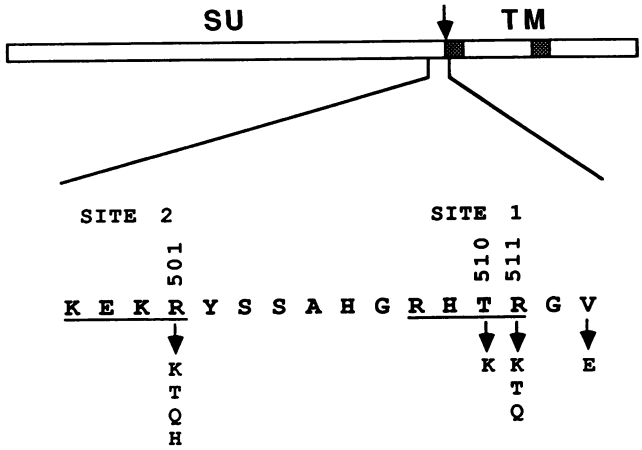

FIG. 3. Single amino acid mutations introduced into the HIV-2 envelope precursor processing region. The positions of mutated residues are indicated. Site 1 and site 2 are the two potential sites of precursor processing. The shaded regions represent hydrophobic domains; the arrow above the bar indicates the site of envelope precursor cleavage.

Arg residues to Lys did not block syncytium formation. In contrast, mutation of the site 1 Arg to Thr or Gln and mutation of the site 2 Arg to Thr or Gln completely abolished syncytium formation. Mutation of the site 2 Arg to His reduced syncytium formation to 1% of wild-type levels. Creating a basic (Lys-Arg) pair at site 1 by introducing the 510TK mutation had no effect on syncytium formation. Finally, in the hydrophobic amino terminus of the HIV-2 TM glycoprotein, a Val-to-Glu mutation (2VE) completely abolished syncytium formation induced by the HIV-2 envelope glycoprotein.

Effects of HIV-2 mutations on the infection process. To examine the ability of the mutant HIV-2 envelope glycoproteins to function in the infection process, we performed *trans*-complementation assays with the replication-defective HIV-1 proviral clone pGB108 (see Materials and Methods). pGB108, which lacks a functional *env* gene, encodes the

TABLE 1. Effects of HIV-2 envelope glycoprotein mutations on syncytium formation[a]

Mutation	Change	Relative syncytium formation[b]
None (wild-type control)		100
308PR	Pro→Arg	<1
316IV	Ile→Val	96
321GE	Gly→Glu	<1
325HD	His→Asp	<1
340CS	Cys→Ser	<1
501RK	Arg→Lys	139
501RT	Arg→Thr	<1
501RQ	Arg→Gln	<1
501RH	Arg→His	1
510TK	Thr→Lys	106
511RK	Arg→Lys	31
511RT	Arg→Thr	<1
511RQ	Arg→Gln	<1
2VE	Val→Glu	<1

[a] All data represent averages of at least six assays.
[b] Standard deviations: 316IV, 20; 501RK, 93; 510RH, 2; 510TK, 16; 511RK, 24.

TABLE 2. Effects of HIV-2 envelope glycoprotein mutations on the formation of infectious virus[a]

Mutation	Change	Relative Hyg^r (CFU/ml)[b]
None (wild-type control)		100
308PR	Pro→Arg	0
316IV	Ile→Val	143
321GE	Gly→Glu	0
325HD	His→Asp	0
340CS	Cys→Ser	0
501RK	Arg→Lys	10
501RT	Arg→Thr	0
501RQ	Arg→Gln	0
501RH	Arg→His	0
510TK	Thr→Lys	102
511RK	Arg→Lys	7
511RT	Arg→Thr	0
511RQ	Arg→Gln	0
2VE	Val→Glu	0

[a] All data represent averages of at least three assays.

[b] HeLa cells were cotransfected with 10 μg of wild-type or mutant pCMVHIV-2env plus 5 μg of pGB108. Virus pools were harvested and used to infect HeLa T4 cells. Standard deviations: 316IV, 40; 501RK, 10; 510TK, 34; 511RK, 6.

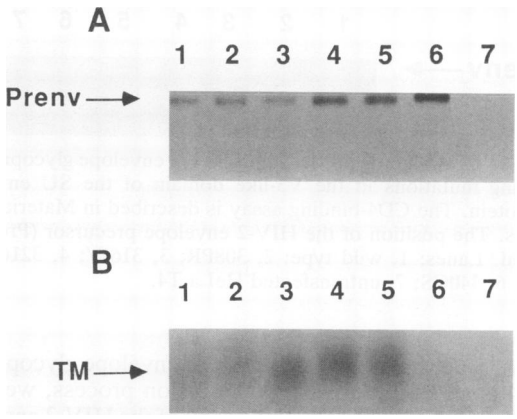

FIG. 4. Radioimmunoprecipitation of HIV-2 envelope glycoproteins containing mutations in the V3-like domain of the SU envelope glycoprotein. Radioimmunoprecipitation analysis was conducted as described in Materials and Methods. (A) HIV-2 envelope precursor (Prenv); (B) a longer exposure of the lower portion of the gel shown in panel A. TM indicates the position of the HIV-2 TM envelope glycoprotein. Lanes: 1, wild type; 2, 308PR; 3, 316IV; 4, 321GE; 5, 325HD; 6, 340CS; 7, untransfected HeLa T4.

gene conferring resistance to hygromycin B. *trans* complementation by pCMVHIV-2env allows transfer of the hygromycin B resistance gene to the infected cell. Selection of infected cultures with hygromycin B and scoring the number of hygromycin B-resistant colonies provide a measure of the ability of the envelope glycoprotein provided in *trans* to function in the infection process. The results of this analysis are provided in Table 2. Those mutants which were blocked for syncytium formation (Table 1) were unable to produce infectious virus particles. The 316IV and 510TK mutations had no effect on infectivity, while the 501RK and 511RK mutations greatly reduced but did not block the formation of infectious virus particles.

Expression and processing of mutant HIV-2 envelope glycoproteins. To analyze the effects of the *env* mutations on envelope glycoprotein expression and processing, HeLa T4 cells were transfected, metabolically labeled with [^{35}S]methionine, lysed, and immunoprecipitated with serum from a rhesus macaque infected with SIVmac. Of the eight macaque serum samples tested that were reactive with the HIV-2 envelope glycoprotein, all bound the HIV-2 envelope precursor (Prenv) and the TM glycoprotein, while none bound the mature HIV-2 SU glycoprotein (data not shown). Thus, our analysis of envelope precursor processing relies upon the presence or absence of the mature TM glycoprotein. Data for the V3-like region mutants are presented in Fig. 4, with the HIV-2 envelope precursors (Prenv) shown in panel A and the TM glycoproteins shown in panel B. The 308PR, 316IV, 321GE, and 325HD mutations had no significant effect on the expression or processing of the HIV-2 envelope glycoprotein (Fig. 4, lanes 2 to 5). The 340CS mutation, however, abolished proteolytic processing of the HIV-2 envelope glycoprotein precursor (Fig. 4, lane 6). These results indicated that mutations in the V3-like region of the HIV-2 SU envelope glycoprotein did not affect envelope precursor processing. However, mutation of the putative disulfide bond-forming Cys at amino acid 340 apparently disrupted envelope glycoprotein structure sufficiently to block precursor processing.

We also analyzed the effects of the processing region mutations on envelope expression and processing (Fig. 5). The 501RK, 501RT, 501RQ, and 501RH mutations reduced but did not abolish precursor processing. The 510TK mutation had no effect on precursor processing. The 511RK mutation reduced precursor processing, while the 511RT and 511RQ mutations completely blocked processing of the HIV-2 envelope precursor (Fig. 5B, lanes 8 and 9). The observation that site 1 (amino acid 511) mutations blocked precursor processing supports the hypothesis that site 1 is the primary site of HIV-2 envelope precursor processing.

The 2VE mutation (located in the hydrophobic amino terminus of the HIV-2 TM glycoprotein) did not affect HIV-2 envelope expression or processing (Fig. 5, lane 10).

CD4-binding properties of mutant HIV-2 envelope glyco-

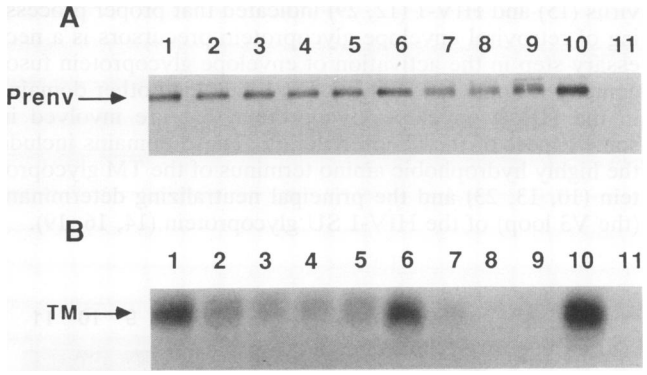

FIG. 5. Radioimmunoprecipitation of envelope glycoproteins containing mutations in the precursor processing region and in the hydrophobic amino terminus of the TM envelope glycoprotein. Radioimmunoprecipitation analysis was conducted as described in Materials and Methods. (A) HIV-2 envelope precursor (Prenv); (B) a longer exposure of the lower portion of the gel shown in panel A. TM indicates the position of the HIV-2 TM envelope glycoprotein. Lanes: 1, wild type; 2, 501RK; 3, 501RT; 4, 501RH; 5, 501RQ; 6, 510TK; 7, 511RK; 8, 511RT; 9, 511RQ; 10, 2VE; 11, untransfected HeLa T4.

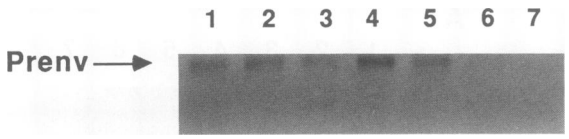

FIG. 6. CD4-binding properties of HIV-2 envelope glycoproteins containing mutations in the V3-like domain of the SU envelope glycoprotein. The CD4-binding assay is described in Materials and Methods. The position of the HIV-2 envelope precursor (Prenv) is indicated. Lanes: 1, wild type; 2, 308PR; 3, 316IV; 4, 321GE; 5, 325HD; 6, 340CS; 7, untransfected HeLa T4.

proteins. Since binding of the HIV SU envelope glycoprotein to CD4 is an essential step in the fusion process, we were interested in determining whether any of the HIV-2 envelope glycoprotein mutations disrupted binding between the HIV-2 envelope and CD4. Our method for analyzing CD4 binding has been previously reported (13, 14) and is described in Materials and Methods. The CD4-binding assays were performed with the same lysates used in the immunoprecipitations shown in Fig. 4 and 5. Figure 6 presents CD4-binding data for the V3-like region mutants. The envelope glycoproteins detected in this assay represent CD4-bound envelope glycoproteins. The 308PR, 316IV, 321GE, and 325HD mutations did not block binding between the HIV-2 envelope glycoprotein and CD4. The 340CS mutation, however, disrupted CD4 binding (Fig. 6, lane 6).

The CD4-binding data for the processing region mutants are presented in Fig. 7. Since CD4-bound envelope is evident in all lanes, these results indicate that none of the processing region mutations blocks CD4 binding. Because this CD4-binding assay is not highly quantitative, we cannot exclude the existence of subtle differences in CD4-binding affinities.

DISCUSSION

The fusion function of retroviral envelope glycoproteins plays an essential role in the infection process and may also contribute to the pathogenic properties of primate immunodeficiency viruses. Studies conducted with murine leukemia virus (15) and HIV-1 (12, 29) indicated that proper processing of retroviral envelope glycoprotein precursors is a necessary step in the activation of envelope glycoprotein fusogenicity. Mutational analyses have identified other domains in the HIV-1 envelope glycoprotein that are involved in some aspect of the fusion reaction. These domains include the highly hydrophobic amino terminus of the TM glycoprotein (10, 13, 23) and the principal neutralizing determinant (the V3 loop) of the HIV-1 SU glycoprotein (14, 16, 19).

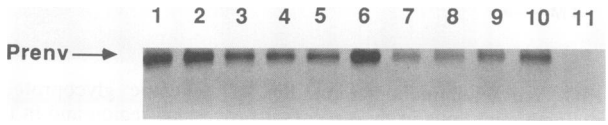

FIG. 7. CD4-binding properties of HIV-2 envelope glycoproteins containing mutations in the precursor processing region and in the hydrophobic amino terminus of the TM envelope glycoprotein. The CD4-binding assay is described in Materials and Methods. The position of the HIV-2 envelope precursor (Prenv) is indicated. Lanes: 1, wild type; 2, 501RK; 3, 501RT; 4, 501RH; 5, 501RQ; 6, 510TK; 7, 511RK; 8, 511RT; 9, 511RQ; 10, 2VE; 11, untransfected HeLa T4.

To identify and characterize domains of the HIV-2 envelope glycoprotein that are involved in fusion and envelope precursor processing, we introduced mutations in the region of the HIV-2 SU glycoprotein corresponding to the principal neutralizing determinant (the V3 loop) of HIV-1, the predicted HIV-2 envelope precursor-processing sequence, and the hydrophobic amino terminus of the HIV-2 TM glycoprotein.

Nonconservative mutations throughout the V3-like region of the HIV-2 SU glycoprotein completely abolished syncytium formation. A conservative mutation in this region (at amino acid 316) had no effect on fusion. Using an envelope glycoprotein *trans*-complementation system, we determined that the nonconservative mutations in the V3-like domain blocked the formation of infectious virus particles, while the conservative mutation at amino acid 316 had no effect on infectivity. These results suggest that the V3-like region of the HIV-2 SU glycoprotein plays a role in the membrane fusion process.

The results of radioimmunoprecipitation analysis indicated that mutations at amino acids 308, 316, 321, and 325 had no effect on envelope expression or precursor processing, nor did they abolish binding to CD4. Because the antisera used in this study do not detect the HIV-2 SU glycoprotein, we cannot rule out the possibility that the association between the SU and TM glycoproteins may be affected by some of these mutations. Linker insertion mutations at a number of positions in the HIV-1 SU and TM can disrupt SU/TM association (23). Our previous studies with the V3 loop of HIV-1, however, indicated that single amino acid mutations in this region have no effect on the association between the SU and TM glycoproteins (14, 16). We must also emphasize that our CD4-binding assay is not quantitative and therefore might not detect subtle differences in CD4-binding affinity.

Mutation of the Cys residue at amino acid 340 completely abolished precursor processing and greatly reduced CD4 binding. We previously obtained a similar result upon mutation of the Cys at the analogous position in the HIV-1 SU glycoprotein (14). This Cys residue in HIV-1 has been shown to be involved in disulfide bond formation (26). The fact that mutation of the amino acid 340 Cys of the HIV-2 SU glycoprotein results in defects in both precursor processing and CD4 binding suggests, but certainly does not prove, that this residue is involved, as it is in HIV-1, in disulfide bond formation. Elimination of this disulfide bond presumably results in a global perturbation of SU glycoprotein structure, thereby interfering with precursor processing and CD4 binding.

Comparison of the amino acid sequence of the HIV-1 V3 loop and the corresponding region of HIV-2 (Fig. 1A) reveals no extensive sequence conservation. The central portion of this region, which is highly conserved among HIV-1 isolates (25), is particularly poorly conserved between HIV-1 and HIV-2. The absence of significant sequence conservation between the tip of the HIV-1 V3 loop and the corresponding region of HIV-2 is intriguing in light of the fact that these regions appear to have similar functions. As we demonstrated here, the V3-like region of HIV-2 appears to play a role in membrane fusion induced by the HIV-2 envelope glycoprotein, and several recent studies suggest that, as is the case for HIV-1, this region may be an immunodominant epitope and a neutralizing determinant of the HIV-2 envelope glycoprotein (3, 6, 9, 30). Other investigators, however, demonstrated that peptides corresponding to the V3-like region of HIV-2 do not elicit neutralizing antibodies (32).

This latter report suggests that loop conformation may play an important role in antibody recognition of this region.

The HIV-1 and HIV-2 envelope glycoprotein precursors contain two sites, site 1 and site 2, which resemble sequences utilized by the host protease to proteolytically process retroviral envelope glycoprotein precursors (Fig. 1B). The sequence at site 1 in certain strains of HIV-2 (for example, HIV-2_{ROD} and HIV-2_{NIH-Z} [18, 36]) contains a Thr-Arg pair instead of the Lys-Arg or Arg-Arg (dibasic) pair typically found at the site of envelope precursor proteolytic processing. To characterize the precursor-processing sequence of the HIV-2 envelope glycoprotein, we introduced single amino acid mutations at both the site 1 and site 2 Arg residues (Fig. 3). Conservative and nonconservative mutations were introduced. We also created a basic pair at site 1 by mutating the amino acid Thr-510 to Lys. Our results indicated that conservative (Arg to Lys) mutations at site 1 or site 2 did not block syncytium formation induced by the HIV-2 envelope glycoprotein, whereas nonconservative mutations at either position greatly reduced or completely abolished syncytium formation. The mutations that blocked syncytium formation also abolished the ability of the mutant envelope glycoproteins to *trans* complement an envelope-defective HIV-1 proviral clone. Proteolytic processing of the HIV-2 envelope precursor was more sensitive to mutations at site 1 than at site 2, consistent with the hypothesis that site 1 is the primary site of precursor processing. Creation of a basic pair at site 1 had no effect on syncytium formation, infectivity, or processing. None of the mutations in the processing region affected envelope glycoprotein binding to the CD4 receptor molecule.

It is interesting that mutations at site 2 had such a major impact on envelope precursor processing and syncytium formation. There are, however, precedents for upstream mutations greatly affecting proteolytic processing reactions. Mutations at site 2 of the HIV-1 envelope glycoprotein reduced processing of the HIV-1 envelope glycoprotein precursor (5, 22). Also, mutations upstream of the presomatostatin cleavage site (a Lys-Arg pair) greatly affected formation of the mature products (17). Furin, or other members of the subtilisin family of mammalian proteases, is thought to mediate the cleavage of viral envelope precursors at Arg-X-Lys-Arg sequences (where X is any amino acid) (2, 20). These enzymes generally prefer to cleave at basic pairs of amino acids, rather than at single basic residues (2). Perhaps the lack of a basic pair of amino acids at site 1 makes HIV-2 envelope precursor cleavage particularly sensitive to upstream mutations. We cannot, however, rule out more-complex interpretations of the results presented here. For example, complete processing of the HIV-2 envelope precursor and the resulting activation of the TM glycoprotein fusion domain might require cleavage reactions at both site 1 and site 2. Thus, mutations at either site would dramatically affect the efficiency of the processing reaction and the activation of the fusion domain. Nor can we exclude the possibility that alternative cleavage sites can be utilized to process mutant HIV-2 envelope glycoprotein precursors. It is also possible that the block in processing observed for some of these mutants may result from impaired envelope transport. Further analysis of this region will provide more information concerning the role of site 1 and site 2 sequences in precursor processing. In particular, it would be interesting to determine whether creation of a basic pair at site 1 (with the 510TK mutation, for example) would render the processing reaction less sensitive to mutations at site 2.

The hydrophobic amino termini of the HIV-1 (10, 13, 23) and SIVmac (4) TM glycoproteins play a role in the fusion process. Because of the close relationship between SIVmac and HIV-2 (8), one would predict that the hydrophobic amino terminus of the HIV-2 TM glycoprotein is also a fusion domain. To test this prediction, we introduced a polar substitution mutation (Val to Glu) in the amino-terminal hydrophobic region of the HIV-2 TM glycoprotein. This mutation (2VE) completely abolished syncytium formation without affecting envelope precursor processing or CD4 binding. The 2VE mutation also blocked the ability of the HIV-2 envelope glycoprotein to *trans* complement an *env*-minus HIV-1 proviral clone. These results suggest that the hydrophobic amino terminus of the HIV-2 TM glycoprotein functions in the fusion process.

The data presented here identify the V3-like region of the HIV-2 SU envelope glycoprotein and the hydrophobic amino terminus of the HIV-2 TM glycoprotein as fusion domains. This study also characterizes the effects of single amino acid mutations in the two sequences of the HIV-2 envelope glycoprotein which resemble sites of envelope precursor processing. Further studies will elucidate the mechanism by which these domains function in the membrane fusion process.

ACKNOWLEDGMENTS

We thank R. Axel for the HeLa T4 cell line, G. L. Buchschacher, Jr., and A. T. Panganiban for pGB108, M. Emerman for pSP275, and K. Schultz for the rhesus macaque serum. We also thank H. M. Temin for helpful discussions.

This work was supported by Public Health Service grant CA22443 from the National Institutes of Health.

REFERENCES

1. **Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin.** 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J. Virol. **59**:284–291.
2. **Barr, P. J.** 1991. Mammalian subtilisins: the long-sought dibasic processing endoproteases. Cell **66**:1–3.
3. **Bjorling, E., K. Broliden, D. Bernardi, G. Utter, R. Thorstensson, F. Chiodi, and E. Norrby.** 1991. Hyperimmune antisera against synthetic peptides representing the glycoprotein of human immunodeficiency virus type 2 can mediate neutralization and antibody-dependent cytotoxic activity. Proc. Natl. Acad. Sci. USA **88**:6082–6086.
4. **Bosch, M. L., P. L. Earl, K. Fargnoli, S. Picciafuoco, F. Giombini, F. Wong-Staal, and G. Franchini.** 1989. Identification of the fusion peptide of primate immunodeficiency viruses. Science **244**:694–697.
5. **Bosch, V., and M. Pawlita.** 1990. Mutational analysis of the human immunodeficiency virus type 1 *env* gene product proteolytic cleavage. J. Virol. **64**:2337–2344.
6. **Bottiger, B., A. Karlsson, P.-A. Andreasson, A. Naucler, C. M. Costa, E. Norrby, and G. Biberfeld.** 1990. Envelope cross-reactivity between human immunodeficiency virus types 1 and 2 detected by different serological methods: correlation between cross-neutralization and reactivity against the main neutralizing site. J. Virol. **64**:3492–3499.
7. **Brun-Vezinet, F., C. Katlama, D. Roulot, L. Lenoble, M. Alizon, J. J. Madjar, M. A. Rey, P. M. Girard, P. Yeni, F. Clavel, S. Gadelle, and M. Harzic.** 1987. Lymphadenopathy-associated virus type 2 in AIDS and AIDS-related complex. Lancet **i**:128–132.
8. **Chakrabarti, L., M. Guyader, M. Alizon, M. D. Daniel, R. C. Desrosiers, P. Tiollais, and P. Sonigo.** 1987. Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses. Nature (London) **328**:543–547.

8a. **Delwart, E. L., G. L. Buchschacher, Jr., E. O. Freed, and A. T.**

Panganiban. Analysis of HIV-1 envelope mutants by genetic complementation. AIDS Res. Hum. Retroviruses, in press.

9. **de Wolf, F., R. H. Meloen, M. Bakker, F. Barin, and J. Goudsmit.** 1991. Characterization of human antibody-binding sites on the external envelope of human immunodeficiency virus type 2. J. Gen. Virol. **72:**1261–1267.
10. **Felser, J. M., T. Klimkait, and J. Silver.** 1989. A syncytia assay for human immunodeficiency virus type 1 (HIV-1) envelope protein and its use in studying HIV-1 mutations. Virology **170:**566–570.
11. **Freed, E. O., E. L. Delwart, G. L. Buchschacher, Jr., and A. T. Panganiban.** 1992. A mutation in the human immunodeficiency virus type 1 transmembrane glycoprotein gp41 dominantly interferes with fusion and infectivity. Proc. Natl. Acad. Sci. USA **89:**70–74.
12. **Freed, E. O., D. J. Myers, and R. Risser.** 1989. Mutational analysis of the cleavage sequence of the human immunodeficiency virus type 1 envelope glycoprotein precursor gp160. J. Virol. **63:**4670–4675.
13. **Freed, E. O., D. J. Myers, and R. Risser.** 1990. Characterization of the fusion domain of the human immunodeficiency virus type 1 envelope glycoprotein gp41. Proc. Natl. Acad. Sci. USA **87:**4650–4654.
14. **Freed, E. O., D. J. Myers, and R. Risser.** 1991. Identification of the principal neutralizing determinant of human immunodeficiency virus type 1 as a fusion domain. J. Virol. **65:**190–194.
15. **Freed, E. O., and R. Risser.** 1987. Role of envelope glycoprotein processing in murine leukemia virus infection. J. Virol. **61:**2852–2856.
16. **Freed, E. O., and R. Risser.** 1991. Identification of conserved residues in the human immunodeficiency virus type 1 principal neutralizing determinant that are involved in fusion. AIDS Res. Hum. Retroviruses **7:**807–811.
17. **Gomez, S., G. Boileau, L. Zollinger, C. Nault, M. Rholam, and P. Cohen.** 1989. Site-specific mutagenesis identifies amino acid residues critical in prohormone processing. EMBO J. **8:**2911–2916.
18. **Guyader, M., M. Emerman, P. Sonigo, F. Clavel, L. Montagnier, and M. Alizon.** 1987. Genome organization and transactivation of the human immunodeficiency virus type 2. Nature (London) **326:**662–669.
19. **Helseth, E., M. Kowalski, D. Gabuzda, U. Olshevsky, W. Haseltine, and J. Sodroski.** 1990. Rapid complementation assays measuring replicative potential of human immunodeficiency virus type 1 envelope glycoprotein mutants. J. Virol. **64:**2416–2420.
20. **Hosaka, M., M. Nagahama, W.-S. Kim, T. Watanabe, K. Hatsuzawa, J. Ikemizu, K. Murakami, and K. Nakayama.** 1991. Arg-X-Lys/Arg-Arg motif as a signal for precursor cleavage catalyzed by furin within the constitutive secretory pathway. J. Biol. Chem. **266:**12127–12130.
21. **Hoxie, J. A.** 1991. Hypothetical assignment of intrachain disulfide bonds for HIV-2 and SIV envelope glycoproteins. AIDS Res. Hum. Retroviruses **7:**495–499.
22. **Kieny, M. P., R. Lathe, Y. Riviere, K. Dott, D. Schmitt, M. Girard, L. Montagnier, and J.-P. Lecocq.** 1988. Improved antigenicity of the HIV env protein by cleavage site removal. Protein Eng. **2:**219–225.
23. **Kowalski, M., J. Potz, L. Basiripour, T. Dorfman, W. C. Goh, E. Terwilliger, A. Dayton, C. Rosen, W. Haseltine, and J. Sodroski.** 1987. Functional regions of the envelope glycoprotein of the human immunodeficiency virus type 1. Science **237:**1351–1355.
24. **Kunkle, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
25. **LaRosa, G. J., J. P. Davide, K. Weinhold, J. A. Waterbury, A. T. Profy, J. A. Lewis, A. J. Langlois, G. R. Dreesman, R. N. Boswell, P. Shadduck, L. H. Holley, M. Karplus, D. P. Bolognesi, T. J. Matthews, E. A. Emini, and S. D. Putney.** 1990. Conserved sequence and structural elements in the HIV-1 principal neutralizing determinant. Science **249:**932–935.
26. **Leonard, C. K., M. W. Spellman, L. Riddle, R. J. Harris, J. N. Thomas, and T. J. Gregory.** 1990. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. J. Biol. Chem. **265:**10373–10382.
27. **Lifson, J. D., M. B. Feinberg, G. R. Reyes, L. Rabin, B. Banapour, S. Chakrabarti, B. Moss, F. Wong-Staal, K. S. Steimer, and E. G. Engleman.** 1986. Induction of CD4-dependent cell fusion by the HTLV-III/LAV envelope glycoprotein. Nature (London) **323:**725–728.
28. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47:**333–348.
29. **McCune, J. M., L. B. Rabin, M. B. Feinberg, M. Lieberman, J. C. Kosek, G. R. Reyes, and I. L. Weissman.** 1988. Endoproteolytic cleavage of gp160 is required for the activation of human immunodeficiency virus. Cell **53:**55–67.
30. **Norrby, E., P. Putkonen, B. Bottiger, G. Utter, and G. Biberfeld.** 1991. Comparison of linear antigenic sites in the envelope proteins of human immunodeficiency virus (HIV) type 2 and type 1. AIDS Res. Hum. Retroviruses **7:**279–285.
31. **Perez, L. G., and E. Hunter.** 1987. Mutations within the proteolytic cleavage site of the Rous sarcoma virus glycoprotein that block processing to gp85 and gp37. J. Virol. **61:**1609–1614.
32. **Robert-Guroff, M., K. Aldrich, R. Muldoon, T. L. Stern, G. P. Bansal, T. J. Matthews, P. D. Markham, R. C. Gallo, and G. Franchini.** 1992. Cross-neutralization of human immunodeficiency virus type 1 and 2 and simian immunodeficiency virus isolates. J. Virol. **66:**3602–3608.
33. **Sodroski, J., W. C. Goh, C. Rosen, K. Campbell, and W. A. Haseltine.** 1986. Role of the HTLV-III/LAV envelope in syncytium formation and cytopathicity. Nature (London) **322:**470–474.
34. **Stein, B. S., S. D. Gowda, J. D. Lifson, R. C. Penhallow, K. G. Bensch, and E. G. Engleman.** 1987. pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. Cell **49:**659–668.
35. **Wain-Hobson, S., P. Sonigo, O. Danos, S. Cole, and M. Alizon.** 1985. Nucleotide sequence of the AIDS virus, LAV. Cell **40:**9–17.
36. **Zagury, J. F., G. Franchini, M. Reitz, E. Collalti, B. Starcich, L. Hall, K. Fargnoli, L. Jagodzinski, H.-G. Guo, F. Laure, S. K. Arya, S. Josephs, D. Zagury, F. Wong-Staal, and R. C. Gallo.** 1988. Genetic variability between isolates of human immunodeficiency virus (HIV) type 2 is comparable to the variability among HIV type 1. Proc. Natl. Acad. Sci. USA **85:**5941–5945.