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Role of the Fusogenic Peptide Sequence in Syncytium Induction and Infectivity of Human Immunodeficiency Virus Type 2

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Syncytium induction is a characteristic feature of infection by human immunodeficiency virus (HIV) in vitro. The hydrophobic amino terminus of the transmembrane glycoprotein of HIV type 1 is an essential determinant of virus entry into the target cell population and the formation of syncytia in cell culture. To define the role of the HIV type 2 fusion peptide during infection and syncytium formation, we introduced 8 amino acid substitutions into the hydrophobic amino terminus of gp41, changing either the hydrophobicity, the charge, or the polarity of the amino acid. Viruses containing the envelope mutations were analyzed for their syncytium-inducing capacities, levels of infectivity, and envelope processing and expression. Mutations that increased the hydrophobic nature of the fusion peptide increased syncytium formation, whereas mutations which increased the charge and the polarity and/or decreased the hydrophobicity of the fusion domain severely reduced the capacity of the virus to induce syncytia. However, viruses severely compromised for syncytium formation exhibit only slightly lower levels of infectivity.

Human immunodeficiency virus types 1 (HIV-1) and 2 (HIV-2) are the causative agents of AIDS (1, 5, 16). Various mechanisms have been proposed to explain the characteristic depletion of $CD4^+$ helper lymphocytes observed throughout the course of the disease. One mechanism of cytopathicity and CD4 depletion is the formation of multinucleated syncytial cells mediated by the interaction between the target cell population expressing CD4 and HIV-infected cells expressing the envelope glycoproteins gp120 and gp41 on their cell surfaces (17, 18, 20). The transmembrane protein gp41 associates with gp120, anchoring itself into the lipid membrane while holding gp120 onto the surface of the virus. The gp41 protein is also implicated in the fusion between the viral envelope and the host cell membrane after the initial steps of infection (10, 15). HIV-simian immunodeficiency virus (SIV) gp41 resembles the transmembrane envelope glycoprotein of ortho- and paramyxoviruses in the context of a hydrophobic amino-terminal amino acid sequence, which is also responsible for membrane fusion during infection (10, 11, 22). Site-directed mutagenesis performed at the amino terminus of HIV-1 or SIV gp41 either abolishes or modulates cell fusion (6, 7, 9, 13).

In order to define the role of the fusogenic domain of the $HIV\text{-}2_{sbl/isy}$ gp41 transmembrane protein in the context of an infectious virus, we introduced a series of nucleotide changes into the 3′ half of an infectious HIV-2 genome by site-directed mutagenesis, changing either the hydrophobicity, the charge, or the polarity of each amino acid. Previous studies by Bosch et al. (2) identified similar amino acid residues in the conserved fusion peptide region of SIV_{mac} gp32 transmembrane glycoprotein necessary for membrane fusion and syncytium induction. The amino acid substitutions (Fig. 1) are as follows: Gly to Ala at position 1 (vGLY1), Gly to Asp at position 1 (vGLY1.1), Gly to Ala at position 9 (vGLY9), Gly to Asp at position 9 (vGLY9.1), Phe to Ser at position 7 (vPHE7), Phe to Ser at position 10 (vPHE10), Leu to His at position 5 (vLEU5), and Leu to His at position 11 (vLEU11). All mutations were confirmed by DNA sequence analysis, and the fragments containing the fusion peptide region were subcloned into plasmid KF-3 (8). HIV-2 gp41 mutants were generated by ligating plasmids EGP and KF-3 and transfecting the ligated DNA into either COS-1 or HeLaT46.C cells, according to the procedure previously described by Hattori et al. (12).

To assess the effects of the mutant envelope glycoproteins on syncytium formation, HeLaT46.C cells (4) were transfected with HIV-2 proviral constructs containing the mutated *env* gene. Three days following transfection, cells were stained with crystal violet and scored for syncytium formation essentially as described by Tschachler et al. (21). As shown in Table 1, increasing the hydrophobic nature of the amino terminus by the substitution of alanine for glycine at position 9 (vGLY9) significantly enhanced the number of syncytia formed, whereas the same substitution at position 1 (vGLY1) had minimal enhancement effect on syncytium formation when compared with that by the wild type. The introduction of charged aspartic acid residues at these positions (vGLY1.1 and vGLY9.1) reduced the formation of syncytia. The substitution of the polar serine residue for phenylalanine at position 7 (vPHE7) severely reduced syncytium formation, whereas the phenylalanine-to-serine substitution at position 10 (vPHE10) had a minimal effect. Substituting a highly charged histidine residue for leucine at positions 5 and 11 (vLEU5 and vLEU11) moderately reduced the abilities of both viruses to induce syncytium formation. In all experiments, the production of virus particles was quantitated by p26 antigen capture and was found to be roughly proportional to the amount of DNA transfected, suggesting that the effects on syncytium formation observed were due to the mutations in the fusogenic domain rather than to a variation in DNA uptake during transfection (Table 1).

To examine whether the syncytium-inducing capacities of

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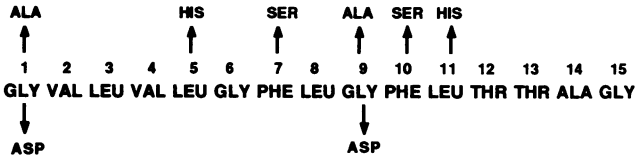

FIG. 1. Amino acid substitutions introduced into the fusion peptide sequence of HIV-$2_{sbl/isy}$. Arrows indicate the amino acid changes at the numbered positions on the sequence, in which the initial glycine residue is at position 1.

different HIV-2 *env* mutants observed in the syncytium formation assays were comparable to those of cell-free infection of $CD4^+$ cells, we infected H9 cells with equivalent p26 units of transfected COS-1 cell supernatants and observed for syncytium formation over a 3-week period. As shown in Table 2, virus vGLY9 formed syncytia to a greater extent and demonstrated higher infectivity than the wild type. In contrast, viruses vGLY1.1, vGLY9.1, vPHE7, vLEU5, and vLEU11 showed marked reduction to complete absence of the formation of syncytia. To measure the relative levels of infectivity of the mutant viruses after 3 weeks, aliquots of infected cell supernatants were assayed for p26 activity over a 3-week period. All mutant viruses retained some infectivity on H9 cells (Table 2), demonstrating that the requirement for fusogenic function is not as stringent for infectivity as for syncytium induction. However, there is some correlation of infectivity with syncytium induction. For example, the highly fusogenic vGLY9 gave the highest yield of p26, while the least fusogenic viruses, vGLY9.1 and vPHE7, gave the lowest yields.

To determine whether mutations within the fusogenic region of gp41 affected expression of the envelope glycoproteins, we examined by using radioimmunoprecipitation the expression, processing, and secretion of the mutant glycoproteins. Several attempts to analyze the expression of mutant envelope glycoproteins by using the HIV-2 proviral ligation and transfection procedure resulted in barely detectable bands as determined by radioimmunoprecipitation. In order to optimize the expression of HIV-2 envelope glycoproteins, we cloned the envelope-coding sequences of wild type and mutant glycoproteins into the eucaryotic expression vector pSVL. Radioimmunoprecipitations of transfected COS-1 cell lysates were done with patient serum from an HIV-2-infected individual. No apparent differences between wild-type and mutant envelope glycoprotein synthesis were observed (Fig. 2A). Previous studies analyzing metabolically labeled proteins of HIV-$2_{sbl/isy}$-infected cells demonstrated poor resolution between the uncleaved precursor gp160 and the mature gp120 (3, 8, 19). We observed similar results with metabolically labeled cells transfected with the HIV-$2_{sbl/isy}$ pSVLenv constructs.

The detection of the surface glycoprotein gp120 in the cell medium indicates proteolytic cleavage of the precursor envelope glycoprotein gp160 into the products gp120 and gp41 and the subsequent transport of the mature envelope

TABLE 1. Effects of amino-terminal gp41 mutations on syncytium formation[a]

Virus[b]	Mutation	No. of syncytia[c]	No. of SFU pg of $p26^{-1}$[d]
Mock		0 ± 0	0
Wild type		27 ± 8.3	3.7×10^{-2}
vGLY1	Gly→Ala	33 ± 7.4	5.1×10^{-2}
vGLY1.1	Gly→Asp	16 ± 4.9	1.9×10^{-2}
vGLY9	Gly→Ala	47 ± 7.7	5.4×10^{-2}
vGLY9.1	Gly→Asp	8 ± 2.8	1.1×10^{-2}
vPHE7	Phe→Ser	6 ± 2.0	8.8×10^{-3}
vPHE10	Phe→Ser	23 ± 4.4	3.1×10^{-2}
vLEU5	Leu→His	17 ± 4.5	2.6×10^{-2}
vLEU11	Leu→His	12 ± 4.7	1.5×10^{-2}

[a] Results represent an average of four experiments.
[b] Lowercase "v" represents HIV-2. The number after each amino acid represents the position of the mutation in the fusogenic peptide sequence of HIV-2 (refer to Fig. 1).
[c] All values are expressed with standard deviations.
[d] SFU pg of $p26^{-1}$, syncytium-forming units per picogram of p26 antigen. Raw data from each transfection ranged from 650 to 800 pg of activity per ml.

TABLE 2. Phenotypic analysis of gp41 mutants[a]

Virus	Syncytium formation at day 21 postinfection in H9 cells[b]	p26 activity (pg/ml) in H9 supernatant at day 21 postinfection[c]
Mock	−	<100
Wild type	++	5,165
vGLY1	++	4,072
vGLY1.1	+	2,327
vGLY9	+++	12,921
vGLY9.1	−	1,356
vPHE7	−	1,585
vPHE10	++	6,682
vLEU5	+	3,860
vLEU11	+	2,090

[a] Results represent an average of two experiments.
[b] H9 cells were infected with equivalent p26 units from transfected COS-1 cell supernatants. Syncytia were scored as follows: −, no syncytia; +, 0 to 5 syncytia; ++, 5 to 15 syncytia; +++, 15 to 25 syncytia.
[c] p26 values represent the highest recorded activities from a three-point assay at day 21 postinfection.

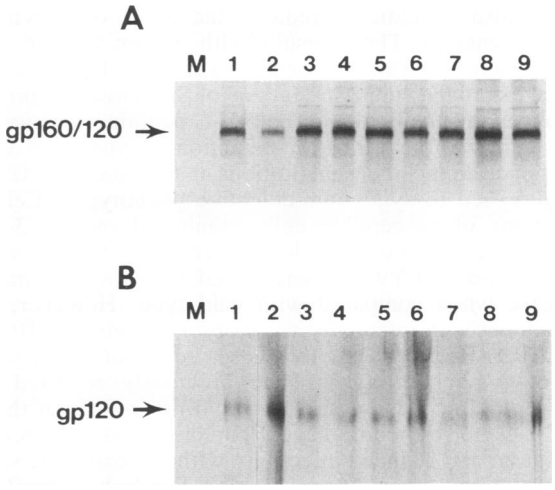

FIG. 2. Expression of HIV-2 envelope glycoprotein mutants in COS-1 cells. Cells were transfected with pSVLenv constructs expressing wild-type and mutant HIV-2 envelope glycoproteins. Immunoprecipitation was performed with serum from a patient with AIDS. (A) Intracellular envelope glycoproteins from transfected COS-1 cell lysates. Lanes: M, untransfected COS-1 cells; 1, wild-type pSVLenv; 2, pSVLgly1; 3, pSVLgly1.1; 4, pSVLgly9; 5, pSVLgly9.1; 6, pSVLphe7; 7, pSVLphe10; 8, pSVLleu5; and 9, pSVLleu11. (B) Secreted envelope glycoproteins from transfected COS-1 cell supernatants. Lanes are as designated for panel A.

glycoproteins to the cell surface (9, 15). To investigate the processing and transport of the mutant surface glycoproteins, we observed for extracellular HIV-2 gp120 in the cell medium. The medium of labeled COS-1 cells transfected with the HIV-2 pSVLenv constructs was immunoprecipitated with HIV-2 patient serum and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. In all lanes, gp120 is evident in the medium of the transfected cells, indicating that none of the transmembrane mutations affected processing and transport of the mature envelope protein (Fig. 2B).

To determine whether the ability of the envelope glycoproteins to bind CD4 has been affected by the gp41 transmembrane mutations, we performed radioimmunoprecipitations on transfected COS-1 cell lysates by using soluble CD4 coupled with OKT4-protein A-Sepharose (6). No differences between wild-type and mutant envelope glycoproteins in CD4 binding were observed (data not shown). These results demonstrate that the mutations in the amino terminus of the transmembrane gp41 glycoprotein did not interfere with gp120 binding to CD4.

The results of the present study confirm and extend previous observations which defined a fusion peptide domain existing within the amino terminus of the glycoprotein that is primarily responsible for mediating the fusion between the viral envelope and the host cell membrane (2, 6, 9, 14). The fusion peptide of the HIV-2 transmembrane glycoprotein comprises a 15-amino-acid sequence at the amino terminus of the transmembrane protein, taking the form GVLVLGFLGFLTTAG, with a highly conserved tripeptide, F-L-G, in the middle. The introduction of amino acids (glycine to alanine at positions 1 and 9) which increased the overall hydrophobicity of the fusion peptide increased the capacity of virus to induce syncytia. Disruption of the hydrophobic residues by the insertion of charged amino acids at those positions (glycine to aspartic acid at positions 1 and 9) greatly reduced the capacity of the virus to induce syncytia. A polar substitution at position 7 (phenylalanine to serine) also dramatically reduced the ability of the virus to induce syncytia. These results with mutant viruses are in general agreement with those from a study by Bosch et al. (2) which examined similar mutations of SIV envelope proteins in subgenomic constructs. The F-L-G peptide sequence may play a critical role during the induction of syncytium formation, since amino acid substitutions at positions 7 (PHE) and 9 (GLY) led to syncytium-defective phenotypes. Cell-free infections of susceptible cells monitored over a 3-week period revealed a complete loss of syncytium formation with vPHE7 and vGLY9.1, whereas vGLY9 showed a marked increase when compared with wild type. However, only partial losses in infectivity were observed with vPHE7 and vGLY9.1. Reduced infectivity as a result of a syncytium-defective phenotype has been previously reported with HIV-1 (6, 14). Dedera and Ratner (6) have shown that an HIV-1 clone (F6) could have a complete loss of syncytium formation and reduced infectivity without losing the single-cell cytopathic effect during infection. Similarly, Kowalski et al. (14) have described a mutation within the HIV-1 gp41 amino terminus important to the induction of the cytopathic effect on target cells. Both attenuated syncytium formation and reduced single-cell lysis were phenotypic consequences of the amino-terminal mutations in the transmembrane glycoprotein of the virus.

Differences in gp120-CD4 binding could have resulted from the amino acid substitutions within the fusion peptide and therefore affected gp120-CD4-mediated membrane fusion. As a control to determine whether mutations within the transmembrane protein affected the receptor-binding interaction between gp120 and CD4, we immunoprecipitated the mutant envelope glycoproteins by using recombinant soluble CD4. Qualitatively, a band corresponding to the gp120-CD4 complex could be seen in each lane. However, quantitative differences in affinity cannot be excluded, since the CD4-binding experiments were not done with intact $CD4^+$ cells. Also, mutations within the fusion peptide did not affect expression or transport of the envelope proteins, as evidenced by the shedding of gp120 into the cell medium. Thus, critical amino acids composing the fusogenic region did not block the transport of the envelope glycoproteins to the cell surface.

The amino termini of the HIV-1 and SIV transmembrane glycoproteins have been previously shown to be responsible for mediating the membrane fusion events leading to syncytium formation. Here, we describe a similar observation for HIV-2. It will be of interest to see whether this region can be exploited to generate a more pathogenic clone of $HIV\text{-}2_{sbl/isy}$ for in vivo studies with rhesus macaques.

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