

Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Oligomerization Requires the gp41 Amphipathic α -Helical/Leucine Zipper-Like Sequence

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Received 4 March 1996/Accepted 26 November 1996

Human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein (Env) oligomerization was investigated by coexpressing wild-type and truncated envelope glycoproteins to determine the minimum sequence required for mutant-wild-type hetero-oligomerization. The gp41 putative amphipathic α -helix, Leu-550 to Leu-582, was essential for hetero-oligomer formation. Alanine substitution of 9 of the 10 residues composing the gp41 amphipathic α -helix 4-3 hydrophobic repeat sequence was required to inhibit mutant-wild-type hetero-oligomerization and to render the envelope glycoprotein precursor, gp160, monomeric. This indicates that multiple hydrophobic contacts contribute to the stable envelope glycoprotein oligomeric structure. Single alanine substitutions within the hydrophobic repeat sequence did not affect gp160 oligomeric structure but abolished syncytium-forming function. Some mutations also diminished gp160 processing efficiency and the association between gp120 and gp41 in a position-dependent manner. These results indicate that the gp41 amphipathic α -helix 4-3 hydrophobic repeat sequence plays a central role in HIV-1 envelope glycoprotein oligomerization and fusion function.

The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein precursor, gp160, undergoes chaperonin-assisted folding and oligomerization in the endoplasmic reticulum (ER) (15). The gp160 oligomer is transported to the Golgi complex, where proteolytic cleavage yields the fusogenic transmembrane subunit, gp41, and the receptor-binding subunit, gp120 (15, 53, 59). The gp120-gp41 complex is translocated to the cell surface and incorporated into budding virions as a gp41 tetramer or trimer anchoring gp120 through noncovalent interactions (42, 44, 51, 55). Infection follows the binding of gp120 to cell surface CD4 receptors (9, 28, 29, 33, 34) and Env glycoprotein-mediated fusion with the target cell membrane (30, 54).

Secondary-structure predictions indicate that retroviral transmembrane proteins share conserved structural motifs (19) that have been observed in the influenza virus hemagglutinin (HA) glycoprotein crystal structure (60). One such motif is a sequence of hydrophobic amino acids at the N terminus of the transmembrane protein (18). The analogous sequence in influenza virus HA plays a pivotal role in membrane fusion (3, 60), and mutagenic analysis of the corresponding sequence in HIV-1 gp41 indicates a conserved fusion role (16, 17, 29, 50). Another striking feature is an extended amphipathic α -helical sequence that contains a hydrophobic amino acid 4-3 repeat, characteristic of a leucine zipper (10, 19). An oligomerization function was initially postulated for the corresponding motifs in retroviral transmembrane proteins (10, 19), since assembly of monomeric proteins into oligomers frequently involves coiled-coil interactions mediated by 4-3 hydrophobic repeats (39, 60). The most striking example is the influenza virus HA trimer, which oligomerizes through a 54-residue amphipathic α -helix present in the transmembrane protein HA2 (60).

Recent data obtained from several experimental approaches suggest more complex structure/function roles for the HIV-1 gp41 amphipathic α -helical sequence. The potential for this sequence to form an α -helix is supported by the finding that a synthetic peptide analog exhibits solution-phase α -helical secondary structure (57, 58). This synthetic peptide has been shown to assemble into a four-stranded coiled coil in solution (47), and the corresponding sequence can assemble protein A or maltose binding protein monomers into oligomers when fused to the C termini of these bacterial proteins (2, 52). Whereas mutations that adversely affect the secondary structure or thermostability of the peptide analog in solution (57) also diminish the oligomerization efficiency of the bacterial protein-gp41 fusion constructs (2, 52), these same mutations do not affect gp160 oligomerization but abrogate fusion function (12, 57). For this reason, it was suggested that the gp41 amphipathic α -helix does not play a role in gp160 oligomerization but is involved in subsequent membrane fusion events, forming a coiled coil following fusion activation (2, 47, 52, 57). Further evidence supporting this idea is provided by Lu et al. (31), who used proteinase digestion of an insoluble, nonglycosylated gp41 ectodomain fragment to release a soluble complex between the extended amphipathic α -helix and a predicted C-terminal amphipathic α -helix. This complex was reconstituted from the component synthetic peptide analogs, and a stable α -helical trimeric structure was inferred. A model for the fusion-activated gp41 oligomeric core was proposed in which the amphipathic α -helix packs into a three-stranded coiled coil, thereby providing a hydrophobic site for interaction with the C-terminal α -helix (31). This association between the two predicted α -helices has also been postulated to generate the gp41 ectodomain fusion-competent structure (4). Proline mutation of the extended amphipathic α -helix or deletion of part of the C-terminal α -helix from a nonglycosylated recombinant gp41 ectodomain fragment leads to disruption of a conformational antibody epitope. This epitope is reconstituted

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when a synthetic peptide analog of the C-terminal α -helix is mixed with the C-terminal α -helix deletion mutant.

In this report, we demonstrate an oligomerization role for the gp41 extended amphipathic α -helix for Env expressed in mammalian cells. We show that the gp41 amphipathic α -helix is essential for the assembly of coexpressed wild-type and truncated gp160 monomers into hetero-oligomers. Alanine substitution mutagenesis of gp41 amphipathic α -helix 4-3 hydrophobic repeat residues indicates that multiple hydrophobic residues within this sequence mediate Env glycoprotein assembly and that each of these residues is essential for a fusion-competent Env glycoprotein structure.

MATERIALS AND METHODS

Plasmid constructs. The recombinant vaccinia virus-T7 polymerase system (36) was used for expression of wild-type and mutant Env. The expression plasmid pTMenv.2 (44) contains the full-length *env* gene (BH8 clone of HIV-1_{LAI} [48]) and was used to construct a dual T7 promoter-driven expression plasmid, pPT7-. The BH8 clone numbering system (48) is used throughout this report. The *StuI* (nucleotides 608 and 2572) and *BamHI* (nucleotide 2251) sites in pTMenv.2 were abolished by oligonucleotide-directed in vitro mutagenesis (Amersham International, Little Chalfont, United Kingdom). The 896-bp T7 expression cassette comprising the T7 promoter, murine encephalomyocarditis virus untranslated sequence, polycloning site, and T7 transcription termination sequence (36) was PCR amplified with *Pfu* polymerase (Stratagene, La Jolla, Calif.) from the pTM.1 expression vector with the oligonucleotide primers 5'-G GTATCGATGACGGCCGGTCTTCC and 5'-CTATTTTCCTTCGTCGG CCGTACGTC. The PCR product was ligated into the unique *EagI* site (36) in the mutated pTMenv.2 vector to yield pPT7-. Genes encoding C-terminally truncated Env mutants were PCR amplified from pPT7- with *Pfu* polymerase and ligated into the *NcoI-StuI* sites of the duplicated expression cassette of pPT7-. The inclusion of an *NcoI* site at the 5' ends of PCR products required an Arg-2-to-Ala (Arg-2→Ala) substitution, while inclusion of a 3' *StuI* site required an Asn-672→Gln substitution for the Env glycoprotein truncated at residue 672 (pP672) and an additional alanine at the C termini of all truncated mutants. Substitution mutants were prepared by oligonucleotide-directed in vitro mutagenesis (44), and mutations were confirmed by the Sequenase method (U.S. Biochemicals, Cleveland, Ohio). The sequences of oligonucleotide primers will be provided on request.

Cells and virus. HeLa-T4 cells were obtained from P. J. Maddon (33), and the recombinant vaccinia virus, vTF7-3, was obtained from T. M. Fuerst and B. Moss (36) through the AIDS Research and Reference Reagents Program, National Institute for Allergy and Infectious Diseases, Bethesda, Md. HeLa-T4 cells were maintained in Dulbecco's modification of minimal essential medium-10% fetal calf serum-G418 (500 μ g/ml; Life Technologies, Gaithersburg, Md.) while HeLa cells were cultured in the absence of G418. Wild-type and mutant Env glycoproteins were expressed following infection of cells with vTF7-3 and transfection with plasmid DNA using lipofectin (Life Technologies).

Antibodies. Human antibodies specific for synthetic peptides corresponding to the gp41 immunodominant epitope, human α -588-599 (21), and predicted gp41 amphipathic α -helix, human α -548-586 (19), or for recombinant gp120, human α -rgp120, were affinity purified from pooled HIV-positive human plasma as described previously (44, 46). Monoclonal antibodies (MAbs) obtained through the AIDS Research and Reference Reagent Program, National Institute for Allergy and Infectious Diseases, included 902 from B. Chesebro (8), ches8 (8) from G. Lewis (1), F105 from M. Posner (43), 50-69 and 126-6 from S. Zoller-Pazner (61), and Md-1 from R. Myers (37). MAb ICR39.3b was obtained from J. McKeating (35) through the Medical Research Council AIDS Directed Programme (Herts, United Kingdom), and MAb OKT4 (26) was obtained from the American Type Culture Collection, Rockville, Md.

Radioimmunoprecipitation. HeLa cells expressing wild-type and mutant Env were biosynthetically labeled with Tran-³⁵S-label for various times and then chased with complete medium for 4 to 5 h to allow intracellular transport and processing of Env glycoproteins. Cells expressing biosynthetically labeled Env glycoproteins and culture supernatants were processed as described previously (44). Cell lysates and culture supernatants were precleared overnight with bovine serum albumin immobilized on cyanogen bromide-activated Sepharose CL 4B (Pharmacia, Uppsala, Sweden). Radioimmunoprecipitation was performed with protein A-sepharose (Pharmacia) coated with human antibodies, with rabbit immunoglobulin G (IgG) to mouse IgG plus murine MAbs, or with rabbit IgG to rat IgG plus rat MAbs. CD4-binding assays were performed in the presence of MAb OKT4 (6 μ g) and soluble, recombinant CD4 (sCD4; 2 μ g) obtained through the AIDS Research and Reference Reagent Program, National Institute for Allergy and Infectious Diseases, from R. Sweet, SmithKline Beecham. Deglycosylations were performed with PNGase F (New England Biolabs, Beverly, Mass.) according to the manufacturer's instructions. Immunoprecipitated glycoproteins were analyzed following reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography or PhosphorImager

SF analysis using the Imagequant program (Molecular Dynamics, Sunnyvale, Calif.).

Sucrose density gradient centrifugation. HeLa cells expressing biosynthetically labeled wild-type or mutant Env were lysed in phosphate-buffered saline containing 1% Triton X-100 and sedimented over 5 to 20% (wt/vol) sucrose density gradients in a Beckman SW41Ti rotor at 35,000 rpm for 19 h at 4°C as described previously (44). Twenty-four fractions were collected from each gradient, and even-numbered fractions immunoprecipitated with MAb C8 were immobilized on cyanogen bromide-activated Sepharose CL 4B (Pharmacia) prior to reducing SDS-PAGE. Alternatively, HeLa cells expressing unlabeled Env glycoproteins were lysed and Env glycoproteins were sedimented as described above. Twenty-two fractions were collected from each gradient, and Env glycoproteins were trichloroacetic acid precipitated from each gradient fraction and subjected to reducing SDS-PAGE. Proteins were transferred to nitrocellulose, immunoblotted with human α -588-599, and then probed with radioiodinated protein A. Env glycoproteins were visualized with a PhosphorImager SF (Molecular Dynamics). Sucrose gradients were calibrated with the molecular weight markers catalase (11.3S), ¹²⁵I-thyroglobulin (19.4S), ¹²⁵I-aldolase (7.3S), and ¹²⁵I-ovalbumin (3.55S) (Pharmacia). Protein A and calibration markers were radioiodinated by the chloramine T procedure (22).

HeLa-T4 syncytium assay. HeLa-T4 cells were infected with vTF7-3, transfected with plasmid DNA, and then stained by the May-Grünwald-Giemsa technique 24 h later (12).

RESULTS

The gp41 amphipathic α -helix is essential for mutant-wild-type Env hetero-oligomer assembly. The sequences involved in gp160 oligomerization were identified by coexpressing wild-type Env with a series of C-terminally truncated Env mutants (Fig. 1) in HeLa cells and then assessing their ability to form mutant-wild-type hetero-oligomers. The anti-gp120 V3 loop MAb, 902 (8), immunoprecipitated pulse-chase biosynthetically labeled gp160 and gp120 from cells transfected with pPT7-, indicating normal synthesis and processing of wild-type Env (Fig. 2A, 902 [cell-associated] panel). Transfection of HeLa-T4 cells with pPT7- resulted in the formation of 11 ± 1.4 (mean \pm standard deviation) multinucleated syncytia per microscope field (magnification, $\times 200$), confirming that the mature Env glycoprotein complex expressed from this vector is fusion competent (data not shown). A series of progressively larger glycoprotein fragments was immunoprecipitated by MAb 902 from cells transfected with the various dual-expression constructs, in addition to gp160 and gp120, confirming that all glycoprotein species were expressed efficiently. Since none of the fragments contain the gp41 cytoplasmic domain, hetero-oligomers were detected with the gp41 cytoplasmic-domain-specific MAb, C8 (1), which efficiently immunoprecipitated gp160 from cells transfected with pPT7- (Fig. 2A, C8 panel). Mutant-wild-type hetero-oligomers did not form when the gp120 domain was coexpressed with the wild type (pP504), consistent with the finding that gp120 is secreted as a monomer when expressed in the absence of gp41 (14). Hetero-oligomers were not evident with extension of the mutant Env C terminus to Gln-547 (pP547), whereas further extension to include part (pP562) or all (pP586) of the gp41 amphipathic α -helix led to increasing levels of hetero-oligomer assembly. Small but significant increases in the efficiency of hetero-oligomer formation were observed with extension to Gln-616 (pP616) or to residue 672 on the N-terminal side of the transmembrane domain (pP672), suggesting that a nonessential oligomerization determinant is located C terminal to Leu-587. These results indicate that the sequence Gln-547 to Gln-586, which encompasses the gp41 amphipathic α -helix, is essential for stable mutant-wild-type hetero-oligomer assembly.

The conformational integrity of the CD4-binding domain of mutant glycoproteins was confirmed when all truncation mutants, as well as gp160 and gp120, were immunoprecipitated by sCD4 plus MAb OKT4 (Fig. 2A, sCD4 panel). Mutant glycoproteins truncated to Glu-504, Gln-547, and Gln-562 were also

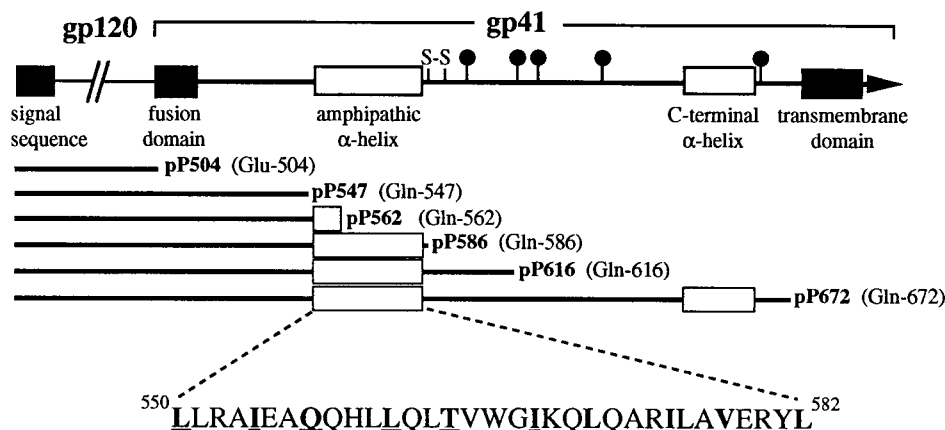


FIG. 1. Linear map of HIV-1 Env. The dual T7 promoter-driven expression plasmids (pP504-pP672) were constructed for coexpression of wild-type Env and truncated Env mutants in HeLa cells. The numerical designations of dual-expression plasmids correspond to the C-terminal residue (shown in parentheses) of the truncation mutant expressed from each plasmid. The gp41 amphipathic α -helical sequence is shown in single-letter code. Residues in the 4-3 hydrophobic repeat that were substituted with alanine are underlined. The positions of N-linked glycosylation sites (●) and the gp41 disulfide bond (S-S) are indicated.

immunoprecipitated by the conformational MAbs ICR.39.3b and F105 (data not shown). These results indicate that a conformationally intact gp120 domain is not sufficient for hetero-oligomer assembly. A number of studies have shown that misfolded Env proteins are retained in the ER, irreversibly bound to the chaperonin BiP (for a review, see reference 11). The transport competence of truncation mutants was therefore confirmed by immunoprecipitation of culture supernatants with MAb 902 followed by deglycosylation with PNGase F. Figure 2A (902 [secreted]) shows that expression from pP504, pP547, and pP562 resulted in secreted glycoproteins that comigrated with gp120 shed from cells transfected with pPT7-. Deglycosylation of these species (Fig. 2A, 902 [secreted, deglycosylated] panel) resulted in a core protein derived from pP504 which comigrated with deglycosylated gp120 (59 kDa), whereas core proteins derived from mutants with C-terminal extensions beyond Glu-504 migrated at progressively higher-molecular-weight positions. Transfection of cells with pP586 did not give rise to a secreted mutant glycoprotein, consistent with the coexpressed mutant being recruited into intracellular membrane-anchored or plasma membrane-anchored hetero-oligomers. However, this mutant is transport competent since it is secreted when expressed in the absence of the wild type from vector pS586 and deglycosylation indicates that it is also cleaved to gp120. Furthermore, an immunoblot assay with MAb C8 revealed no significant difference in the efficiency of gp160 cleavage to gp41 when expressed in the presence (pP586) or absence (pPT7-) of the gp160(1-586) construct in HeLa cells (data not shown). This indicates that export of gp160 from the ER to the *trans*-medial Golgi compartments where cleavage occurs (15, 59) is not affected by the presence of the gp160(1-586) mutant. Reduced secretion of mutants truncated to Gln-616 and residue 672 was also observed and is also consistent with hetero-oligomer formation.

To confirm that coexpressed gp160(1-586) and wild-type glycoproteins do not form nonspecific aggregates following synthesis, immunoprecipitations were performed following 18 min of pulse-labeling, when gp160 is monomeric, or following a 4-h chase, when gp160 is oligomeric (15). The control MAb, 902, immunoprecipitated both glycoprotein species at both labeling times. In contrast, MAb C8 immunoprecipitated only wild-type Env following the 18-min pulse but coimmunoprecipitated gp160(1-586) together with wild-type Env following the 4-h

chase (Fig. 2B). These results indicate that gp160(1-586)-wild-type Env hetero-oligomerization does not occur postsynthesis but requires an extended chase time, as is the case with wild-type Env (15). To rule out the possibility that gp160(1-586) and wild-type glycoproteins aggregate following detergent lysis, these glycoproteins were expressed separately from the vectors pS586 and pTMenv.2, respectively, and the lysates of pulse- or pulse-chase-labeled singly transfected cells were mixed prior to immunoprecipitation experiments. Expression of both species was confirmed when MAb 902 immunoprecipitated both glycoprotein species at both labeling times (Fig. 2B, MIX lanes). In contrast, MAb C8 immunoprecipitated only wild-type Env, indicating that these glycoproteins do not aggregate postlysis. The conformation of the gp41 domain of gp160 within the gp160(1-586)-wild-type Env hetero-oligomer was next assessed by immunoprecipitation with MAbs 126-6 and Md-1, which were isolated from HIV-1-infected individuals (37, 61). These MAbs recognize conformational epitopes within oligomeric but not monomeric gp41 as assessed by immunoblot assay (37, 45) and pulse-chase analysis (see Fig. 5). Figure 2B shows that neither MAb recognized Env glycoproteins following the 18-min pulse whereas both MAbs efficiently immunoprecipitated gp160 and gp160(1-586)-wild-type Env hetero-oligomers following the 4-h chase. That singly expressed gp160(1-586) was not recognized by these MAbs is consistent with the epitope of MAb 126-6 mapping to Ser-639-Leu-658 (61) in a C-terminal putative α -helical sequence (19) and the epitope of Md-1 mapping to the gp41 ectodomain fragment, Leu-560-Phe-680 (37). These results indicate that gp160 monomers within the hetero-oligomer acquire native conformation.

The oligomerization of influenza virus HA monomers occurs through the symmetrical packing of amphipathic α -helix hydrophobic repeat residues (60). To determine if a similar mechanism operates for HIV-1 Env, 9 of the 10 residues (excepting Gln-557) composing the gp41 amphipathic α -helix hydrophobic repeat sequence (Fig. 1) were substituted with alanine in the context of the gp160(1-586) truncation mutant. Alanine was chosen because of its strong α -helix-forming propensity and minimal disruptive effects when substituted into the α -helices of proteins and model peptides (25, 32, 38, 62). Immunoprecipitation with MAb 902 indicates comparable expression of glycoproteins truncated to Gln-586 containing either wild-type (pP586) or multiply substituted (pP586.Ala.9)

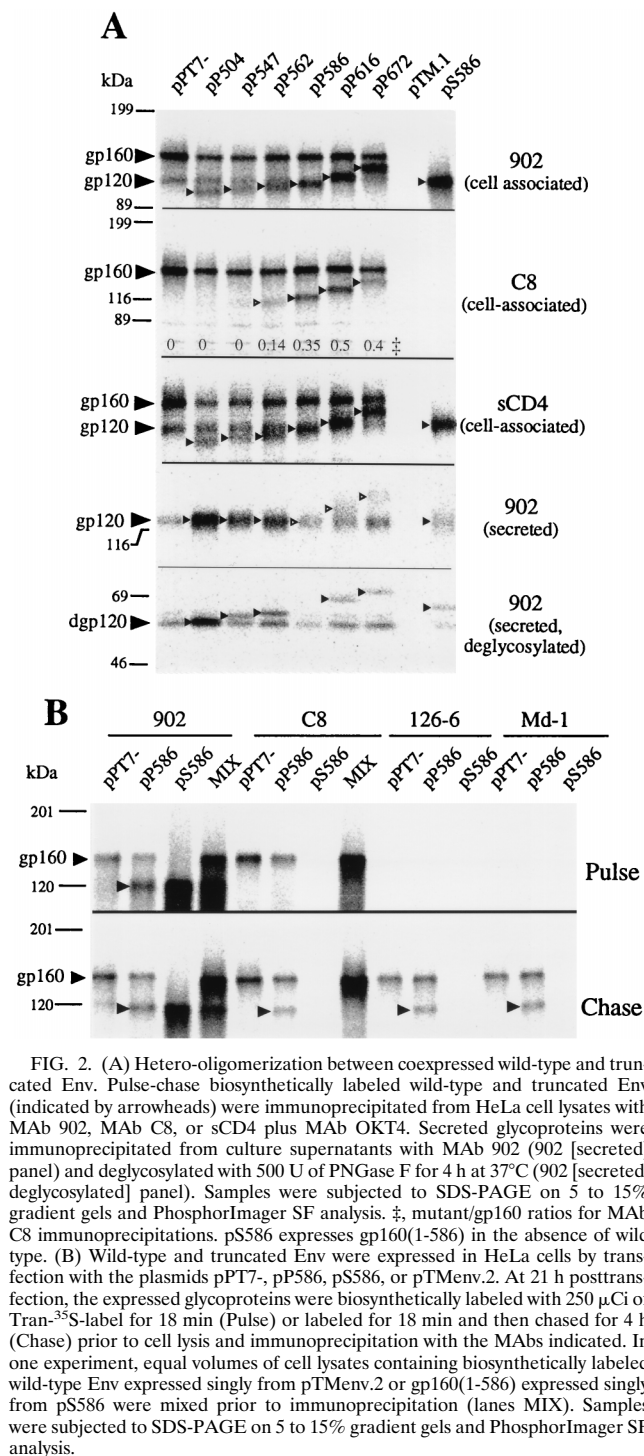


FIG. 2. (A) Hetero-oligomerization between coexpressed wild-type and truncated Env. Pulse-chase biosynthetically labeled wild-type and truncated Env (indicated by arrowheads) were immunoprecipitated from HeLa cell lysates with MAb 902, MAb C8, or sCD4 plus MAb OKT4. Secreted glycoproteins were immunoprecipitated from culture supernatants with MAb 902 (902 [secreted] panel) and deglycosylated with 500 U of PNGase F for 4 h at 37°C (902 [secreted, deglycosylated] panel). Samples were subjected to SDS-PAGE on 5 to 15% gradient gels and PhosphorImager SF analysis. ‡, mutant/gp160 ratios for MAb C8 immunoprecipitations. pS586 expresses gp160(1-586) in the absence of wild type. (B) Wild-type and truncated Env were expressed in HeLa cells by transfection with the plasmids pPT7-, pP586, pS586, or pTMenv.2. At 21 h posttransfection, the expressed glycoproteins were biosynthetically labeled with 250 μ Ci of Tran-³⁵S-label for 18 min (Pulse) or labeled for 18 min and then chased for 4 h (Chase) prior to cell lysis and immunoprecipitation with the MABs indicated. In one experiment, equal volumes of cell lysates containing biosynthetically labeled wild-type Env expressed singly from pTMenv.2 or gp160(1-586) expressed singly from pS586 were mixed prior to immunoprecipitation (lanes MIX). Samples were subjected to SDS-PAGE on 5 to 15% gradient gels and PhosphorImager SF analysis.

amphipathic α -helices (Fig. 3). While truncated Env containing the wild-type α -helical sequence (pP586) was efficiently coimmunoprecipitated with gp160 by MAb C8, the multiple alanine substitution resulted in a marked decrease in mutant-wild-type hetero-oligomerization and a corresponding increase in the amount of secreted glycoprotein.

Multiple alanine substitution of the gp41 amphipathic α -helix hydrophobic repeat disrupts gp160 oligomerization. The results of coexpression experiments support the concept that

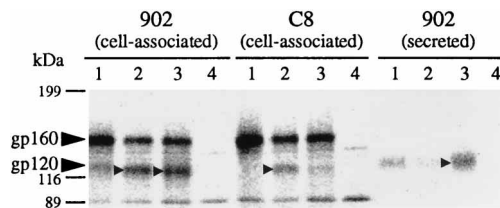


FIG. 3. Hetero-oligomerization between wild-type and truncated Env is inhibited by a multiple alanine substitution in the gp41 amphipathic α -helix hydrophobic repeat. Wild-type gp160 was expressed individually (lanes 1 [pPT7-]) or coexpressed with a mutant glycoprotein truncated at Gln-586 and containing an unmodified α -helix (lanes 2 [pP586]) or multiply substituted α -helix (lanes 3 [pP586.Ala.9]) in HeLa cells. Pulse-chase-labeled Env glycoproteins were immunoprecipitated from cell lysates with MAb 902 or with MAb C8 or from culture supernatants with MAb 902 (902 [secreted]). Samples were analyzed by SDS-PAGE on 5 to 15% gradient gels followed by PhosphorImager SF analysis. Lanes 4, control transfection with pTM.1.

gp41 amphipathic α -helix hydrophobic repeat residues mediate oligomerization through multiple intermonomer interactions. To determine if this was also the case for full-length Env, we assessed the effects of single and multiple alanine substitutions on gp160 oligomerization by sucrose density gradient centrifugation, immunoprecipitation with MAb C8-Sepharose, and SDS-PAGE (Fig. 4). Following 15 min of pulse-labeling, preassembled gp160 monomers (15) peaked at fraction 16 (7.5S), while following a 4-h chase, oligomeric gp160 sedimented further into the gradient, with a major peak in fractions 10 to 12 (11.6 to 13.6S), corresponding to dimers and trimers, and a minor peak in fraction 2 (19.4S), corresponding to tetramers (44). As described previously (12, 57), the Ile-to-Ala mutation at position 568 (I568A mutation) did not affect the oligomeric structure of gp160. The peak for gp160 bearing the triple mutation L550A-I554A-L561A (Ala.3) was consistently observed in fraction 8 (15.6S), indicating that the predominant species in this case was trimeric (44). The mutants Ala.5 (L550/I554/L561/L571/V578A) and Ala.6 (L550/I554/L561/I568/I575/L582A) have additional alanine substitutions in their hydrophobic repeat sequences, which results in the presence of both oligomeric and monomeric gp160 in sucrose

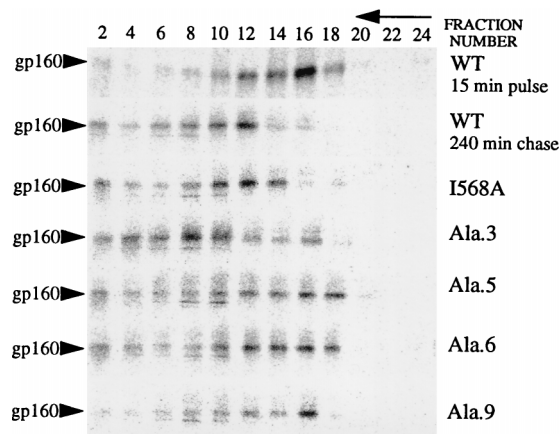


FIG. 4. Sucrose density gradient sedimentation of gp160 bearing multiple alanine substitutions. HeLa cells expressing wild-type (WT) or mutated Env were pulsed with 200 μ Ci of Tran-³⁵S-label for 15 min and lysed immediately (WT, 15-min pulse) or chased with complete medium for 4 h before lysis (WT, 240-min chase; I568A; Ala.3; Ala.5; Ala.6; Ala.9). Lysates were sedimented over 5 to 20% sucrose density gradients. Even-numbered fractions were immunoprecipitated with MAb C8-Sepharose prior to SDS-PAGE on 5 to 15% gradient gels and PhosphorImager SF analysis.

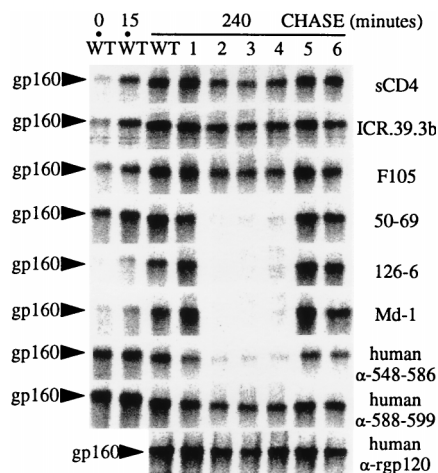


FIG. 5. Recognition of Env mutants by MABs and peptide-specific antibodies. HeLa cells expressing wild-type or mutated Env were pulsed with 250 μ Ci of Tran- 35 S-label for 15 min and chased for 0, 15, or 240 min. Precleared cell lysates were immunoprecipitated with sCD4 plus MAB OKT4 or the antibodies indicated prior to SDS-PAGE on 5 to 15% gradient gels and PhosphorImager SF analysis. WT, wild type; lane 1, Ala.3; lane 2, Ala.5; lane 3, Ala.6; lane 4, Ala.9; lane 5, I554A; lane 6, L550A. Wild-type gp160 was not immunoprecipitated by MAB OKT4 alone or control human IgG at the chase times tested (data not shown).

gradients, indicating partial disruption of gp160 oligomer formation and/or stability. Alanine substitutions at all 4-3 hydrophobic repeat positions except Gln-557 (Ala.9) effected almost complete disruption of Env oligomerization, indicating that oligomerization is mediated through multiple intermonomer contacts within the gp41 amphipathic α -helix hydrophobic repeat. Since the Ala.9 mutation did not abolish all oligomeric gp160 in sucrose gradients, additional minor assembly domains could also be present.

Effect of multiple alanine substitutions on acquisition of conformational epitopes in the gp41 ectodomain. Otteken et al. (40) recently investigated the time course of acquisition of gp160 native conformation by comparing the ability of conformational MABs to immunoprecipitate pulse-chase biosynthetically labeled gp160-folding intermediates. Their results suggest that gp160 oligomerization precedes the native folding of the gp41 ectodomain. It was of interest, therefore, to assess whether the multiple alanine substitutions, which disrupt gp160 oligomerization, affected the conformational maturation of the gp41 domain. Soluble, recombinant CD4 and a panel of conformational MABs were used to probe the maturation of alanine substitution mutants by radioimmunoprecipitation. Figure 5 indicates minimal binding of sCD4 and the panel of MABs to unfolded wild-type gp160 following 15 min of pulse-labeling with no chase. Soluble CD4 and the anti-gp120 MABs ICR.39.3b and F105 exhibited intermediate binding to wild-type Env following the 15-min chase, which is the half-time required for acquisition of CD4-binding competence but precedes oligomerization (15). Maximal binding followed the 4-h chase, at which point oligomerization and conformational maturation is complete (15, 40). The anti-gp41 MABs 126-6 and Md-1 exhibited a stricter requirement for oligomeric gp160, consistent with their recognition of oligomeric gp41 but not monomeric gp41 in immunoblot assays (37, 45). In contrast, MAB 50-69, human α -548-586, and human α -588-599 recognized epitopes that are not affected by oligomeric status. We examined the effect of the Ala.3, Ala.5, Ala.6, and Ala.9 mutations on antibody recognition following the 4-h chase. Im-

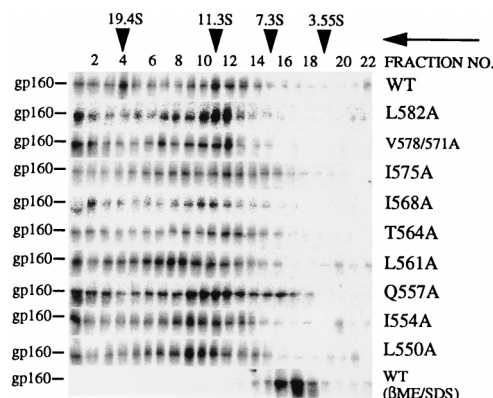


FIG. 6. Sucrose density gradient sedimentation of gp160 bearing single alanine substitutions. Lysates of HeLa cells expressing wild-type (WT) or mutated Env were sedimented over 5 to 20% sucrose density gradients. Fractions were subjected to trichloroacetic acid precipitation prior to SDS-PAGE, electrophoretic transfer to nitrocellulose, and immunoblot assay with human α -588-599 and radioiodinated protein A. Env glycoproteins were visualized by PhosphorImager SF analysis. β ME/SDS, HeLa cell lysate containing wild-type Env treated with β -mercaptoethanol and SDS at 100°C for 5 min before sedimentation.

munoprecipitation of gp160 alanine substitution mutants by sCD4 plus MAB OKT4 and the anti-gp120 MABs ICR39.3b and F105 was comparable to that of the wild type, indicating that the gp120 domain of the mutants folds normally. However, the Ala.5, Ala.6, and Ala.9 mutants were not immunoprecipitated by MABs 50-69, 126-6, and Md-1. These results indicate that the mutations affecting oligomeric structure had also blocked the formation of epitopes recognized by MAB 50-69, which is dependent on disulfide bond formation between Cys-593 and Cys-599 (61), and MABs 126-6 and Md-1, which have been mapped to the C-terminal portion of the gp41 ectodomain and require oligomeric structure for optimal binding (37, 45, 61). Recognition of Ala.5, Ala.6, and Ala.9 mutants by human α -548-586 was also decreased, indicating alteration of a linear epitope in the gp41 amphipathic α -helix. In contrast, the Ala.3, I554A, and L550A mutants were immunoprecipitated by sCD4 and all Env glycoprotein-specific antibodies at wild-type levels. These results indicate that mutations affecting Env oligomeric structure also block the conformational maturation of the gp41 ectodomain.

Alanine-scanning mutagenesis of the gp41 amphipathic α -helix hydrophobic repeat. Individual amino acids composing the gp41 amphipathic α -helix hydrophobic repeat (Fig. 1) were substituted with alanine to assess their contribution to Env oligomeric structure and function. The single substitutions L582A, I575A, I568A, T564A, Q557A, L561A, I554A, and L550A and the double substitution V578A/L571A did not alter gp160 oligomeric structure as determined by sucrose density gradient sedimentation (Fig. 6). However, position-dependent effects on precursor processing and subunit association were observed. Immunoprecipitation of cell-associated gp120 following a 5-h chase was observed for mutants L582A, V578A, I575A, I568A, and Q557A, indicating the maintenance of an associated gp120-gp41 complex following precursor cleavage. In contrast, mutants L571A, T564A, L561A, I554A, and L550A did not maintain gp120-gp41 association. Furthermore, an accompanying increase in the amount of gp120 immunoprecipitated from culture supernatants was not observed for these mutants, indicating decreased gp160 processing efficiency (Fig. 7A). Mutants I554A and L550A, however, were efficiently immunoprecipitated by sCD4 and the panel of conformational MABs (Fig. 5). These data suggest that only subtle

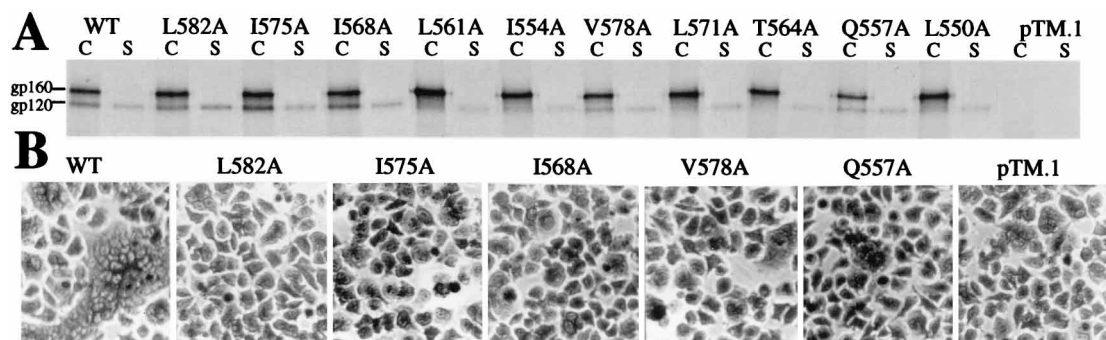


FIG. 7. (A) Effect of alanine substitutions on gp41-gp120 association. HeLa cells expressing mutant and wild-type (WT) Env were pulsed with 150 μ Ci of Tran-³⁵S-label for 20 min and then chased with complete medium for 5 h before lysis. Preacted cell lysates (C) and culture supernatants (S) were immunoprecipitated with human α -gp120 antibody and protein A-Sepharose. Samples were analyzed by SDS-PAGE on 4 to 12% gradient gels followed by autoradiography. (B) Effect of alanine substitutions on syncytium-forming function. ν TF7-3-infected HeLa-T4 cells were transfected with pTM.1 containing either wild-type or mutated *env* genes. At 24 h posttransfection, cells were stained by the May-Grünwald-Giemsa technique. Magnification, \times 130.

effects on Env structure can result in the observed decrease in cleavage efficiency. This may be due to a decreased rate of transport of mutant gp160 oligomers to the *trans*-medial Golgi or alteration of the processing site and a resultant gp41 structure that is unable to anchor gp120. All of the mutations abolished syncytium-forming function as determined in HeLa-T4 cells (Fig. 7B; also data not shown), but this is not due to loss of CD4-binding function, since all mutants were coimmunoprecipitated by sCD4 plus MAb OKT4 (data not shown). These results indicate that all residues in the gp41 amphipathic α -helix hydrophobic repeat contribute to a fusion-competent Env structure.

DISCUSSION

Mutagenic and immunochemical studies have indicated that the gp41 ectodomain is important for the assembly of HIV-1 Env oligomers (13, 14, 44, 46). We report herein that the conserved 33-residue amphipathic α -helix, Leu-550–Leu-582, within the gp41 ectodomain, is essential for efficient hetero-oligomerization between truncated and full-length Env monomers. Alanine substitution of 9 of the 10 residues composing the gp41 α -helix hydrophobic repeat blocks gp160 oligomerization and inhibits the ability of the truncated glycoprotein, gp160(1-586), to form hetero-oligomers with wild-type Env. These results indicate a primary role for the gp41 amphipathic α -helix in Env precursor oligomerization and are consistent with our earlier finding that deletions in this sequence result in gp160 oligomer disruption (44).

Truncation and alanine substitution mutagenesis of the gp41 α -helix hydrophobic repeat indicate that Env oligomerization involves multiple hydrophobic repeat residues throughout the length of the helix. We observed that truncation of the entire helix was required to block mutant-wild-type Env hetero-oligomerization, and nine alanine substitutions were necessary to generate gp160 monomers. Mutants containing truncated heptad repeat sequences exhibited drastically reduced oligomerization efficiency and/or stability. Thus, gp160(1-562) contains the first 12 α -helical residues and has reduced hetero-oligomer-forming ability compared with gp160(1-586), which contains the entire α -helix. Furthermore, significant but incomplete oligomerization disruption is observed for the Ala.5 and Ala.6 mutants, which contain three and two C-terminal hydrophobic heptad repeat residues, respectively. In contrast, oligomeric structure is maintained for the Ala.3 mutant which contains the C-terminal two-thirds of the heptad repeat. Sev-

eral structural studies have shown that alanine has the highest α -helix-forming propensity of the amino acids (32, 38, 62), the most striking example being that of T4 lysozyme, in which alanine substitution of 10 consecutive residues within the interdomain α -helix does not block α -helix formation and results in only minor effects on the tertiary structure, activity, and thermostability of the protein (25). There are also examples of α -helices that contain heptad repeats of alanines on one face (20). The secondary structure of the gp41 α -helical sequence is therefore likely to be maintained in the gp160 alanine substitution mutants while potential hydrophobic contact sites are removed from the heptad repeat. The residual oligomerization observed for the gp160(1-562), Ala.5, and Ala.6 mutants may therefore be mediated through the limited packing of remaining heptad repeat residues [Leu-550, Ile-554, and Leu 561 in gp160(1-562); Ile-568, Ile-575, and Leu-582 in Ala.5; and Leu-571 and Val-578 in Ala.6] into an abbreviated coiled coil if they are kept in register by maintenance of α -helical structure. These observations are paralleled by previous work on the *Lac* repressor (5). A heptad repeat of three leucines is sufficient to assemble dimers into tetramers through an abbreviated coiled coil, and extension of this heptad repeat sequence by one and two leucine heptad repeat units strengthens subunit association. Our observations suggest that HIV-1 Env oligomerization occurs via coiled-coil-dependent oligomerization, as seen for other proteins. For example, seven hydrophobic repeat residues pack the amphipathic α -helix of each influenza virus HA monomer into a triple-stranded coiled coil to stabilize the trimer (60) while GCN4 transcription factor dimerization occurs through the packing of one monomer's leucine zipper hydrophobic repeat against the corresponding sequence of a second monomer (39). Furthermore, such multiple intermonomer hydrophobic interactions in twofold, threefold, and fourfold symmetry pack model leucine zipper peptides into dimeric, trimeric, and tetrameric coiled coils, respectively (23, 24). Analytical equilibrium ultracentrifugation studies indicate tetrameric structures for a gp41 amphipathic α -helix synthetic peptide analog (47) and a maltose binding protein-gp41 amphipathic α -helix fusion construct (52). The gp41 α -helix could therefore symmetrically pack gp160 monomers into its various oligomeric forms and maintain gp41 in tetrameric form through coiled-coil formation. Our results, however, do not preclude the presence of other minor determinants that could contribute to Env oligomerization and/or oligomer stability, because extension of the gp160(1-586) fragment to Gln-616 or amino acid 672 resulted in a minor increase in the efficiency of

mutant-wild-type Env hetero-oligomer assembly and because nine alanine substitutions in the 4-3 hydrophobic repeat of this mutant did not completely inhibit its hetero-oligomer-forming ability.

Lu et al. (31) recently used protein dissection and peptide reconstitution techniques to identify the putative fusion-activated gp41 oligomeric core. Their data are consistent with a model in which the sequence Gln-535 to Gln-585, containing the gp41 amphipathic α -helix, packs into a three-stranded coiled coil through the hydrophobic 4-3 repeat. Furthermore, expression of gp41 ectodomain fragments lacking the N-terminal fusion peptide in insect cells resulted in α -helical rod-like oligomers (56). Our data now provide direct evidence that HIV-1 Env oligomerization is mediated through multiple hydrophobic interactions involving amphipathic α -helix hydrophobic repeat residues. However, gp41 cleaved from gp160 when expressed in mammalian cells appears to adopt tetrameric structure as defined by chemical cross-linking experiments (41, 44, 46, 51) or by subjecting viral gp41 to SDS-PAGE in differing SDS concentrations (42, 46). In contrast, the putative oligomeric core released by proteolysis of the insoluble, nonglycosylated recombinant gp41 ectodomain fragment, Gln-535 to Trp-665 (31), and gp41 ectodomain fragments lacking the N-terminal fusion peptide expressed in insect cells (56) are trimeric. Accommodation of glycosylated and nonglycosylated Env glycoprotein sequences that are N and C terminal to the two gp41 α -helices within native gp160 and gp41 oligomeric structures may favor coiled-coil packing in fourfold symmetry. In contrast, trimerization may be favored for the packing of the amphipathic α -helix present in gp41 ectodomain fragments lacking these N- and C-terminal sequences.

Otteken et al. (40) recently demonstrated that oligomer-specific, conformational MAbs directed against the gp41 ectodomain recognize gp160-folding intermediates with half-times longer than that required for gp160 oligomerization. Furthermore, conformational anti-gp41 MAbs that recognize both monomeric and oligomeric Env are directed to epitopes which form with a half-time of 30 min, which coincides with oligomerization. These results suggest that gp160 oligomerization is required for the conformational maturation of the gp41 ectodomain. Consistent with these findings, we observed that the multiple mutations Ala.5, Ala.6, and Ala.9, which disrupted oligomerization to varying degrees, also blocked the acquisition of conformational epitopes recognized by the human anti-gp41 MAbs 126-6 and Md-1. Whereas a component of the loss in recognition results from loss of oligomeric structure (MAbs 126-6 and Md-1 require Env oligomerization for optimal binding [37, 45]), the mutations appear to have also directly affected the folding of the gp41 domain, because the Ala.5 and Ala.6 mutants are partially oligomeric but are not recognized at all by these MAbs. Multiple alanine substitutions in the α -helix hydrophobic repeat Leu-550 to Leu-582 presumably disrupt Env oligomerization, because the coiled-coil packing of this helix is disrupted. This could modify (Ala.5 and Ala.6) or ablate (Ala.9) a site on the exterior of the coiled coil required for interaction with other gp41 regions that generate the gp41 tertiary and quaternary structure. One potential site for interaction is a C-terminal α -helical sequence, Ile-641 to Trp-665 (19). Lu et al. (31) proposed that the coiled-coil packing of the α -helix Leu-550 to Leu-582 provides a hydrophobic face for association with the C-terminal α -helix Asn-631 to position 661, while Chen et al. (4) suggested that an interaction between the two α -helices is essential to generate the gp41 ectodomain structure. Previous studies have shown that proline substitutions in the gp41 amphipathic α -helix disrupt the proximal MAb 50-69 epitope without affecting gp160 oligomerization (6, 7), and conversely, Cys-593 \rightarrow Ser and Cys-

599 \rightarrow Ser substitutions, which would disrupt the epitope of this MAb, do not affect oligomeric structure (14). Formation of the disulfide bond between Cys-593 and Cys-599 appears to also be inhibited in the multiple alanine mutants, since they are not recognized by MAb 50-69. This is likely to be a direct effect of the mutations on the structure of this site that is independent of gp160 oligomerization disruption and suggests a functional interaction between the α -helix and the disulfide-bonded domain. Significantly, mutant-wild-type Env hetero-oligomer assembly can occur in the absence of the sequence Leu-587 to Asn-672, which contains the epitopes of MAbs 50-69, 126-6, and Md-1. The inhibition of oligomerization resulting from the multiple alanine substitutions cannot therefore be simply attributed to accompanying conformational effects in the gp41 ectodomain beyond Gln-586 and is more likely to be a direct result of alterations in the gp41 amphipathic α -helix.

Wild et al. (57) postulated that gp41 amphipathic α -helices from adjacent monomers form a coiled coil as part of the structural changes associated with Env-mediated fusion rather than directly initiating gp160 oligomerization. This was based on the findings that I568A, I568S, and I568P mutations increasingly disrupt the solution-phase α -helical structure of a synthetic peptide analog of the amphipathic α -helix but do not block gp160 oligomerization, affecting only fusion function. Results presented in this study do not support this concept but indicate a direct oligomerization role for the gp41 amphipathic α -helical sequence. gp160 and gp41 oligomers are partially resistant to boiling in the presence of SDS and reducing agents (13, 42, 46); this suggests a stable oligomeric structure that is assembled by extensive intermonomer contacts, as has been observed for other oligomeric proteins (27, 60). It is therefore not surprising that essentially the entire gp41 4-3 hydrophobic repeat sequence must be ablated to effect gp160 oligomer disruption. Proline substitutions introduced into the gp41 amphipathic α -helix (7, 31, 57) probably have subtle effects on gp41 structure and can be accommodated without disrupting oligomerization, whereas fusion competence, which is exquisitely sensitive to mutations in the hydrophobic repeat sequence, is abolished by such substitutions. This interpretation is consistent with the detailed studies on T4 lysozyme, in which a proline substitution in the 23-residue interdomain α -helix results in only modest effects on tertiary structure, thermostability, and catalytic activity, with a 5.5° bend at the substitution site (49).

Whereas data presented in this study indicate a direct oligomerization role for the gp41 amphipathic α -helical sequence, results obtained with single alanine substitution mutants suggest that the α -helical sequence is also involved in other aspects of Env function since such mutants do not disrupt gp160 oligomeric structure but abolish syncytium-forming function and cause position-dependent decreases in precursor-processing efficiency and gp120-gp41 subunit association. Residues Leu-571, Thr-564, Leu-561, Ile-554, and Leu-550 are required for normal precursor-processing efficiency and subunit association and could play a critical role in Env glycoprotein structure stabilization. In contrast, mutation of Leu-582, Val-578, Ile-575, Ile-568, and Gln-557 affects only syncytium-forming function, without having detectable effects on gp120-gp41 structure or cell surface expression (6, 57). A recent study has shown that gp41 amphipathic α -helix synthetic peptide analogs bind directly to membranes (47), suggesting that the hydrophobic repeat sequence serves as a secondary membrane-binding site following insertion of the N-terminal fusion peptide into the target cell membrane. Thus, decreasing the hydrophobicity of the α -helical sequence by introducing alanine substitutions could decrease its membrane-binding poten-

tial, thereby abolishing syncytium-forming function. However, in the case of the influenza virus HA, the N-terminal one-third of the HA2 amphipathic α -helix maintains fusion-activated monomers in trimeric form through its hydrophobic repeat sequence while the N terminus of the α -helix is extended by approximately 100Å to project the fusion domain from the core of the trimer towards the target membrane (3). By analogy, gp41 hydrophobic repeat residues could mediate Env oligomerization and also stabilize the fusion-activated gp41 oligomer. The residues Leu-582, Val-578, Ile-575, Ile-568, and Gln-557 could then function in facilitating the conformational changes in gp41 that accompany membrane fusion. It will be of great interest to determine whether gp41 and HA2 amphipathic α -helices play similar roles in the fusion process.

ACKNOWLEDGMENTS

We are grateful to Frosa Katsis for oligonucleotide and peptide synthesis.

This work was supported by the National Health and Medical Research Council and the Commonwealth AIDS Research Grants Program. P.P. is a CARG fellow, and B.E.K. is an NHMRC fellow.

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