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Functional Role of the Zipper Motif Region of Human Immunodeficiency Virus Type 1 Transmembrane Protein gp41

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To study the functional role of the zipper motif region, located in the N-terminal region of the envelope transmembrane protein of human immunodeficiency virus type 1, a series of vaccinia virus-expressed mutant proteins containing a proline substitution in this region were characterized. All of the mutant proteins showed partial or no inhibition in gp160 cleavage, demonstrated impaired ability of gp120 to associate with gp41, and were unable to mediate syncytium formation with $CD4^+$ cells. Moreover, mutants 580 and 587 secreted excessive gp120 into the medium compared with the wild type. Mutations in this region affected the conformation of the local or proximal sequence but did not alter the conformation conferred by a distal site. These studies reveal the crucial role of the C-terminal segment of the zipper motif region in envelope heterodimeric association and suggest that this sequence forms a gp120 contact site.

The envelope (*env*) gene product of human immunodeficiency virus type 1 (HIV-1) is synthesized as a gp160 precursor in the rough endoplasmic reticulum. After synthesis, the Env precursor is folded into a form competent to bind the viral receptor CD4 and is oligomerized in the rough endoplasmic reticulum (8, 12, 13, 15, 35). gp160 is proteolytically cleaved, presumably in the Golgi compartment (10, 13, 38, 41), and forms the mature surface protein gp120 and the transmembrane (TM) protein gp41. Precursor processing and the presence of a noncovalent gp120-gp41 complex on the cell surface are essential for virus infectivity and syncytium formation with target cells expressing the CD4 receptor (1, 16, 25, 30, 31, 39).

Retroviral TM proteins have common structural features, such as the N-terminal hydrophobic fusion domain, leucine zipper-like motif (termed zipper motif hereafter), cysteine loop, and TM region (9, 17, 18, 34). The HIV-1 zipper motif spans amino acid residues 559 to 587 (on the basis of the sequence of the HXB2 proviral DNA clone) and shows a periodic repeat of a leucine or isoleucine residue at every seventh position over eight helical turns. The region containing the zipper motif, called the zipper motif region, is highly conserved among HIV-1 isolates. The zipper motif region is explicit as a heptad repeat sequence, an extensive region containing seven residue repeats of amino acids in a sequence periodicity (*a*, *b*, *c*, *d*, *e*, *f*, *g*) with nonpolar residues in all *a* positions and in most *d* positions when displayed on an alpha-helical wheel. Heptad repeat sequences are conserved in the fusion proteins of paramyxoviruses, influenza viruses, coronaviruses, and retroviruses (4). All of these heptad repeat sequences are predicted to form long amphipathic alpha-helices (4, 18). Indeed, the synthetic peptide DP107, which contains the zipper motif region, forms a stable alpha-helical structure in solution (40).

Nonconservative amino acid substitutions for the middle isoleucine located in the zipper motif of HIV-1 gp41 have been shown to affect virus infectivity and membrane fusion (11). In addition, peptide DP107 blocks infectivity and the syncytium-forming ability of HIV-1 (40). Nevertheless, the precise role(s) of the zipper motif region in Env protein function has not been addressed in detail.

In a previous study, four proline substitution virus mutants were generated to examine whether the zipper motif in gp41 was critical to the virus life cycle (6). Mutant Env proteins still formed oligomers, but the virus mutants all showed severely impaired infectivity. In this study, the zipper motif region of gp41 was further studied as a model system to address the structure-function relationship of viral envelope heptad repeat sequences.

To further examine the role of the zipper motif region in virus infectivity, three mutants in addition to the four virus mutants previously studied (6) were constructed. Three highly conserved leucine or isoleucine residues at 565, 576, and 580 were each replaced by a proline residue. COS-1 cells were transfected by the DEAE-dextran method with equal amounts of wild-type (wt) or mutant plasmid DNA (6). Three days posttransfection, comparable amounts of virion-associated reverse transcriptase (RT) activity were detected in the culture supernatants of cells transfected with wt or mutant plasmid DNA. wt and mutant viruses with equivalent amounts of RT activity were assayed for infectivity on SupT1 cells. As shown in Table 1, wt virus produced significant levels of virion-associated RT activity and p24 in the medium during the culture period. In contrast, mutant viruses did not produce any detectable levels of RT activity or p24 during the 43-day culture period. Moreover, cytopathic effects, including syncytium formation, were observed in wt but not mutant viruses. These results together with the previous study (6) indicate that the zipper motif region in gp41 might have an important role in the virus life cycle.

The vaccinia virus (VV) vector system provides an approach to expressing biologically functional HIV-1 Env protein and to studying the specific interaction between Env subunits in the absence of other viral proteins. The detailed kinetics of biosynthesis and maturation of the Env protein expressed by a

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TABLE 1. Construction and phenotypes of mutant viruses

Construct[a]	Amino acid sequence[b]	Infectivity		Syncytium formation[c]
		RT[d]	p24[e]	
$HXB2_{gpt}$(wt)	IEAQQHLLQLTVWGIKQLQARILAVERYL	+	+	+
$HXB2_{gpt}$(565)	------P----------	–	–	–
$HXB2_{gpt}$(576)	----------P----------	–	–	–
$HXB2_{gpt}$(580)	----------P-------	–	–	–

[a] The following synthetic oligonucleotides were used as primers to generate mutants: $HXB2_{gpt}$(565), GAGGCGCAACAGCATCCGTTGCAACTCACAGT CTGGGGC; $HXB2_{gpt}$(576), GTCTGGGGCATCAAGCAGCCCCAGGCAAGAATCCTGGC; and $HXB2_{gpt}$(580), GGCATCAAGCAGCTCCAGGCAAGACC CCTGGCTGTGGAAAGA. Underlining indicates the nucleotides that encode proline substitutions.

[b] The amino acid sequences of wt and mutant Env proteins between residues 559 and 587 are shown. The residues that constitute the zipper motif are underlined. A dash indicates that the residue in that position in the mutant protein is the same as that in the wt protein. The one-letter code is used for amino acids.

[c] Cells infected with wt virus showed extensive syncytium formation 3 days after infection. No syncytium formation was seen in cells treated with mutant viruses over the 43-day culture period.

[d] wt virus produced 1.7×10^5 and 2.7×10^6 cpm of RT activity per ml at days 6 and 12 postinfection, respectively. None of the mutant viruses produced RT activity above the background level over the 43-day culture period.

[e] In a separate experiment, wt virus produced $>1 \times 10^3$ and $>2.3 \times 10^5$ pg of p24 antigen per ml at days 7 and 11 postinfection, respectively. Mutant viruses did not produce any detectable amounts of p24 over the 35-day period.

VV vector have previously been reported (13). A VV expression system using pV*Env*(A) that synthesizes HIV-1 Env protein (BH10 strain [20]) under the control of the P_{11} promoter (5) was therefore employed to address the role of this region in Env protein function.

The *env* genes from wt and mutant $HXB2_{gpt}$ were subcloned into this vector. A plasmid that synthesizes Env protein with a leucine-to-proline substitution at 568 was also constructed (5). CV-1 cells infected with strain WR of VV were transfected with equal amounts of wt or mutant plasmids by a liposome-mediated method (14) (Bethesda Research Laboratories, Gaithersburg, Md.). VV was propagated in HeLa cells as previously described (28). Six hours posttransfection, cells were labeled with Tran^{35}S-label (ICN, Costa Mesa, Calif.) for 16 h. Equal portions of the cell lysate were precipitated with pooled anti-HIV sera and *Staphylococcus aureus* protein A (the Enzyme Center, Inc., Malden, Mass.) and were separated by sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gel electrophoresis (PAGE). As expected, transfection of the wt plasmid produced gp160, gp120, and gp41 (Fig. 1A, top, lane 3) (3-day exposure), and gp41 migrated as a broad band in the gel. Transfection of 3PrV*Env*, a plasmid that contains the complete *env* gene with an ATG initiation codon about 480 bp downstream from the P_{11} promoter (5), also produced these

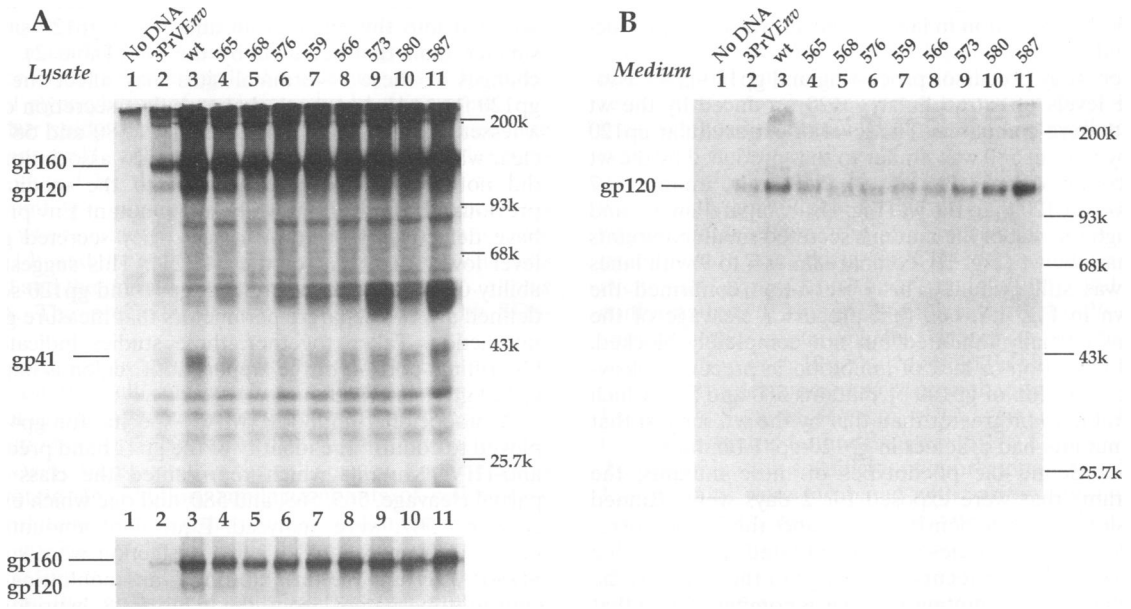

FIG. 1. Immunoprecipitation of lysate and culture medium from cells transfected with wt or mutant *env*-expressing plasmids. CV-1 cells grown in six-well plates were infected with VV at a multiplicity of infection of 5 at 37°C. One hour postinfection, cells were transfected with 3 μg of wt or mutant plasmids in the presence of 20 μg of lipofectin reagent according to procedures described by the manufacturer. Six hours after cotransfection, monolayers were washed twice with phosphate-buffered saline. One milliliter of cysteine- and methionine-free Modified Eagle medium containing 2% dialyzed fetal calf serum and Tran^{35}S-label (at a specific activity of 100 μCi/ml during labeling) was added to the cultures, and cells were incubated at 37°C for 16 h. Cell lysate (A) and culture medium (B) were incubated with 5 μl of pooled anti-HIV antisera and precipitated with prewashed *S. aureus* protein A. Proteins eluted from the washed immune complexes were separated by SDS–7.5% PAGE and then subjected to fluorography. Molecular sizes, in kilodaltons, are also shown. (A) Autoradiograms after 3 days (top) or 1 day (bottom) of exposure. (B) Autoradiogram after 3 days of exposure. Notice the expression of gp160 and gp120 only after 1 day of exposure.

three species but at reduced levels (Fig. 1A, top panel, lane 2). This may be due to the decreased promoter strength for expression of a gene at a promoter-distal site. These Env proteins were not detected during mock transfection (Fig. 1A, top panel, lane 1).

All mutants synthesized cell-associated gp160 at a level similar to that produced by the wt (Fig. 1A, top panel), indicating that proline substitution in this region did not affect Env precursor synthesis. Intracellular gp120 was evident in cells expressing wt protein (Fig. 1A, top panel, lane 3). Only a trace amount of gp120 was identified in cells synthesizing mutant 565 protein or 568 protein (Fig. 1A, top panel, lanes 4 and 5). Virtually no discrete cell-associated gp120 bands were detected in the six other mutants (Fig. 1A, top panel, lanes 6 to 11). When the film was exposed for 1 day, wt intracellular gp120 was still visible but mutant intracellular gp120 was not detected (Fig. 1A, bottom). Mutations in gp120 or gp41 can completely dissociate gp120 from the heterodimeric complex, resulting in all of the gp120 being secreted into the culture medium. Thus, intracellular gp120 represents the predominant gp120 fraction in cells that is associated with gp41 (25). The observation that mutants produced much less gp120 than the wt in the cells suggests that cleavage of mutant Env proteins was inhibited and/or that the mutants had impaired gp120-gp41 association.

To differentiate these two possibilities, the levels of cell-associated gp41 produced by the mutants were compared with that produced by the wt. Mutant 587 synthesized an amount of cell-associated gp41 similar to that synthesized by the wt (Fig. 1A, top panel; compare lanes 3 and 11), indicating that precursor processing of this mutant was not much affected. The rest of the mutants produced smaller amounts of cell-associated gp41 than the wt (Fig. 1A, top panel; compare lanes 4 to 10 with lane 3), suggesting that these mutant proteins had reduced precursor cleavage. A strong band that migrated at about the 55-kDa position in lanes 9 and 11 was not reproducibly observed.

To further study precursor processing and gp120-gp41 association, the levels of extracellular gp120 produced by the wt and mutants were compared. The level of extracellular gp120 produced by mutant 580 was similar to that produced by the wt (Fig. 1B; compare lanes 3 and 10). Strikingly, mutant 587 secreted more gp120 than the wt (Fig. 1B; compare lanes 3 and 11). Although the rest of the mutants secreted smaller amounts of gp120 than the wt (Fig. 1B; compare lanes 4 to 9 with lanes 3), gp120 was still evident. This observation confirmed the result shown in Fig. 1A, i.e., that precursor cleavage of the mutants was partially inhibited but not completely blocked. The partial inhibition or lack of inhibition in precursor cleavage and the secretion of gp120 by mutants 580 and 587, which was comparable to or greater than that by the wt, suggest that these two mutants had a defect in gp120-gp41 binding.

To further define the phenotypes of these mutants, the autoradiograms that were exposed for 2 days were scanned two-dimensionally by a densitometer, and the areas corresponding to each Env species were quantitated. To determine the degree of inhibited precursor cleavage of the mutants, the ratio of mutant gp41 to mutant gp160 was compared with that of the wt. Except for mutant 587, which had slightly greater gp160 cleavage than the wt, all mutants showed a 35 to 67% inhibition in precursor cleavage (Table 2). The mutants' processing index, a measure of the conversion of mutant gp160 to gp120 relative to that of the wt, and the association index, a measure of the ability of mutant gp120 to associate with gp41 relative to that of the wt protein, were calculated (21). As shown in Table 2, all of the mutants except for mutant 587 had a reduced processing index compared with that of the wt. All of the mutants had either a slightly or a markedly reduced association index. In addition, mutations in the C-terminal part had a greater effect on the ability of gp120 to associate with gp41 than those in the N-terminal region.

TABLE 2. Phenotypes of Env protein mutants

Envelope protein[a]	Relative gp160 cleavage[b]	Processing index[c]	Association index[d]	Relative gp120 secretion[e]
wt	1.0	1.0	1.0	1.0
559	0.41	0.22	0.12	1.06
565	0.43	0.48	0.91	1.18
566	0.52	0.27	0.52	0.72
568	0.33	0.28	0.37	1.29
573	0.65	0.41	0.33	1.01
576	0.44	0.25	0.29	0.96
580	0.60	0.69	0.21	2.08
587	1.17	1.10	0.14	1.86

[a] Autoradiograms after 2 days of exposure of the gels in Fig. 1 were scanned with an LKB Ultrascan XL laser densitometer. The relative intensity of each Env species was quantitated by LKB GelScan software.

[b] Calculated as the ratio of $[gp41]_{mutant}/[gp160]_{mutant}$ to $[gp41]_{wt}/[gp160]_{wt}$.

[c] Analyzed as the ratio of $[\text{total gp120}]_{mutant}/[gp160]_{mutant}$ to $[\text{total gp120}]_{wt}/[gp160]_{wt}$.

[d] Measured as the ratio of $[\text{mutant gp120}]_{cell}/[\text{mutant gp120}]_{supernatant}$ to $[\text{wt gp120}]_{cell}/[gp120]_{supernatant}$.

[e] Defined as the ratio of $[\text{mutant gp120}]_{supernatant}/[\text{mutant gp41}]$ to $[\text{wt gp120}]_{supernatant}/[\text{wt gp41}]$.

To study whether the mutants secreted gp120 more readily than the wt, the ratio of mutant-secreted gp120 to cell-associated gp41 relative to that of the wt was determined. Mutants 580 and 587 had approximately twofold-greater gp120 secretion than the wt (Table 2). This is consistent with their striking impairment in gp120 association with gp41, as reflected in the association index. The remainder of the mutants secreted into the medium an amount of gp120 similar to or smaller than that secreted by the wt (Table 2). Therefore, changes at these N-terminal sites may affect the affinity of gp120 for gp41, but these changes affect secretion of gp120 to a lesser degree than the mutations in 580 and 587. It is not clear why mutant 559 had a relatively low association index but did not secrete excessive gp120 into the medium. It was previously observed that while some mutant Env proteins may have defective association indices, they secreted gp120 at a level lower than that of the wt (21). This suggests that the ability of gp120 to associate with gp41 and gp120 secretion as defined are two separate parameters that measure gp120-gp41 interaction. Taken together, these studies indicate that the C-terminal portion of the zipper motif region is important for gp120-gp41 interaction.

A monoclonal antibody (MAb) specific for gp41 was employed to confirm the identity of the gp41 band precipitated by anti-HIV. Mutants which represented the class possessing partial cleavage, 565, 568, and 580, and one which exhibited wt cleavage, 587, were analyzed. Equivalent amounts of lysate prepared from cells 48 h posttransfection were subjected to SDS-PAGE and Western blotting (immunoblotting) using the culture supernatant from the Chessie 8 hybridoma. Mock transfection did not show significant Env protein-specific signals (Fig. 2, lane 1). All mutant precursors were synthesized at a level similar to that of the wt gp160. Similar amounts of cell-associated gp41 were detected in mutant 587 and the wt (Fig. 2, lanes 2 and 6). The remainder of the mutants synthesized smaller amounts of cell-associated gp41 than the wt (compare lanes 3 to 5 with lane 2). Densitometric analysis of mutant gp160 cleavage relative to that of the wt was as follows:

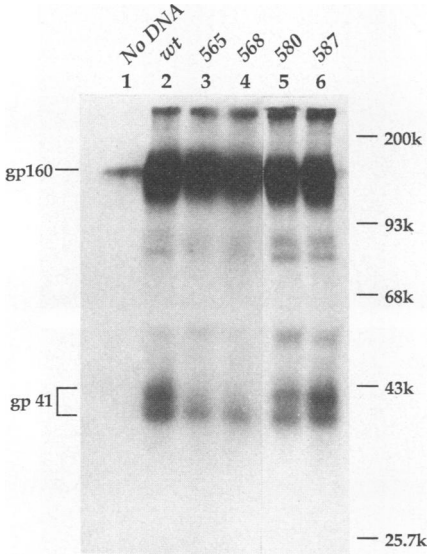

FIG. 2. Expression of mutant Env proteins. CV-1 cells were infected with VV and subsequently transfected with wt or mutant plasmids. Forty-eight hours posttransfection, cell lysates were prepared and subjected to gel electrophoresis, blotted onto a nitrocellulose membrane (0.45-μm pore size), and analyzed with Chessie 8 mouse MAb anti-gp41. The immune complex was visualized by using a biotin-streptavidin detection system coupled with an enhanced chemiluminescence method (Amersham).

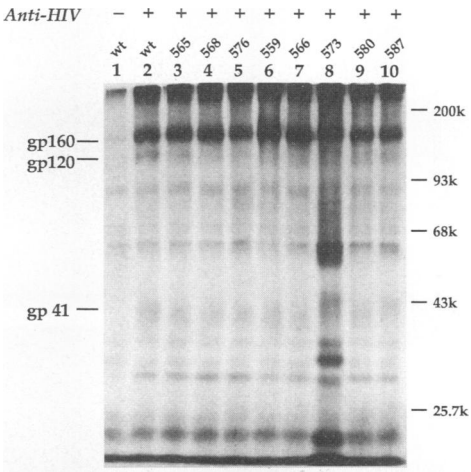

FIG. 3. Cell surface expression of mutant Env proteins. CV-1 cells were infected, transfected, and metabolically labeled. Cells were washed twice with phosphate-buffered saline and incubated with 0.5 ml of phosphate-buffered saline containing 2% dialyzed fetal calf serum and 1:33-diluted preimmune human serum (lane 1) or human anti-HIV (lanes 2 to 10) at 4°C for 1 h. The cells were washed and lysed. The lysates were incubated with *S. aureus* protein A, and the bound proteins were analyzed by SDS-PAGE.

mutant 565, 0.45; mutant 568, 0.41; mutant 580, 0.69; and mutant 587, 1.03. These results were similar to those obtained by radioimmunoprecipitation (Table 2). The band that precipitated with anti-HIV and migrated slightly ahead of the 43-kDa molecular mass standard was, indeed, the gp41 band. The appearance of the smeared gp41 doublet may result from the differential glycosylation of gp41 detected by this system.

To further study the impaired ability of gp120 to associate with mutant gp41, cell surface-accessible gp120-gp41 complexes produced by the wt and mutants were detected by antibody binding as previously described (22). ^{35}S-labeled cells producing wt or mutant proteins were incubated with anti-HIV at 4°C for 1 h, and the unbound antibody was washed. The cells were lysed and the immune complexes were bound to *S. aureus* protein A. The precipitates were then separated by SDS-PAGE.

Preimmune human serum did not bind to wt Env proteins expressed on the cell surface (Fig. 3, lane 1). Comparable amounts of cell surface-accessible gp160 were produced by the wt and mutants (Fig. 3, lanes 2 to 10), suggesting that mutant precursors were transported to the cell surface as efficiently as the wt protein. In addition, similar amounts of cell surface gp41 were detected in all of the mutants and in the wt (Fig. 3). However, gp120 was clearly detected only on the surfaces of cells expressing wt protein (Fig. 3, lane 2). It was faintly detected on the surfaces of cells synthesizing mutant 565 protein (Fig. 3, lane 3). The rest of the mutants did not show discrete cell surface-accessible gp120 bands (Fig. 3, lanes 4 to 10). Although some shadows were observed ahead of the gp160 precursor in some of the mutants, these diffuse species migrated faster than the wt gp120 (Fig. 3, lane 2). Densitometric quantitation showed that all of the mutants had a 46 to 83% reduction in cell surface gp120 levels compared with that of the wt. This result indicates that mutants formed gp120-gp41 complexes on the cell surface less sufficiently than the wt.

To determine whether mutant proteins expressed on the cell surface were functional, the ability of mutant proteins to induce syncytium formation with CD4-bearing cells was studied. VV-infected cells were transfected with an equal amount of wt or mutant plasmids. Six hours posttransfection SupT1 cells were added to the cultures. Cocultures were photographed 18 h posttransfection for syncytium formation under a light microscope. As shown in Fig. 4A, mock transfection did not result in any visible syncytia. Expression of wt protein resulted in extensive syncytium formation (Fig. 4B). In contrast, synthesis of mutant proteins did not mediate any visible syncytium formation (Fig. 4C to J).

To examine whether the phenotype of mutant Env proteins was directly attributable to the effects of the mutations in the zipper motif region, the overall structures of the mutant proteins were assessed. gp120-CD4 interaction is a high-affinity event and depends on the correct folding, and the overall conformation, of the gp120 molecule (8, 15, 26, 29, 31). To study whether mutations in this region affected the gross conformation of gp120, the CD4-binding ability of the mutant proteins was determined by coprecipitation with OKT4 (Ortho Diagnostic Systems, Raritan, N.J.), a mouse MAb that binds CD4 at an epitope other than the gp120-CD4 interaction site. To determine the specificity of coprecipitation, ^{35}S-labeled wt Env proteins were incubated with various amounts of SupT1 cell lysate at 4°C overnight. The complex was then precipitated with OKT4 and *S. aureus* protein A. As a control, wt gp160, gp120, and gp41 were precipitated with anti-HIV (Fig. 5A, lane 1). In the absence of SupT1 cell lysate the wt Env species were not precipitated by OKT4 (Fig. 5A, lane 2). wt gp160 and gp120, but not gp41, were coprecipitated with OKT4 in the presence of SupT1 cell lysate (Fig. 5A, lanes 3 to 5).

To examine whether mutant proteins bound CD4, aliquots of cell lysate containing similar amounts of ^{35}S-labeled wt or mutant proteins were incubated with excessive SupT1 lysate and reacted with OKT4. Mutant precursors were coprecipitated with OKT4 to an extent similar to that of the wt (Fig. 5B). To further address this issue, equal portions of extracellular

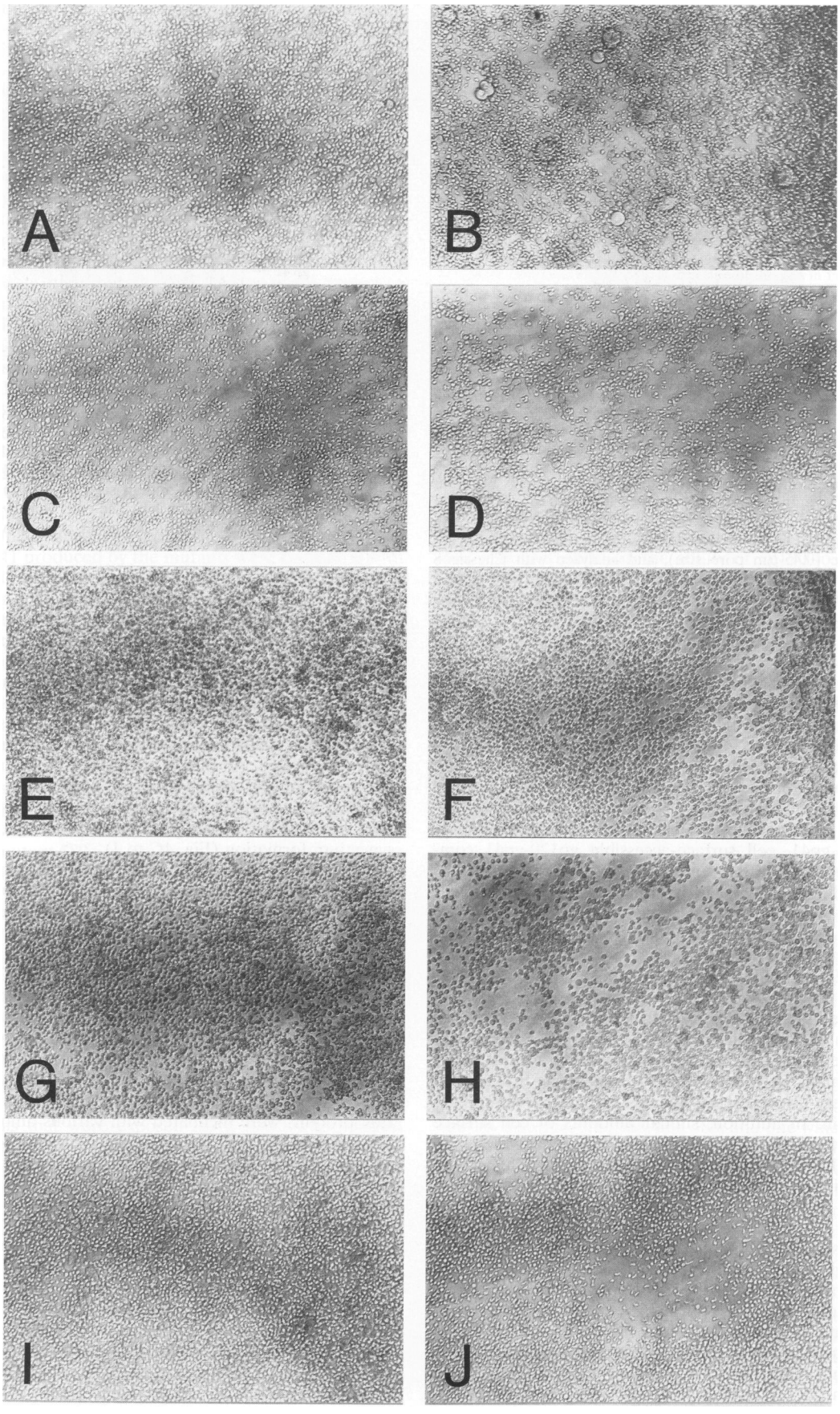
A
B
C
D
E
F
G
H
I
J

FIG. 4. Photomicrograph of wt and mutant Env proteins in induction of syncytium formation with SupT1 cells. CV-1 cells grown in 24-well plates were infected with VV and transfected with 1 μg each of various plasmids in the presence of 5 μg of lipofectin reagent. Six hours posttransfection, 5×10^5 SupT1 cells were added to each well and cultures were incubated at 37°C for 18 h before photographs were taken under a light microscope with a magnification of ×100. (A) No DNA; (B) wt; (C to J) mutants 565, 568, 576, 559, 566, 573, 580, and 587.

fractions from the wt and mutants were analyzed for CD4 binding of secreted gp120. As shown in Fig. 5C, secreted gp120 from all of the mutants bound CD4 substantially. Taken together, these experiments demonstrate that the CD4-binding ability of mutants was not compromised.

To determine whether mutations in this region affected the conformation of mutant gp41, binding of mutant proteins to conformation-specific MAbs was compared with that of wt protein by enzyme-linked immunosorbent assay (ELISA). Because mutations in this region did not affect the overall conformation of gp120 (Fig. 5), the binding of mutant proteins to mouse MAb 902, directed against an epitope in gp120 (7), was used as a control. Indeed, all of the mutant proteins efficiently bound to this MAb (Table 3).

To examine whether mutations in this region affected the conformation of an overlapping or proximal sequence, binding to human MAb 50-69 was assessed. This MAb is mapped to the region between residues 579 and 614 and is specific for the conformation conferred by the extracellular disulfide linkage of gp41 (42). As indicated in Table 3, mutants 565, 566, and 568, each with mutations in the N-terminal part of this region, retained their MAb-binding ability. In contrast, mutants 576, 580, and 587 had considerably reduced MAb binding. This result suggests that mutations in the C-terminal part of the zipper motif region altered the conformation of this epitope. The decreased binding of mutant 559 to MAb 50-69 suggests the spatial proximity of this residue to the conformational epitope.

To find out whether proline substitutions altered the conformation conferred by a distal sequence located in gp41, binding to two human MAbs, 98-6 and 126-6, was performed. These two human independent clones are both reactive with a discontinuous, conformational determinant located at amino acids 644 to 663 (42). All mutant proteins bound to these MAbs efficiently (Table 3), indicating that a point mutation in the zipper motif region did not cause a significant conformational change in the C-terminal sequence distal to the altered region.

In this study, the majority of cell-associated wt Env protein expressed by a VV vector was in the form of gp160 (Fig. 1A, lanes 3). The level of gp120 produced by the wt was about 30 to 36% of that of gp160. Only about 5 to 15% of synthesized gp160 is cleaved to form gp120 in cells infected with HIV derived from a molecular clone (41). The degree of processing has been shown to be cell type dependent (13). Cell-associated gp120 is about 30% of the total amount of gp160 and gp120 produced in CV-1 cells (1). A substantial amount of wt gp160 was detectable on the cell surface (Fig. 3). Similar observations were made when Env proteins were expressed by other viral vectors (19, 22, 30, 33). Because gp160 contains all of the

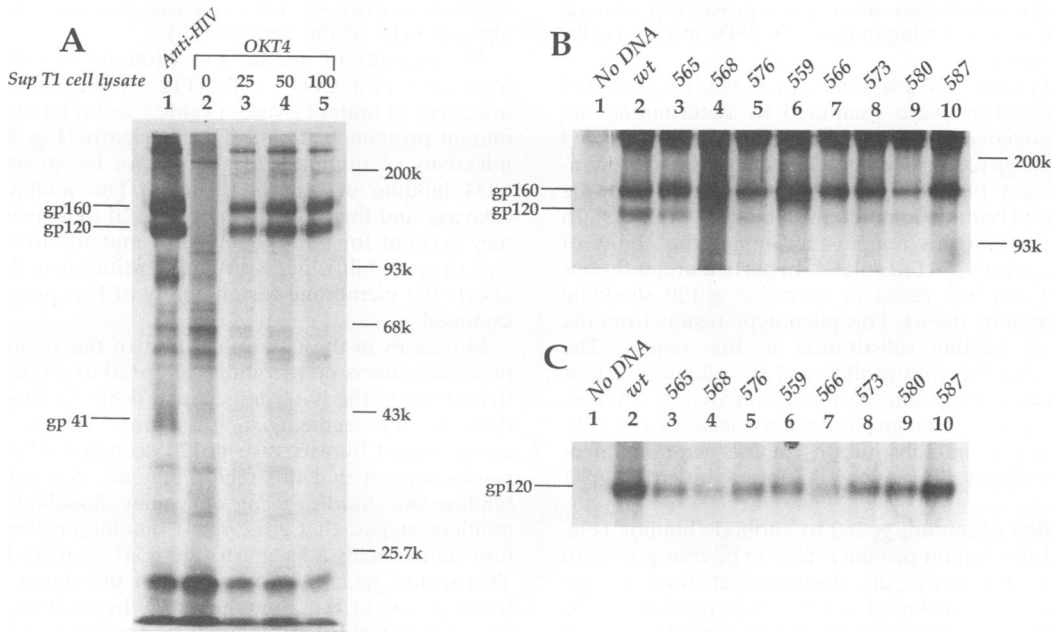

FIG. 5. CD4 binding of Env proteins. (A) Coprecipitation of wt Env protein with OKT4 via binding to CD4. SupT1 cells (1.4×10^8) were lysed with 1 ml of lysis buffer and used as the CD4 source. Aliquots of lysates from ^{35}S-labeled cells synthesizing wt protein were incubated with the indicated amounts (in microliters) of SupT1 lysate at 4°C overnight. One microgram of OKT4 was added to the complex, and mixtures were incubated at 4°C for 1 h (13) and then precipitated with *S. aureus* protein A. The precipitates were subjected to SDS-PAGE. Labeled cell lysate precipitated with anti-HIV as shown in lane 1. (B) Binding of mutant proteins to CD4. Equal portions of lysate from ^{35}S-labeled CV-1 cells (2×10^5) expressing wt or mutant proteins were incubated with 0.1 ml of SupT1 lysate and precipitated with OKT4. The immune complexes were fractionated by SDS-PAGE. (C) CD4 binding of gp120 secreted by mutants. Equal portions of culture supernatant from labeled transfected cells expressing wt or mutant proteins were analyzed for CD4 binding as described for panel B.

TABLE 3. Binding of mutant proteins to conformation-specific MAbs[a]

Mutant	Relative binding to:			
	MAb 902	MAb 50-69	MAb 98-6	MAb 126-6
wt	100	100	100	100
559	98	60 ± 7	98 ± 2	92 ± 4
565	114	97 ± 4	96 ± 3	132 ± 1
566	110	109 ± 9	90 ± 4	82 ± 3
568	140	122 ± 7	110 ± 3	89 ± 5
573	94	81 ± 6	96 ± 10	132 ± 28
576	122	58 ± 3	105 ± 7	136 ± 15
580	92	9 ± 1	103 ± 3	113 ± 4
587	89	1 ± 1	94 ± 1	84 ± 4

[a] One hundred microliters of diluted lysate from cells expressing similar amounts of wt or mutant proteins were coated on microplates and incubated at 4°C overnight. Antigens were then incubated with excess mouse MAb 902 (1:50), human MAb 50-69 (1:50), human MAb 98-6 (1:20), human MAb 126-6 (1:20), or pooled human anti-HIV (1:100) at 37°C for 1 h. The immune complex was determined by a biotin-streptavidin detection system coupled with a horseradish peroxidase-catalyzed reaction. The A_{492} was read with a DuPont ELISA microplate reader. The absorbance for each binding was first normalized to the same Env antigen content by calculating the absorbance ratio of MAb binding to anti-HIV binding. The ratio for the wt protein in each case was arbitrarily defined as 100%, and the relative binding of mutant proteins to that of the wt protein is expressed as a percentage. Except for values for the binding of MAb 902, which are from a typical experiment, values are the averages of data from two representative experiments.

intracellular transport information located in gp120 and gp41, it is expected to be transported to the cell surface. Therefore, it is likely that the cell surface gp160 represented the uncleaved gp160 fraction that accumulated on the cell surface during metabolic labeling. Nevertheless, the possibility that mutations in the zipper motif region may affect gp160 processing, making it different from that occurring in naturally HIV-infected cells, cannot be eliminated.

The effects of proline substitution on precursor cleavage and gp120-gp41 association were examined by determining the levels of cell-associated and extracellular Env species and cell surface-accessible gp120-gp41 complexes. Densitometric quantitation shows that precursor cleavage of mutants may be partially decreased but not completely eliminated (Table 2). In addition, mutations in this region may impair the ability of gp120 to associate with gp41 (Table 2). In particular, mutations at residues 580 and 587 result in excessive gp120 shedding compared with that by the wt. This phenotype results from the specific effect of proline substitution in this region. The replacement of Asp-368 with proline and the addition of four amino acids, including a proline residue, immediately C terminal to Ala-517 do not alter gp120-gp41 association (24, 33). Moreover, some proline substitutions in the ectodomain of gp41 outside the zipper motif region do not inhibit gp120-gp41 binding (3).

The accessibility of mutant gp160 to antibody binding (Fig. 3) indicates that the mutant protein is able to be transported to the cell surface. Therefore, the decreased cleavage of the mutant precursors is attributed not to their inability to be transported to the cleavage site but to their reduced sensitivity to protease cleavage. The decreased cleavage may result from conformational changes induced by mutations. The cell surface transport of mutant proteins and extracellular secretion of gp120 by these mutants also suggest that these mutant Env species are efficiently glycosylated.

Protein folding and oligomerization play an essential role in the transport of viral envelope glycoproteins to the plasma membrane (for reviews, see references 23 and 36). The transport of mutant gp160 to the cell surface (Fig. 3) indicates that these mutant proteins are oligomeric. This is supported by sucrose gradient centrifugation analysis (6). Point mutations in this region do not seem to greatly affect the overall conformation of gp120, as measured by CD4-binding ability (Fig. 5). Conformation-specific MAb binding shows that a proline substitution affects, in a distance-converse manner, the conformation conferred by the changed amino acid and its proximal sequences, but it does not change the conformation conferred by a distal site in gp41 (Table 3). The conformation-specific epitope recognized by MAbs 98-6 and 126-6 is adjacent to the TM region (residues 684 to 705) (18). It is expected that mutations in the zipper motif region do not affect the conformation conferred by the TM and cytoplasmic domains.

The present genetic analysis shows that a single proline substitution in the zipper motif region does not affect the synthesis, transport, cell surface expression, or global structure of the Env protein or the overall conformation of gp120, nor does substitution alter the conformation conferred by a distal site in gp41. However, a proline substitution appears to change the local conformation, presumably the alpha-helix, of the zipper motif region of gp41 (Table 3). This is supported by the finding that a proline substitution for the middle isoleucine in peptide DP107 destabilizes the alpha-helical structure of the peptide (40).

Substitutions with nonconservative amino acids other than proline for Ile-573 in the zipper motif region do not affect precursor cleavage or gp120-gp41 binding (11). Such mutations seem to alter the alpha-helical structure of the zipper motif region less than proline, a strong alpha-helix destabilizer. Therefore, the secondary structure conferred by the zipper motif region of gp41 appears to be critical for Env protein function. In support of this notion is the observation that the antiviral activity of DP107 correlates with the degree of alpha-helicity of the peptide (40).

The inability of mutant Env proteins to induce syncytium formation with $CD4^+$ cells (Fig. 4) parallels the impaired infectivity of mutant viruses (Table 1 and reference 6). Because mutant proteins still bind CD4 efficiently (Fig. 5), the lack of infectivity of mutant viruses may not be attributable to the CD4 binding of mutant proteins. The inhibited precursor cleavage and impaired ability of gp120 to associate with gp41 may account for the phenotype of mutant viruses. Nevertheless, the possibility that a proline substitution in this region also affects the membrane fusion ability of Env protein cannot be excluded.

Mutations in the C-terminal part of this region exert more profound effects on the ability of gp120 to associate with gp41 than those in the N-terminal part (Table 2). Moreover, mutations in this segment, such as those at 580 and 587, are accompanied by excessive gp120 secretion (Table 2). These results suggest that this region may act as a potential gp120-binding site. Studies using molecular modeling and idiotypic mimicry suggest that gp120-gp41 binding involves a stretch of four amino acids adjacent to the gp41 cysteine loop (27, 37). This region, near the C terminus of the zipper motif region, forms a part of the alpha-helix (9). In addition, the segment from 572 to 604 has been implicated in gp120 binding (32). An 11-amino-acid deletion in the zipper motif region of the TM protein of Mason-Pfizer monkey virus results in a noninfectious virus that lacks the surface protein (2). These observations are in accordance with the findings presented here.

The present study illustrates the crucial role of the C-terminal sequence of the zipper motif region in HIV-1 envelope heterodimeric association and provides information to-

ward understanding the mechanism(s) responsible for the impaired infectivity of these virus mutants. Further examination of the zipper motif region in natural virus infection may give insight into the contribution(s) of this region to the virus life cycle.

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