Role of the HIV gp120 Conserved Domain 1 in Processing and Viral Entry*§

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The importance of the N-terminal region of HIV gp120 conserved domain 1 (gp120-C1) to envelope function has been examined by alanine-scanning mutagenesis and subsequent characterization of the mutagenic effects on viral entry; envelope expression, processing, and incorporation; and gp120 association with gp41. With respect to the wild-type gp120, mutational effects on viral entry fall into two classes: functional, as defined by >20% entry with respect to wild type, and impaired, as defined by <20% entry with respect to wild type. Based on Western blot analyses of cell lysates and virions, the entry impairment of W35A, V38A, Y39A, Y40A, G41A, V42A, and I52A is due primarily to disruption of envelope processing. The entry impairment of P43A and W45A is apparently due to a combination of effects on processing and incorporation into virions. In contrast, the entry impairment of V44A and F53A is primarily due to disruption of the gp120-gp41 interaction, which results in dissociation of gp120 from the virion. We present a model for gp120-C1 interactions with gp120-C5 and the gp41 disulfide loop in unprocessed gp160 and processed gp120/gp41.

Human immunodeficiency virus (HIV) entry is mediated by the viral envelope proteins gp120 and gp41 (reviewed in Ref. 1). gp120 mediates attachment of the virus to the appropriate target cells through interactions with CD4 and chemokine co-receptors (2, 3). gp41, which is tethered to the viral membrane by a transmembrane domain, mediates fusion of the viral and target cell membranes (4). gp120 and gp41 are formed by cleavage of the precursor gp160 by cellular furin-like proteases (5, 6). Specifically, the amino acid sequence REKR located at the C terminus of gp120 forms the furin recognition site. Mutations of this site result in unprocessed mature HIV gp120. However, the structure of HIV gp120-C5 (residues 489–511) has been determined as an isolated domain by NMR spectroscopy (13).

Materials and Methods

Mutagenesis and Viral Entry Assays—Mutants were prepared from plasmid pCONBgp160opt (22) using the Stratagene QuikChange II site-directed mutagenesis kit with subsequent verification by DNA sequencing. The functionality of gp120 mutants was determined in a luciferase-based entry assay (23). For this assay, plasmids pCONBgp160opt (bearing wild-type or mutant gp120) and pNL4.3.Luc.R–E– (24) were co-transfected by Lipofectamine 2000 (Invitrogen) into 293T cells, which were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 1% l-glutamine, and 1% penicillin/streptomycin. Forty-eight hours post-transfection, the medium was harvested and filtered through a 0.45-μm filter to make the virus stock. For assay of viral entry, U87.CD4.CCR5 cells (3), which were maintained in Dulbecco’s medium with 15% fetal bovine serum supplemented with 1 μg/ml puromycin, 300 μg/ml G418, 1% l-glutamine, and 1% penicillin/streptomycin, were seeded to 1 x 10⁵ cells/well of a 12-well cell culture plate in a volume of 1 ml. The following day, 500 μl of the virus stock was added to each of the wells of the U87 cells after removal of the medium. The plates were incubated overnight at 37 °C in a CO₂ incubator. After ~16 h, the virus was aspirated and replaced with U87 medium, and the cells were allowed to rest for another 24 h. Luciferase activity was measured using the luciferase assay system from Promega and a Berthold FB12...
luminometer running Sirius software. The experiments were run in triplicate from transfection to assay of luciferase activity, and thus the uncertainties represent all stages of the experiment. In all cases, the viral entry levels fell within the linear range of detection (i.e. the values of the wild-type and mutants never exceeded $3 \times 10^6$ relative light units) (14). Entry levels were normalized to relative p24 levels observed in the Western blot of the virus, as described below.

**Western Blot Analysis**—Cell lysates were collected from 293T producer cells using lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and 0.1% SDS). The virus pellet was prepared by ultracentrifugation on a cushion of 20% sucrose at 55,000 rpm for 35 min using a Beckman SW55T1 rotor. The virus pellets were resuspended in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and 0.1% SDS). After SDS-PAGE, transfer, and blocking of the membranes with 5% milk/TBST for gp41 and SuperBlock (Pierce) for gp120, the blots were probed with either goat anti-HIV-1 gp120 polyclonal antibody (U. S. Biological) or with mouse anti-HIV-1 gp41 monoclonal antibody (Chessie 8; National Institutes of Health AIDS Research and Reference Reagent Program) (22). HIV-1 p24 was detected by probing the membrane with mouse anti-HIV-1 p24 antibody (U. S. Biological). The secondary antibody used was peroxidase-conjugated AffiniPure donkey anti-goat or goat anti-mouse IgG (H+L) from Jackson ImmunoResearch Laboratories, Inc. and developed using the ECL kit (Pierce). The relative amount of envelope or p24 on the Western blots was determined by a densitometric analysis. Briefly, digital scans of the blots were opened in Photoshop 7.0, the image was inverted, the appropriate bands were selected with the Lasso function, and the mean intensity and area were measured with the Histogram function. Subsequently, the relative percentage was determined by the following relationship,

$$
\text{Relative} \% = 100 \times \left( \frac{I_{\text{exp}} \times A_{\text{exp}} - I_{\text{cont}} \times A_{\text{cont}}}{I_{\text{wt}} \times A_{\text{wt}} - I_{\text{cont}} \times A_{\text{cont}}} \right)
$$

where $I$ and $A$ denote mean intensity and band area, respectively, and the subscripts exp, cont, and wt denote experimental, control (i.e. in the absence of envelope or pNL4-3.Luc.R−E− plasmid), and wild-type, respectively. The mean intensity and band area values used in this analysis are shown in Tables S1 and S2.

**RESULTS**

**Design of the HIV gp120-C1 Mutants**—The location of gp120-C1 within the context of gp160 is shown in Fig. 1a. The N-terminal region of gp120-C1 is relatively conserved between HIV-1, HIV-2, and SIV with ~50% sequence identity (9), with the most conserved region, composed of hydrophobic residues, spanning residues 36–45. In the present study, 21 alanine substitutions of HIV-1 gp120-C1 were generated by site-directed mutagenesis (Fig. 1b). We have chosen to use the CONB envelope sequence, a consensus sequence of subtype B that is optimized for expression in mammalian cells and uses CCR5 as the co-receptor (22).

**Viral Entry of the HIV gp120-C1 Mutants**—The effects of the gp120 mutations on viral entry were first tested by a luciferase-based assay, in which viral entry is proportional to the observed luciferase activity (14, 23). Viral entry levels of wild-type and mutants are shown in Fig. 2. The mutational effects on viral entry can be divided into two classes: 1) functional, as defined by >20% entry with respect to wild type (E32A, K33A, I34A, V36A, T37A, K46A, E47A, T49A, T50A, and T51A); 2) impaired, as defined by <20% entry with respect to wild type (W35A, V38A, Y39A, Y40A, G41A, V44A, P43A, V44A, W45A, I52A, and F53A). With respect to the functional mutants, Val36 and Thr37 are highly conserved among HIV-1, HIV-2, and SIV; however, substitution of these residues with alanine is a relatively conservative change. With respect to the impaired mutations, Val36, Tyr40, Gly41, Pro43, Trp45, and Phe53 are highly conserved among HIV-1, HIV-2, and SIV. Interestingly, many of the largest effects to entry occur in the hydrophobic region encompassing Val36–Trp45.

**Envelope Expression and Processing**—A Western blot analysis of wild-type and mutant envelope present in cell lysates was carried out to probe for mutational effects on expression and processing, as shown in Fig. 3. First note that the wild type shows the characteristic bands of gp160, gp120, and gp41, indicating that gp160 is expressed and processed by furin-like pro-
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The positions of gp41, gp120, and gp160 are shown.

FIGURE 3. Western blot analysis of envelope expression and processing for HIV-1 wild type (wt) and the gp120-C1 mutants in lysates from 293T cells. The positions of gp41, gp120, and gp160 are shown.

FIGURE 4. Western blot analysis of virion incorporation and gp120-gp41 association for HIV-1 wild type (wt) and the gp120-C1 mutants. The positions of p24, gp41, gp120, and gp160 are shown.

tees to form mature gp120 and gp41. In the case of the mutants, the relatively high levels of gp160 present in cell lysates suggest that envelope expression has not been significantly affected by any of the substitutions. On the other hand, many of the mutations have reduced gp160 processing, as evidenced by reduced levels of gp120 and gp41. For example, W35A, V38A, Y39A, Y40A, G41A, V42A, P43A, W45A, I52A, and F53A exhibit undetectable or greatly reduced levels of gp120 and gp41 in the cell lysates, presumably due to a lack of processing by furin-like proteases. Not surprisingly, many of the mutations that display reduced gp160 processing also are impaired for viral entry (i.e. entry levels are <20% with respect to the wild type). V44A is a notable exception for which gp160 processing is not significantly affected but it nonetheless is impaired for viral entry.

Virion Incorporation and gp120 Association with gp41—A Western blot analysis of wild-type and mutant envelope present in purified virions was carried out to probe for mutational effects on envelope incorporation into virions and gp120 association with gp41 as well as to confirm the expression and processing results observed in cell lysates. Note that virion incorporation requires surface expression of envelope, which has been shown to be dependent upon proper folding and oligomerization (26–28). The Western blot analysis of wild-type and mutant virions is shown in Fig. 4. The relative amounts of p24 were assayed as a loading control. The wild-type virions show the presence of gp160, gp120, and gp41. The presence of gp160 in virions has been previously attributed to saturation of furin-like proteases by overproduction of envelope (22, 29). Not surprisingly, the functional mutants (E32A, K33A, I34A, V36A, T37A, K46A, E47A, T49A, T50A, and T51A) exhibit the wild-type phenotype, with the difference presumably due to the nature of the substituting group (e.g. the leucine substitution is more bulky than valine and may result in steric hindrance). The impaired mutants W35A, V38A, Y39A, Y40A, G41A, V42A, and I52A exhibit gp160, suggesting virion incorporation, but the absence or greatly reduced level of gp120 and gp41 suggests a processing defect, which is in agreement with the result for the Western blot of the cell lysates (Fig. 3). Impaired mutants V44A and F53A display bands for gp160, suggesting virion incorporation, and gp41, suggesting processing. In the case of F53A, the processing observed in virions is in disagreement with the lack of processing observed in cell lysates (compare Figs. 3 and 4); the underlying reasons for this discrepancy are not apparent at this time. Interestingly, the absence of gp120 in the impaired mutants V44A and F53A suggests that the gp120-gp41 interaction has been disrupted to result in “shedding.”

DISCUSSION

Summary of the gp120-C1 Mutational Effects—The effects of the gp120-C1 alanine substitutions on viral entry, envelope expression, processing, and virion incorporation; and gp120-gp41 association are summarized in Table 1. Based on the data presented herein, a phenotype can be given for each of the mutants. For example, mutants E32A, K33A, I34A, V36A, T37A, K46A, E47A, T49A, T50A, and T51A are functional for virion entry (defined as >20% entry with respect to the wild type), and they exhibit the wild type pattern of gp160, gp120, and gp41 in cell extracts and virions. Consequently, this group is assigned a wild-type phenotype (Table 1). Impaired mutants P43A and W45A show reduced levels of virion incorporation and processing, and thus they exhibit a mixed phenotype. Impaired mutants W35A, V38A, Y39A, Y40A, G41A, V42A, and I52A exhibit a processing defect, as evidenced by decreased levels of gp120 and gp41 in cell lysates and viruses (cf. Figs. 3 and 4 and Table 1). Note that the Y40A substitution is found in some HIV-1 subtype O strains, which suggests that other mutations compensate to allow processing in these strains. Impaired mutants V44A and F53A exhibit a defect in gp120-gp41 association. In the case of V44A, the alanine substitution occurs in the HIV-2 and SIV envelope (Fig. 1), which suggests that other mutations compensate to stabilize gp120-gp41 association.

Previous Studies of gp120-C1—A number of previous studies have characterized the effects of mutations in HIV gp120-C1 on viral function, and thus it is of interest to compare the conclusions of these studies in light of the present work. For example, Ivey-Hoyle et al. (14) observed that deletion of the 31 amino-terminal residues of gp120 (residues 31–57 of gp120-C1) (Fig. 1) did not disrupt envelope processing or CD4 binding but did result in dissociation of gp120 from gp41, which suggested that the gp120-C1 domain directly interacts with gp41. The Y44A and F53A mutants of the present study, which are found within this region, also disrupt gp120 association with gp41 and thus support this notion. In another study, Helseth et al. (15) found that single-site mutants V36L and Y40D were processed and bound CD4 but resulted in dissociation of gp120 from gp41. In the present study, the analogous mutant V36A exhibited a wild-type phenotype, with the difference presumably due to the nature of the substituting group (e.g. the leucine substitution is more bulky than valine and may result in steric hindrance).
The present study represents the first single site mutations to Glu\textsuperscript{32}, Lys\textsuperscript{33}, Ile\textsuperscript{34}, Trp\textsuperscript{35}, Thr\textsuperscript{37}, Val\textsuperscript{38}, Tyr\textsuperscript{39}, Gly\textsuperscript{41}, Val\textsuperscript{42}, Pro\textsuperscript{43}, Val\textsuperscript{44}, Lys\textsuperscript{46}, Gly\textsuperscript{47}, Thr\textsuperscript{49}, Thr\textsuperscript{50}, Thr\textsuperscript{51}, Ile\textsuperscript{52}, and Phe\textsuperscript{53}. Of the novel mutations, mutants W35A, V38A, Y49A, V42A, and I52A exhibit significantly impaired function due to processing defects, mutant P43A exhibits impaired function due to defects in processing and virion incorporation, and mutants V44A and F53A exhibit impaired function due to disruption of the gp120-gp41 association. Taken together, the gp120-C1 region plays important roles in envelope processing and stabilization of the gp120-gp41 interaction.

Interactions of gp120-C1 in gp120/gp41—As mentioned in the Introduction, large regions of the gp120-C1 and gp120-C5 domains are missing from the available structures of the gp120 core (10–12), and thus mutagenesis and immunological studies play important roles in the characterization of these domains, which are highly conserved between HIV-1, HIV-2, and SIV (9). However, it is important to note that single site mutational effects can be due to direct interactions or interactions propagated to more distant sites. Nonetheless, we would like to consider intermolecular interactions between gp120-C1 and gp120-C5 with the gp41 disulfide loop in processed gp160 (Fig. 5). The gp120-C1 domain is shown with the secondary structure predicted by Hansen et al. (13), and the gp41 disulfide loop structure is based on Refs. 25 and 31.

Moreover, the Y40A mutant of the present study disrupted envelope processing, with the difference probably due to the smaller and more hydrophobic nature of alanine with respect to aspartate. In addition, Helseth et al. (15) found that mutant W45S reduced processing and gp120 association with gp41. In agreement, the analogous W45A mutant of the present study also reduced processing; however, the effects on gp120-gp41 association could not be assayed due to the complete absence of processing in this mutant. Finally, we note that the present study represents the first single site mutations to Glu\textsuperscript{32}, Lys\textsuperscript{33}, Ile\textsuperscript{34}, Trp\textsuperscript{35}, Thr\textsuperscript{37}, Val\textsuperscript{38}, Tyr\textsuperscript{39}, Gly\textsuperscript{41}, Val\textsuperscript{42}, Pro\textsuperscript{43}, Val\textsuperscript{44}, Lys\textsuperscript{46}, Gly\textsuperscript{47}, Thr\textsuperscript{49}, Thr\textsuperscript{50}, Thr\textsuperscript{51}, Ile\textsuperscript{52}, and Phe\textsuperscript{53}. Of the novel mutations, mutants W35A, V38A, Y49A, V42A, and I52A exhibit significantly impaired function due to processing defects, mutant P43A exhibits impaired function due to defects in processing and virion incorporation, and mutants V44A and F53A exhibit impaired function due to disruption of the gp120-gp41 association. Taken together, the gp120-C1 region plays important roles in envelope processing and stabilization of the gp120-gp41 interaction.

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Hansen et al. (30). The gp120-C5 structure is taken from Guilhau
dis et al. (13), and the gp41 disulfide loop structure is taken
from Refs. 25 and 31. Note that the gp120-C5 structure has the
caveat that it was determined in the presence of 40% trifluoro-
ethanol, a co-solvent that stabilizes helical structure, and in the
absence of other gp120 and gp41 domains (13). The gp41 disul-
fide loop has the caveat that it is based on the postfuson form of
gp41 (i.e. the six-helix bundle); however, we are not aware of
any evidence for a large structural change of the disulfide loop
during HIV entry. In Fig. 5, residues implicated by mutagenesis
studies in forming intermolecular interactions in processed
shows in green. The interaction between Val44 and
Phe53 of gp120-C1 and gp41 is based on the present work, in
which single site mutations resulted in disruption of the gp120-
gp41 interaction. The intermolecular interactions of Ile591 of
gp120-C5 and Trp596 and Ser618 of the gp41 disulfide loop are
based on previous mutagenesis studies, suggested by dissocia-
tion of gp120 from gp41 (19, 21). The interaction between A501
gp120-C5 and Thr505 of the gp41 disulfide loop is based on the
SOS mutant of Binley et al. (16), in which the double mutant
A501C/T605C was shown to form a nonnative disulfide bond,
and thus a direct interaction between these residues was
implied. As noted above, Val38, Tyr40, and Trp45 have also been
previously implicated in forming intermolecular interactions
with gp41, based on gp120 dissociation from gp41 (15). Impor-
tantly, immunological studies have also suggested interactions
between the gp120-C1, gp120-C5, and the gp41 disulfide loop
(17, 18), thereby supporting the schematic model of processed
gp160. Finally, note that the topology of the gp120 core struc-
tures imply that gp120-C1 and -C5 are in close proximity
(10–12).

Interactions of gp120-C1 in gp160—It is next of interest to
examine potential long range intramolecular interactions
between gp120-C1, gp120-C5, and the gp41 disulfide loop in
unprocessed gp160. In Fig. 5, residues implicated in forming
long range interactions with the furin recognition site in
unprocessed gp160 are shown in blue. Specifically, the interac-
tion between Trp35, Val38, Tyr39, Ty40, Gly41, Val42, Pro43,
Trp45, and Ile52 of gp120-C1 and the furin recognition site of
gp120-C5 is based on the data presented herein, in which ala-
nine substitutions resulted in severely decreased envelope
processing. The interaction between Leu593 of the gp41 disul-
fide loop and the furin recognition site is based on a previous
mutagenesis study (19). The notion that the gp41 disulfide
bond interacts with gp120-C5 in gp160 is also supported by the
mutagenesis study of Sen et al. (20), who showed that the disul-
fide bond within the loop was important to gp160 processing.
Interestingly, the present study suggests that long range inter-
actions between gp120-C1 and gp120-C5, which encompasses
the furin recognition site, occur in unprocessed gp160. Indeed,
the impaired entry of many of the gp120-C1 mutants was due to
a lack of processing, underscoring the importance of gp120-C1
residues to proper presentation of the gp120-C5 furin recogni-
tion site, a site that is ~450 residues away (cf. Fig. 1). Ivey-Hoyle
et al. (14) have shown that deletion of the N-terminal 31 resi-
dues of gp120 (residues 31–57 of gp120-C1) (Fig. 1) does not
significantly reduce envelope processing, which may appear to
be in disagreement with the results presented herein. It is
tempting to speculate that deletion of gp120-C1, in contrast to
single site mutations, may remove residues with the potential to
reduce access to the furin recognition site, thereby allowing
envelope processing.

In summary, the extreme sensitivity of gp120-C1 to alanine
substitutions suggests that this region is an attractive and novel
target for future drug discovery efforts. For example, com-
ounds that bind to gp120-C1 may be expected to disrupt
gp120 function and hence HIV entry, by disrupting either
gp160 processing or gp120 association with gp41.

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