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Mutational Analysis of the Leucine Zipper-Like Motif of the Human Immunodeficiency Virus Type 1 Envelope Transmembrane Glycoprotein

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The N-terminal region of the envelope (*env*) transmembrane protein of human immunodeficiency virus type 1 (HIV-1) has a leucine zipper-like motif. This highly conserved zipper motif, which consists of a heptad repeat of leucine or isoleucine residues, has been suggested to play a role in HIV-1 *env* glycoprotein oligomerization. This hypothesis was tested by replacing the highly conserved leucine or isoleucine residues in the zipper motif with a strong α-helix breaker, proline. We report here that such substitutions did not abolish the ability of *env* protein to form oligomers, indicating that this highly conserved zipper motif does not have a crucial role in *env* protein oligomerization. However, the mutant viruses all showed impaired infectivity, suggesting that this conserved zipper motif can have an important role in the virus life cycle.

The envelope (*env*) glycoprotein of human immunodeficiency virus type 1 (HIV-1) has several important functions. It determines the interaction between the host cell and the virus, it causes cytopathic fusion of the virus with host cells carrying the CD4 receptor (7, 14, 20, 21, 23, 31), and it is the major viral antigen and primary target of immune responses (2). The *env* protein is synthesized as a gp160 precursor and is subsequently processed into gp120, the exterior protein, and gp41, the transmembrane (TM) protein (1, 28, 33). The *env* precursor assembles as oligomers (10, 26).

The leucine zipper-like motif of HIV-1 (termed zipper motif hereafter) is a heptad repeat of leucine or isoleucine residues located in the N-terminal region of gp41 (8) (Fig. 1). It spans from amino acid residue 559 to 587, based on the sequence of an infectious HIV-1 proviral DNA clone, HXB2 (11). When the amino acid sequence of this domain is displayed on an α-helical wheel, this zipper motif contains a periodic repeat of leucine or isoleucine residues at every seventh position over eight helical turns (Fig. 1).

The zipper motif is highly conserved among different HIV-1 isolates. It is located adjacent to other regions in the N terminus of gp41 that are believed to have important roles in viral entry and the host immune response. The zipper motif is immediately followed by an immunodominant domain (residues 598 to 609) (13, 34) and is preceded by a putative membrane fusion domain (amino acid residues 512 through 527) (17). This zipper motif has been suggested to form part of an extended, extramembranous α helix that is structurally homologous to the α helices of influenza virus hemagglutinin HA_2 (12). By circular dichroism, a synthetic peptide corresponding to the zipper motif of gp41 was shown to form a high degree of α-helical structure in solution (36). Interestingly, this motif is highly conserved among the TM proteins of other retroviruses such as human T-cell leukemia virus types I and II, bovine leukemia virus, Moloney murine leukemia virus, mouse mammary tumor virus, Mason-Pfizer monkey virus, and strains A, B, and C of feline leukemia virus (8).

The leucine zipper has been shown to mediate dimerization of a class of DNA-binding proteins, including transforming proteins such as Fos, Jun, and Myc, and several cellular transcription factors such as the rat liver nuclear enhancer protein c/EBP and yeast transcriptional activator GCN4 (16, 19, 30, 32). The zipper motif has also been suggested to play a role in subunit assembly of other membrane-bound glycoproteins (4, 22, 35). By analogy, the retroviral zipper motif was proposed to mediate *env* protein oligomerization (8). However, this hypothesis has not been extensively tested.

To determine whether the zipper motif of gp41 was critical for *env* precursor oligomerization, four of the highly conserved residues in the zipper motif, Ile-559, Leu-566, Ile-573, and Leu-587, were each substituted with a proline residue. These four residues are found in all HIV-1 isolates with known nucleotide sequences (24). Proline is expected to disrupt the α helix more severely than do other amino acids (6).

Oligonucleotide-directed mutagenesis that selects against a single-stranded DNA template containing uracil (18) was performed. The uracil DNA template for mutagenesis was derived from the negative-stranded *Eco*RI-*Bam*HI fragment of HXB2 (2.7 kb), which was cloned in M13mp18, and synthesized by transfecting *Escherichia coli* CJ236 with the M13 vector containing the insert.

The single-stranded DNA template was primed with synthetic oligonucleotides to prepare the replicative form of the recombinant phage that contains the desired mutations in the *env* gene. The oligonucleotides used for mutagenesis were as follows: ACAATTTGCTGAGGGCTCCTGAGGCGCAAC AGCAT (559); GCGCAACAGCATCTGCCGCAACTCAC AGTCTGG (566); CTCACAGTCTGGGGCCCCAAGCAGC TCCAAGC (573); and AGCTGTGGAAAGATACCCAAAG

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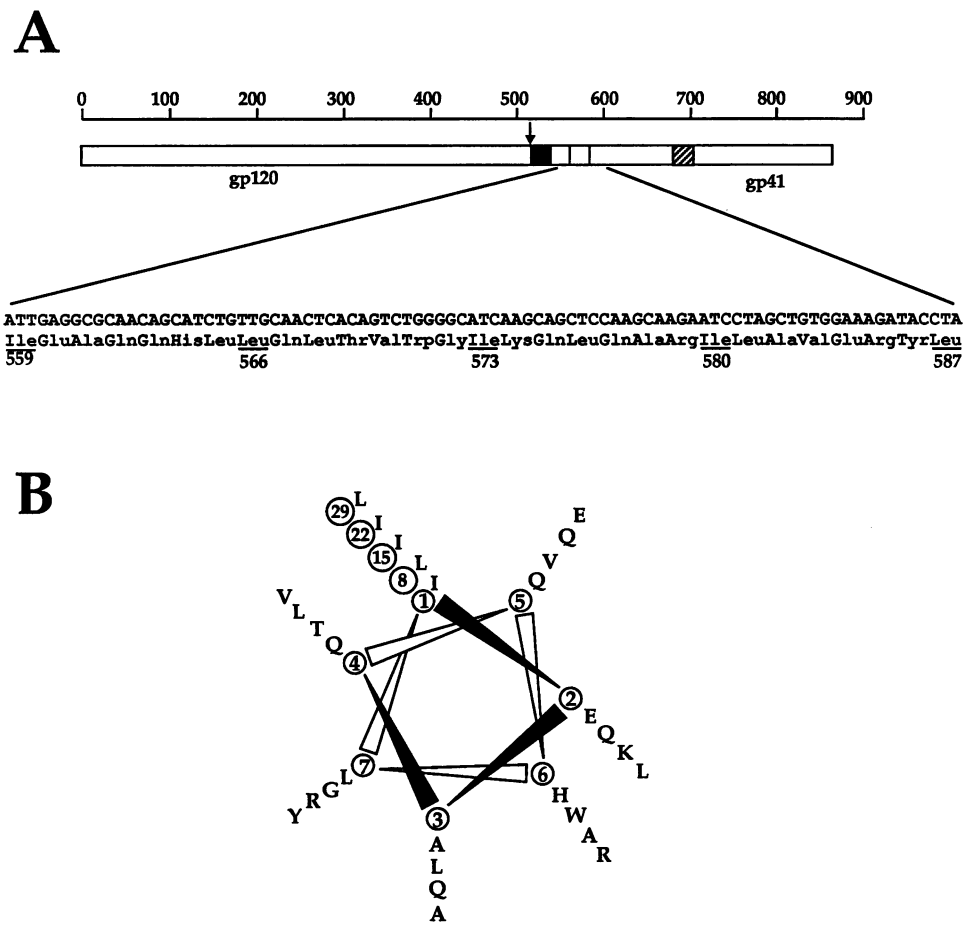

FIG. 1. Hypothetical α-helical structure of the zipper motif and locations of leucine or isoleucine residues in the zipper motif. (A) Schematic diagram of the HIV-1 *env* glycoprotein, with an arbitrary scale showing the number of amino acid residues. The endoproteolytic cleavage site is marked with an arrow. The locations of the fusion peptide, zipper motif region, and TM domain are indicated by closed, open, and hatched boxes, respectively. The detailed nucleotide sequence of HXB2 *env* that contains a zipper motif and the deduced amino acid sequence with the conserved leucine or isoleucine residues are expanded below. (B) Diagram of the amino acid sequence of the zipper motif region on an α-helical wheel.

GATCAACAG (587). The numbers in parentheses indicate the locations of the proline substitutions; the underlines indicate the nucleotides that encode for proline substitutions. The phages were screened by dideoxy-chain termination (29) using T7 Sequenase (United States Biochemicals, Cleveland, Ohio). The sequence primer, a sequence 5′ to the zipper motif, was as follows: GTACAGGCCAGACAATT ATTGTCTGGTATAGT. The mutagenized recombinant M13 was grown, and the mutated *Sal*I-*Bam*HI fragment was substituted for the homologous sequence in $HXB2_{gpt}$ (11). Mutations in this region did not affect the coding sequences of the *tat* and *rev* genes.

A transient expression system using COS-1 cells (11) was employed to express the *env* proteins. The COS-1 cell line, a derivative of CV-1 that expresses simian virus 40 T antigen, was maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. $HXB2_{gpt}$ contains a simian virus 40 origin for propagation in COS-1 cells.

COS-1 cells were transfected with equal amounts of wild-type (wt) or mutant $HXB2_{gpt}$ proviral DNA by the DEAE-dextran method (15). An equivalent amount of cell lysate from each transfected culture was subject to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (5) and analyzed by Western immunoblotting with a sheep anti-gp120 antiserum. Comparable levels of gp160 were synthesized by all of the mutant proviruses (data not shown), indicating that a proline substitution does not greatly affect the synthesis of the *env* precursor.

To determine whether proline substitution affects *env* protein oligomerization, sucrose gradient analysis as described by Earl et al. (10) was performed to separate the mutant *env* monomeric form from the oligomeric forms.

As a control, the proteins produced by wt $HXB2_{gpt}$ in COS-1 cells were sedimented onto a 8 to 20% sucrose gradient. After fractionation, the proteins were separated by SDS-PAGE and analyzed by Western blotting with anti-gp120 serum. As shown in the top panel of Fig. 2A, wt gp120 sedimented predominantly in fractions 9 to 11. These fractions represent the monomeric form of gp120. The oligomeric forms of gp120 are not stable during centrifugation (10). The majority of wt gp160 sedimented faster than gp120 and was distributed in the bottom fractions of the gradient. These fractions might represent the oligomeric forms of gp160. The sucrose gradient separation profile presented here, in terms of the relative sedimentation positions of

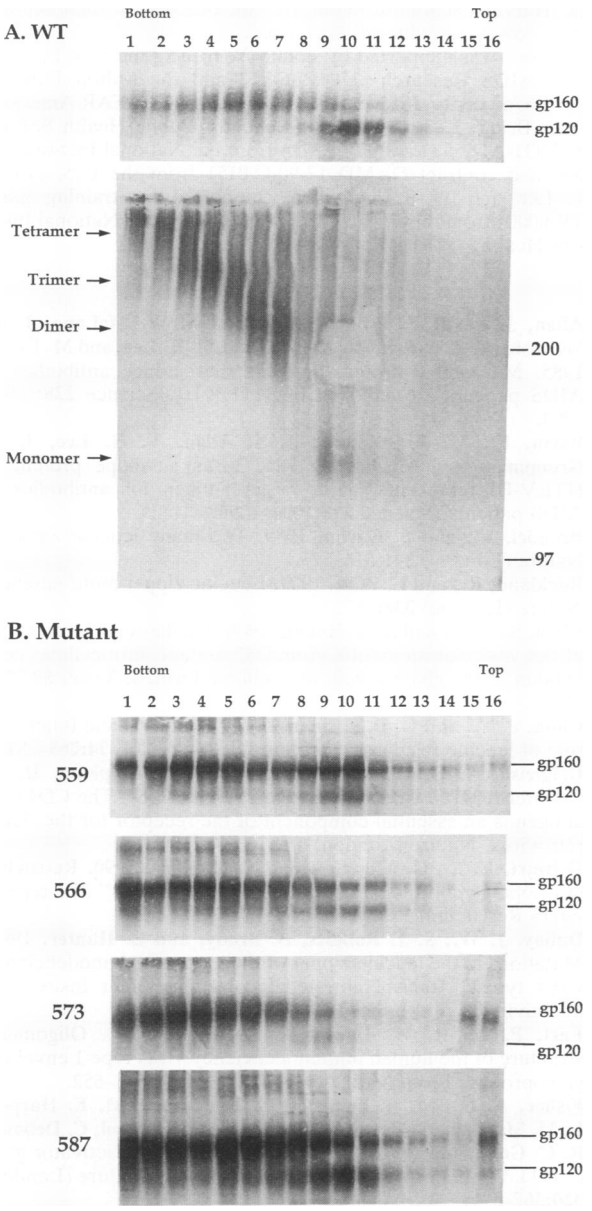

FIG. 2. Oligomeric forms of mutant *env* proteins. (A) Sucrose gradient centrifugation and chemical cross-linking of wt *env* protein. wt $HXB2_{gpt}$-transfected COS-1 cells were lysed with Tris-buffered saline (50 mM Tris [pH 7.5], 150 mM NaCl) containing 1% Triton X-100, left on ice for 10 min, and centrifuged for 10 min to remove cell debris. The supernatant was subjected to sucrose gradient centrifugation as described previously (10) except that 8 to 20% gradients were run on a Beckman SW41 rotor at 40,000 rpm at 4°C for 22 h. Proteins were collected from the bottom of the gradients. Gradient performance was checked by reading the refraction index of sucrose with a Bausch & Lomb refractometer. Proteins from one-half of each fraction were separated by SDS-PAGE (5) and then subjected to Western blot analysis with anti-gp120 (top panel). The immune complexes were visualized by the biotin-streptavidin detection system (Amersham). The other half of each fraction was incubated with 5 mM ethyleneglycobis(succinimidylsuccinate) at room temperature for 30 min and saturated with glycine (pH 8.0) to a final concentration of 0.1 M. The proteins were incubated at room temperature for 15 min, separated by SDS-PAGE, and subjected to Western blot analysis with anti-gp120 (bottom panel). (B) Sucrose gradient analysis of mutant *env* proteins. Lysates from cells transfected with mutant $HXB2_{gpt}$ constructs, as indicated, were analyzed as described for panel A.

oligomeric forms of gp160 to the monomeric form of gp120, is similar to those described previously (10, 25).

To confirm the oligomeric structures of the *env* precursors which sedimented faster than the monomeric form of gp120, the materials in each fraction were cross-linked with 5 mM ethyleneglycobis(succinimidylsuccinate) (Pierce, Rockford, Ill.), separated on a 4% gel containing SDS, and analyzed by Western blotting with anti-gp120 serum. While good resolution of each of the oligomeric species is generally hard to obtain after chemical cross-linking (10, 25), three forms of wt gp160 which sedimented faster than the monomeric form, presumably tetramers (fractions 1 and 2), trimers (fractions 3 to 5), and dimers (fractions 6 and 7), were identified (Fig. 2A, bottom panel).

The mutant proteins that sedimented parallel to the wt protein were similarly analyzed by Western blotting. The majority of four mutant precursors all sedimented faster than the monomeric form of gp120 and were distributed in the bottom gradient fractions (Fig. 2B). This result indicates that the mutant precursors still formed oligomers and shows that the conserved leucine or isoleucine residues were not critical for *env* protein oligomerization.

We also examined whether the zipper motif in gp41 was important for virus infectivity. To study whether mutant constructs produced infectious virions, COS-1 cells were transfected with wt or mutant $HXB2_{gpt}$ proviral DNA and the virion-associated reverse transcriptase (RT) activity was determined. Two days posttransfection, the culture supernatants were harvested and precipitated with 5% polyethyleneglycol and assayed for RT activity, using [^{3}H]TTP as the substrate and poly(A)-oligo(dT) as the template-primer (27). All of the mutant proviruses as well as the wt provirus produced RT activity (data not shown), indicating that a proline substitution in the zipper motif did not greatly affect virus production.

Equal amounts of mutant and wt viruses, as measured by RT activity, were used to infect SupT1 cells. SupT1, a non-Hodgkin's T-lymphoma cell line that expresses a high level of CD4 molecules on the cell surface, was maintained in RPMI 1640 with 10% fetal calf serum. As summarized in Table 1, the wt virus induced cytopathic effects, including syncytium formation, after 3 days of culture. All of the mutant virus cultures remained healthy and showed no syncytium formation over the entire 43-day culture period. Furthermore, the wt virus produced virion-associated RT activity and p24 antigen in the medium. In contrast, these mutant viruses did not produce detectable levels of RT activity and p24 in the medium. These results indicate that the mutant viruses were severely impaired in infectivity.

Recently, Dubay et al. reported that substitutions with nonconservative amino acids, such as Ala, Gly, Ser, Asp, and Glu, for the middle isoleucine residue, Ile-573, in the zipper motif of gp41 did not affect the oligomeric structure of *env* protein (9). This finding suggests that these amino acids may disrupt the hydrophobicity of the predicted α helix of the zipper motif, but the zipper motif is not involved in protein oligomerization. Alternatively, such substitutions at this specific site may not have disrupted the structure of the zipper motif in gp41.

In this study, we substituted a proline residue, the strongest possible α-helix breaker (6), for the conserved leucine or isoleucine residues located in the zipper motif of gp41. A single proline substitution for the leucine residues in the zipper motif of the *fos* oncoprotein is sufficient to abolish its heterodimeric formation with *jun* protein (32). Moreover, substitution of a proline for an isoleucine in a synthetic

TABLE 1. Summary of the characteristics of mutant viruses

Construct[a]	Syncytium formation[b]	Infectivity	
		RT[c]	p24 production[d]
$HXB2_{gpt}$ (wt)	+	+	+
$HXB2_{gpt}$ (559)	−	−	−
$HXB2_{gpt}$ (566)	−	−	−
$HXB2_{gpt}$ (573)	−	−	−
$HXB2_{gpt}$ (587)	−	−	−

[a] Mutant $HXB2_{gpt}$ proviral DNA plasmids were constructed as described in the text. The numbers in parentheses indicate the locations in the *env* gene where the conserved leucine or isoleucine residues are replaced with a proline residue.

[b] Cells infected by wt virus showed extensive syncytium formation 3 days postinfection. Cells infected by mutant viruses did not show any syncytium formation over the 43-day culture period.

[c] Culture supernatants of SupT1 cells infected with wt or mutant viruses were assayed for RT activity. The RT activities of the wt virus at 6 and 12 days postinfection were 1.7×10^5 and 2.7×10^6 cpm/ml, respectively. None of the mutant viruses produced RT activity above the background level over the 43-day period.

[d] In separate experiments, aliquots of culture medium at different days postinfection were assayed for p24 antigen production by the DuPont p24 antigen capture assay. wt virus produced significant levels of p24 ($>10^3$ pg/ml at day 7 after infection). Mutant viruses did not produce any detectable amounts of p24 over the 35-day period.

peptide containing the zipper motif was found to significantly destabilize the α-helical structure of the peptide (36). Thus, substitutions of a proline for the conserved leucine or isoleucine residues in the zipper motif, located at positions 559, 566, and 587, as well as the Ile-573 which was examined by Dubay et al. (9), were expected to disrupt the local structure conferred by the zipper motif.

If the zipper motif of gp41 is essential for *env* protein oligomerization, it is expected that all of the mutant proteins would assemble as monomers. Our finding that all four mutant proteins still formed oligomers (Fig. 2) strongly suggests that the zipper motif of gp41 is unlikely to have a crucial role in protein oligomerization. It also raises doubt that structural homologs of the zipper motif found in the TM proteins of other retroviruses are involved in oligomer formation (3, 8).

Although proline substitutions did not affect *env* protein oligomerization, such substitutions affected virus infectivity (Table 1). This finding is in agreement with the observation that replacement of the middle isoleucine in the zipper motif by a nonconservative amino acid interferes with virus infectivity (9). The loss of virus infectivity in both studies could be attributed to the direct effect of a mutation in this region or a global conformational change induced by the amino acid substitutions. However, the recent finding that a synthetic peptide containing the zipper motif of gp41 blocked HIV-1 infectivity and syncytium-forming activity (36) supports the notion that the zipper motif at the N-terminal region of gp41 has a direct role in the HIV-1 life cycle. Further supporting this conclusion is the observation that the antiviral effect of the synthetic peptide was abolished once the middle isoleucine residue in the zipper motif was replaced by a proline residue.

The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: sheep antiserum to HIV-1 gp120 from Michael Phelan (University of California at Santa Cruz) and SupT1 cells from James Hoxie (University of Pennsylvania Hospital, Philadelphia). We are grateful to Alice S. Huang (New York University, New York) and Max Essex (Harvard School of Public Health, Boston) for their support and E. Conway for editorial assistance.

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