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Fusogenic segments of bovine leukemia virus and simian immunodeficiency virus are interchangeable and mediate fusion by means of oblique insertion in the lipid bilayer of their target cells

(bovine leukemia virus envelope/fusogenic segment of bovine leukemia virus and simian immunodeficiency virus/conformational analysis)

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ABSTRACT **Modified bovine leukemia virus (BLV) glycoproteins were expressed by using vaccinia virus recombinants, and their fusogenic capacities were examined by a syncytia-formation assay. This analysis indicates that (*i*) both BLV envelope glycoproteins gp51 and gp30 are necessary for cell fusion; (*ii*) insertion of the N-terminal segment of gp30 (fusion peptide) into the lipid bilayer in an oblique orientation, as predicted by computer conformational analysis, results in fusogenic capacities higher than insertion in a perpendicular or parallel orientation; and (*iii*) replacement of the BLV fusion peptide with its simian immunodeficiency virus counterpart does not modify the fusogenic capacity of the BLV glycoprotein.**

The molecular mechanisms involved in membrane fusion are not well known. Mutational analyses on the envelope genes of various retroviruses, such as human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV), have indicated that the N-terminal segment of the transmembrane region (fusion peptide) of the viral envelope glycoprotein is involved in membrane fusion (1–3). Computer conformational analyses on the fusogenic segment of HIV and Newcastle disease virus envelope glycoproteins have led to the hypothesis that the fusion peptide is obliquely oriented at the lipid–water interface, thereby inducing a local membrane destabilization and promoting cell fusion (4, 5). This hypothesis has also been extended to several other viral systems that display fusogenic properties (6), including bovine leukemia virus (BLV).

BLV is the etiological agent of enzootic bovine leucosis, the most frequent bovine neoplastic disease. The BLV envelope glycoprotein is synthesized as a 72-kDa (gp72) precursor polypeptide that is proteolytically processed into the 51-kDa (gp51) and 30-kDa (gp30) mature forms by a cellular protease. gp51 is the external subunit containing the cell receptor-binding domain, and gp30 is the transmembrane subunit that anchors the gp51–gp30 complex in the plasma membrane of infected cells and the viral envelope obtained by viral budding from the infected cell (7). This BLV envelope glycoprotein complex is essential to infection and syncytium induction, as demonstrated by the ability of polyclonal sera (8) and some monoclonal antibodies (mAbs) (9) directed against gp51 to inhibit BLV-induced cell fusion.

We show here that orientation of the fusogenic segment of the BLV envelope glycoprotein in the lipid bilayer is critical for membrane fusion. When the fusion peptide is inserted in the lipid bilayer obliquely, fusion capacity is higher than when the peptide is inserted in a perpendicular or parallel orientation. In addition, we show that the BLV fusion peptide can be replaced by its SIV counterpart without modification of the fusogenic capacity.

MATERIALS AND METHODS

Cloning of BLV Envelope Gene in M13 Phage and DNA Mutagenesis. A recombinant λ gt10 phage containing the BLV envelope sequence was obtained from a cDNA library constructed from total RNA from bat lung cells producing BLV (10). A *Xho* I–*Xho* I fragment was excised from the recombinant phage and subcloned into the *Sal* I site of plasmid vector pSP64, giving pSP64-ENV.

A *Bam*HI–*Hin*dIII fragment containing the BLV envelope sequence was excised from pSP64-ENV and subcloned into phage M13mp18, giving M13-ENV. Single-stranded DNA from this phage was used as template for *in vitro* mutagenesis, following Amersham protocols based on the Eckstein method (11, 12).

Construction of Vaccinia Virus (VV) Donor Plasmids. An *Eco*RI–*Hin*dIII fragment was excised from each M13-ENV mutant phage (MU0–MU5), filled with *Escherichia coli* DNA polymerase I (Klenow fragment), and cloned into the *Sma* I site of the VV insertion vector pSC11, giving pSC11-MU0 to -MU5 (pSC11 was obtained from B. Moss, National Institutes of Health). The complete sequence of the modified gp51 and gp30 genes was then determined to verify the presence of the introduced mutations and to check for the absence of unwanted modifications.

A *Bam*HI–*Hin*dIII fragment containing the wild-type BLV envelope sequence was excised from pSP64-ENV, filled with Klenow fragments, and cloned into the *Sma* I site of pSC11, giving pSC11-ENV. pSC11 and all constructions derived from it use the VV promoter p7.5.

Construction of VV Recombinants. The procedures used to isolate VV recombinants have been described (13, 14).

Cells, Viruses, and mAbs. Wild-type VV (Elstree strain) and recombinants derived from it were propagated in CV1 cells. VV recombinants vP391 and vP482, encoding gp51 and uncleaved gp72, respectively, have been described (15).

The BLV gp51-specific mAbs (mono A, B, B′, C, D, D′, E, F, G, and H) have been described (9).

ELISA. ELISA determinations to measure recombinant VV infected cell extracts were done as described (16).

Indirect Immunofluorescence Analysis. CV1 and VERO cells grown in 96-well plates were infected with recombinant VV at a multiplicity of infection of 4 plaque-forming units per

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Abbreviations: HIV, human immunodeficiency virus; BLV, bovine leukemia virus; SIV, simian immunodeficiency virus; mAb, monoclonal antibody; VV, vaccinia virus.

cell. Twenty-four hours later, the cells were washed and fixed. Glycoproteins were detected with anti-gp51 antibodies and fluorescein-labeled goat anti-mouse serum (17); the test was also done on nonfixed cells.

Immunoblot Analysis. Proteins from lysates of recombinant VV-infected cells were separated by SDS/12.5% PAGE, blotted, and detected with a panel of anti-gp51 mAbs (mono A, B, B', C, D, and E) (18).

Syncytia-Formation Assay. Two methods were used: (*i*) VERO cells, grown in 96-well plates (10,000 cells per well), were infected with recombinant VV at multiplicity of infection of 1 plaque-forming unit per cell and cocultivated with CC81 cells (40,000 cells per well) 6 hr after infection. Twenty-four hours after infection, cells were washed and fixed. Syncytia were counted microscopically in six different fields at ×400. Fifty cells were counted per field, and multinucleated cells containing more than four nuclei were considered to be syncytium; (*ii*) CV1 cells (50,000 cells per well) were infected with recombinant VV at a multiplicity of infection of 1 plaque-forming unit per cell. Twenty-four hours later, cells were treated as described in *i*.

Fusion-Inhibition Assay. CV1 or VERO cells were incubated with dilutions (1:3, 1:9, 1:27, 1:81, 1:243, and 1:729) of a BLV gp51-specific mAb to epitope H and after infection with various recombinant VVs were analyzed for syncytia formation as described above.

Conformational Analysis. The method used to predict conformational structure of the fusion peptide accounts for the contribution of the lipid–water interface, the concomitant variation of the dielectric constant, and the transfer energy of atoms from a hydrophobic to hydrophilic environment (19). The structure, mode of insertion, and orientation of the fusogenic peptide were studied in a dipalmitoylphosphatidylcholine molecular layer (20). In this model, the interaction energy (sum of contributions from Van der Waals energy interactions, torsional potential energy, electrostatic interactions, and transfer energy between fusogenic peptide and dipalmitoylphosphatidylcholine in the monolayer) is considered and minimized until the lowest energy state of the entire aggregate is reached. We limit this approach to the number of lipids sufficient to surround the peptide, and we keep the structure of molecules (lipids and peptide) frozen during the process of assembly (6, 21, 22). All calculations were made on an Olivetti CP486, by using PC-TAMMO+ (Theoretical Analysis of Molecular Membrane Organization) and PC-PROT+ (Proteins Plus Analysis) software. Graphs were drawn with the PC-MGM+ (Molecular Graphics Manipulation) program.

RESULTS

Both gp51 and gp30 Are Required for Cell Fusion Activity. To study the role that various regions of the BLV envelope glycoprotein have in membrane fusion, recombinant VVs containing modified BLV envelope genes were constructed. A recombinant virus, vP391, containing BLV gp51 without gp30 was constructed to study the importance of gp30 in fusogenic activity of the BLV envelope gene product (15). Another recombinant virus, vP482, containing an envelope gene mutated at the cleavage site (amino acids 268 and 269 converted from Arg-Ser to Gln-Thr) was constructed to determine whether processing of the envelope gp72 precursor glycoprotein into mature gp51 and gp30 forms is required for fusogenic activity (15). As a control, a recombinant ENV containing a wild-type BLV envelope gene was also constructed.

To verify that vP391 and vP482 express and process the modified BLV envelope glycoproteins correctly, immunoblot analysis was done (Fig. 1). This immunoblot shows that cells infected with vP391 and vP482 express proteins with

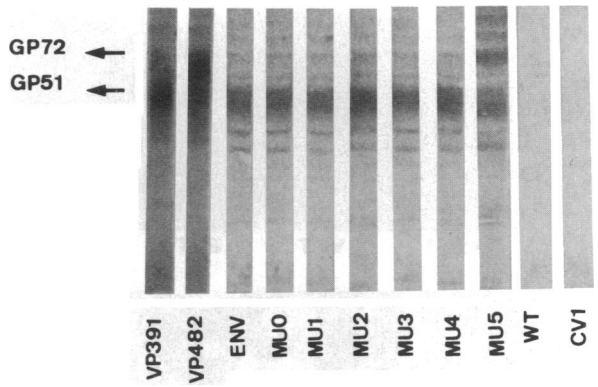

FIG. 1. Immunoblot analysis of BLV envelope glycoproteins. Lysates of recombinant VV-infected CV1 cells were subjected to SDS/PAGE (12.5%), blotted, and incubated with a mixture of mAbs against gp51 (A, B, B', C, D, and E). WT, nonrecombinant VV; CV1, uninfected CV1 cells. Positions of gp72 and gp51 glycoproteins are indicated at left.

molecular masses of ≈50 and 70 kDa, respectively. Immunoblots were also prepared from supernatants of the infected cultures. These blots indicate that more gp51 appears in the supernatant of vP391-infected cells than in the supernatant of ENV-infected cells (data not shown), suggesting that gp51 is shed at a higher rate when expressed without the gp30 transmembrane domain than when gp51 is expressed with gp30.

To determine whether gp51 is expressed at the cell surface, indirect immunofluorescence with a mixture of mAbs against gp51 was done (Fig. 2). These studies show that ENV and vP482-infected cells express gp51 on their surface, suggesting that the intracellular transport of gp51 to the plasma membrane is not affected by a lack of cleavage of the gp72 precursor glycoprotein. However, the amount of gp51 detected at the surface of vP391-infected cells was low in comparison with ENV-infected cells, suggesting once again that gp51 is secreted into the supernatant at a higher rate in cells infected with vP391 than in cells infected with a recombinant expressing wild-type gp51 and gp30.

The antigenic structure of the glycoprotein produced in vP391- and vP482-infected cells was examined by using an ELISA with mAbs against the gp51 conformational epitopes F, G, and H (Fig. 3). These results show that the F, G, and H epitopes are weakly recognized on the uncleaved gp72 precursor produced in vP482-infected cells. This result indicates that the affinity of the mAbs monoF and monoH for gp72 is reduced relative to their affinity for the glycoprotein produced in ENV-infected cells. This observation is consistent with previous results showing that the absence of cleavage affects the generation of native gp51 (15). ELISA data also reveal that epitopes F and H are weakly recognized on the glycoprotein produced in vP391-infected cells. This result, however, may be from the elevated levels of gp51 shed in cells infected by this recombinant virus.

ENV, vP391, and vP482 were tested for their ability to fuse in two cell culture systems (Table 1 and Fig. 2). These experiments reveal that vP391 cannot induce syncytia, suggesting an important role for gp30 in membrane fusion. These results also show that the fusogenic activity of vP482 is very weak (10%) relative to ENV, indicating that the absence of cleavage of gp72 affects fusogenic activity of the BLV envelope gene product.

Inhibition of syncytia formation by a neutralizing antibody against the conformational epitope H of gp51 was examined for vP482 (Table 1). The fusogenic activity of vP482 is completely inhibited by monoH serum, as it is for ENV, indicating that the H epitope plays a crucial role in syncytia

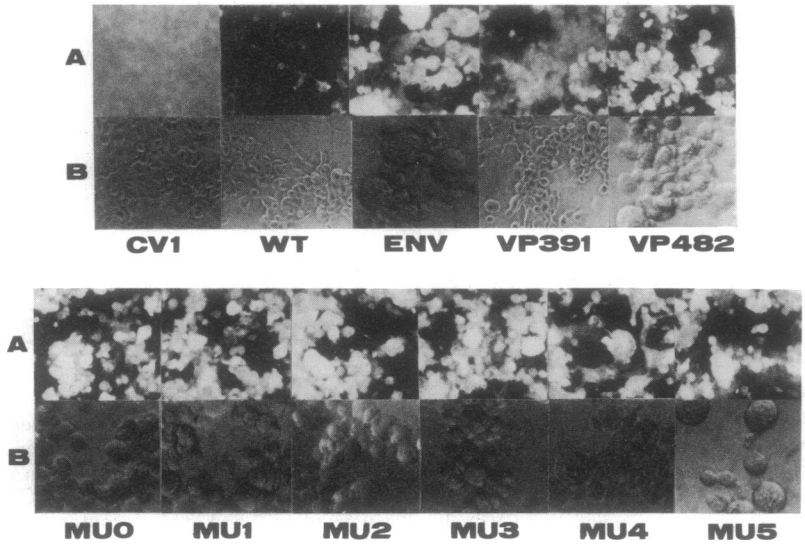

FIG. 2. (*A*) Indirect immunofluorescence analysis of recombinant VV-infected CV1 cells. BLV envelope glycoproteins were detected with a panel of mAbs against gp51 (A, B, B′, C, D, D′, E, F, G, and H) and fluoresceinated goat anti-mouse serum. (×290.) (*B*) Syncytia induced on monolayers of recombinant VV-infected CV1 cells. (×280.)

formation, despite the reduced affinity of monoH for this epitope.

Theoretical Analysis of BLV Fusion Peptide. To create a model of the interaction between the BLV fusion peptide within the lipid bilayer, computer-aided conformational analyses were done. The amino acid sequence of the BLV gp72 precursor has been analyzed according to Eisenberg *et al.* (23, 24). The region immediately after the cleavage site of gp72 contains a hydrophobic protein segment called the fusion peptide. There is a high probability that the BLV fusion peptide will adopt a helical structure after its insertion into a lipid bilayer (5, 25, 29). Two major types of helical structures have been identified so far in proteins that associate with lipids: amphipathic helices that lie at the surface of the lipid phase, and the helical configurations oriented in parallel to the lipid acyl chains adopted by transmembrane domains. A conformational analysis of the first 12 amino acids of BLV gp30, as described (4) for gp41 of HIV, shows that the BLV fusogenic peptide orients obliquely with a 60° angle relative to the lipid–water interface and exhibits membrane-destabilizing properties. The precision of prediction of the peptide orientation is equal to 5° and is due to the method used (6, 21, 22).

Orientation of Fusion Peptide in Lipid Bilayer Determines Fusogenic Activity. To test this hypothesis, recombinants containing point mutations in the fusion peptide were constructed. These point mutations were designed to modify the theoretical angle of insertion of the fusion peptide in the lipid bilayer. Five recombinants were constructed, MU0–MU4, each containing an envelope glycoprotein with a fusion peptide predicted to insert into the lipid bilayer in either one of three different orientations: perpendicular, parallel, or oblique (Table 1).

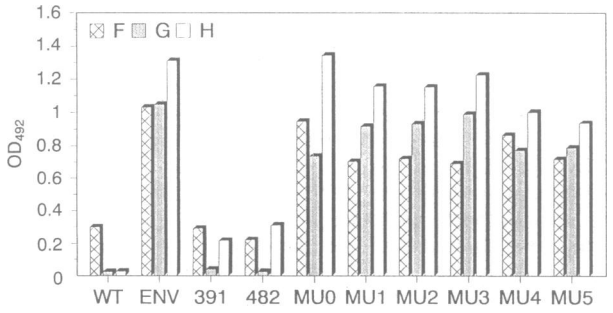

FIG. 3. ELISA analysis of recombinant VV-infected CV1 cell lysates with mAbs against the conformational epitopes of gp51, F, G, and H. vP391 and vP482 were derived from an $F^+G^-H^+$ BLV variant. MU0, MU1, MU2, MU3, MU4, and MU5 were derived from an $F^+G^+H^+$ BLV variant. Results are expressed in terms of maximum OD observed.

In MU0, three amino acid substitutions (Leu-Ala-Leu converted to Arg-Asp-Arg) were introduced at the C terminus of the fusion peptide, producing a fusion peptide with a theoretical insertion angle of 87°. In MU1, amino acids 1 and 4 were changed (Ser-1 converted to leucine and Ala-4 converted to leucine), producing a fusion peptide with a theoretical insertion angle of 4°. In MU2, amino acids 1 and 5 were changed (Ser-1 converted to leucine and Ala-5 converted to leucine), producing a fusion peptide with a theoretical insertion angle of 31°. In MU3, amino acids 4 and 7 were changed (Ala-4 converted to leucine and Thr-7 converted to serine), producing a fusion peptide with a theoretical insertion angle of 85°. In MU4, amino acids 5 and 7 were changed (Ala-5 converted to leucine and Thr-7 converted to serine), producing a fusion peptide with a theoretical insertion angle of 68°. (MU1 and MU2 as well as MU3 and MU4 were designed to have identical global hydrophobicities, yet different theoretical angles of insertion. High hydrophobicity values indicate a high affinity for the lipid bilayer, and low values indicate a low affinity for the lipid bilayer.)

Fig. 4 illustrates the mode of insertion in the lipid matrix of the wild-type BLV fusion peptide (ENV) and the two mutants (MU1 and MU0) that mimic the major types of membrane helical structures, amphipathic and transmembrane protein domains. Fig. 4 illustrates that the highest theoretical membrane-destabilizing capacity is induced by the wild-type fusion peptide (ENV).

Another recombinant virus, MU5, was constructed to study specificity of the BLV and SIV fusion peptides. In this recombinant, the BLV fusion peptide is replaced by its SIV counterpart. The theoretical angle of insertion of the fusion peptide of MU5 is 53° (Table 1).

To verify that these recombinant viruses express and process the modified BLV envelope glycoproteins correctly, immunoblot analysis was done (Fig. 1). This analysis reveals that cells infected with MU0–MU4 express proteins with molecular masses of ≈50 kDa, indicating that the mutations in these recombinants do not affect expression or processing of the modified envelope glycoproteins. This analysis did show, however, that the cleavage efficiency of gp72 in MU5-infected cells is altered.

To determine whether gp51 is expressed at the cell surface, indirect immunofluorescence by using a mixture of mAbs against gp51 was done (Fig. 2). This technique revealed that ENV, MU0, MU1, MU2, MU3, MU4, and MU5-infected

Table 1. Relation between amino acid sequence, predicted orientation, hydrophobicity mean, fusogenic capacity, and fusion inhibition of VV recombinants

Wild type or recombinant	Expressed BLV glycoproteins	Fusogenic peptide: Amino acid sequence	Fusogenic peptide: Viral origin	Fusogenic peptide: Predicted orientation	Angle of insertion	Fusogenic capacity*, %	Fusion inhibition†	Hydrophobicity, mean‡
Wild-type VV						0		
ENV	gp51–gp30	SPVAALTLGLAL	BLV	Oblique	60°	100	27	7.73
VP391	gp51					0		
VP482	gp72					10	81	
MU0	gp51–gp30	SPVAALTLGRDR	BLV	Perpendicular	87°	17	81	0.38
MU1	gp51–gp30	LPVLALTLGLAL	BLV	Parallel	4°	15	27	9.48
MU2	gp51–gp30	LPVALLTLGLAL	BLV	Oblique	31°	23	81	9.48
MU3	gp51–gp30	SPVLALSLGLAL	BLV	Perpendicular	85°	18	27	8.08
MU4	gp51–gp30	SPVALLSLGLAL	BLV	Oblique	68°	86	27	8.08
MU5	gp51–gp30	GVFVLGFLGFLA	SIV	Oblique	53°	100	81	11.15

*Fusogenic capacities are expressed as percentage of syncytial number obtained for ENV, which is arbitrarily 100%.
†Fusion inhibition was seen in a syncytia-formation assay after incubation with anti-gp51 mAbs. Fusion inhibition was expressed as serum titers (reciprocal dilution giving 50% inhibition).
‡Hydrophobicity means were computed according to the Eisenberg consensus scale (21).

cells expressed gp51 on their surface and that the intracellular transport of gp51 to the plasma membrane is not affected by the modifications of the gp30 fusion peptide.

The antigenic structure of the gp51 glycoprotein produced in cells infected with these recombinant viruses was then examined in an ELISA, with mAbs against the gp51 conformational epitopes F, G, and H (Fig. 3). The antigenic structure of the glycoproteins produced in MU0- to MU5-infected cells is not altered by the amino acid substitutions introduced into the gp30 fusogenic segment.

MU0–MU5 were then tested for their fusogenic capacity in two cell culture systems (Table 1 and Fig. 2). The fusogenic activity of MU0, MU1, MU2, and MU3 is reduced (17%, 15%, 23%, and 18%, respectively) relative to ENV, but MU4 and MU5 induce efficient fusion (86% and 100%, respectively).

Inhibition of syncytia formation by a neutralizing antibody against the conformational epitope H of gp51 was examined in these recombinant viruses (Table 1). The fusogenic activities of MU0, MU1, MU2, MU3, MU4, and MU5 are inhibited by similar titers of monoH serum as ENV, indicating that the antigenic structure of the glycoproteins produced in cells infected with these recombinant viruses is not significantly altered with respect to the wild-type gp51–gp30 complex.

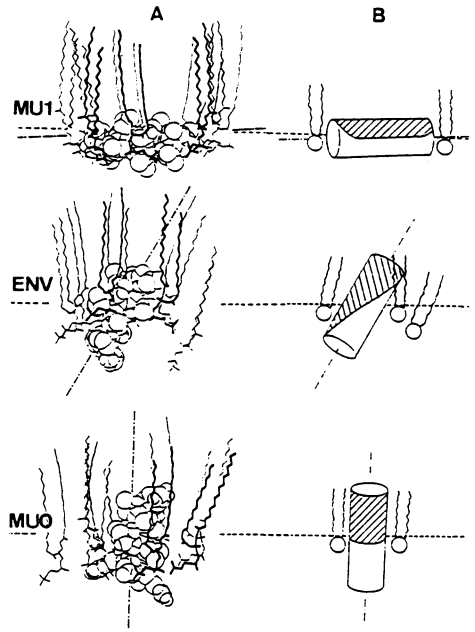

FIG. 4. Molecular modeling representation (*A*) and schematic view (*B*) of the mode of insertion of three types of peptides inserted in a lipid matrix. In *A*, peptides are drawn in a space-filling representation, and lipids are represented in skeleton; lipids in front of the peptides are represented with thick lines. In *B*, hatched regions represent the hydrophobic region of the peptide. Dotted line delineates interface between hydrophobic phase (above) and hydrophilic phase (below). Axis line shows orientation of peptide axis at interface. As illustrated, wild-type peptide ENV inserts into the lipid matrix in an oblique orientation relative to interface and induces a greater lipid destabilization than modified peptides, MU1 (parallel to interface) or MU0 (perpendicular to interface).

DISCUSSION

Studies done on the envelope glycoprotein of syncytia-inducing retroviruses, such as HIV and SIV, have suggested that the N-terminal segment of the transmembrane glycoprotein is involved in membrane fusion (1–3). Corresponding structures have also been found in myxo- and paramyxoviruses (26, 28). This communication reports on the molecular aspects of the fusogenic activity of the BLV envelope glycoprotein.

Our results indicate that both the gp51 and gp30 components of the BLV envelope gene product are important for fusogenic activity. A VV recombinant, vP391, expressing gp51 alone had no fusion activity. Further experiments with this recombinant showed that the conformational epitopes F and H were weakly represented, suggesting that conformational changes in gp51 had occurred. Thus, the native configuration of gp51 appears to depend upon an interaction between gp51 and gp30, and both glycoproteins are necessary for cell fusion.

Furthermore, cleavage of the gp72 precursor glycoprotein into mature gp51 and gp30 forms is also important for inducing fusion. A VV recombinant, vP482, expressing an uncleaved form of BLV glycoprotein exhibited weak fusogenic activity, suggesting that cleavage of gp72 precursor glycoprotein is necessary for fusion. These observations are consistent with results obtained by McCune *et al.* (27), in which an HIV variant expressing an uncleaved form of gp160 was deficient in both infectivity and cell fusion activity.

The decreased fusogenic activity of vP482 does not appear to be due to its lower expression because the uncleaved gp72 glycoprotein was expressed at the cell surface of infected cells at apparently the same level as the wild-type glycoprotein. This decrease most likely is from a conformational change in gp72 glycoprotein. This hypothesis is consistent with the ELISA data, indicating that gp72 reactivity to mAbs against conformational epitopes F and H was significantly

reduced. Thus, the conformational integrity of gp51 and gp30 is apparently important for fusogenic activity.

In the second part of our study, amino acid substitutions were introduced in the fusion domain of gp30. These substitutions were made to modify the orientation of the fusion peptide relative to the lipid bilayer.

The VV recombinants MU0, MU1, and MU3 exhibited very weak fusogenic activities. Based on theoretical calculations, the fusion peptides of these three recombinants minimally disturb the lipid bilayer. The fusion peptides of the recombinants MU0 and MU3 were designed to align in parallel with the lipid acyl chains (angles of insertion are 87° and 85°, respectively), and the fusion peptide of recombinant MU1 was designed to align perpendicularly to the lipid acyl chains (angle of insertion is 4°). The fusogenic activity of these recombinants is consistent with the hypothesis that fusion requires disruption of the dynamic stability of the lipid bilayer, a state highly favored by the oblique insertion of a peptide helical structure (4, 5).

The recombinant MU4 had fusogenic activity of 86%. This recombinant was constructed so that the fusion peptide would be inserted into the lipid bilayer obliquely (angle of insertion was 68°). Mutations in MU4, therefore, had little effect on syncytia formation, suggesting that insertion of the fusion peptide in the lipid bilayer in an oblique orientation is important for membrane fusion.

Recombinants MU3 and MU4 have the same global hydrophobicity; their theoretical orientation in the membrane, however, differs significantly. The fusion peptide of MU3 was predicted to be inserted into the lipid–water interface at a perpendicular angle. The fusion peptide of MU4 was predicted to be inserted obliquely. Because MU3 has a fusogenic activity of 18%, and MU4 has a fusogenic activity of 86%, it appears that together with the global hydrophobicity of the fusion peptide, oblique insertion is a major prerequisite for fusion efficiency.

The fusion peptide of recombinant MU2 was predicted to be inserted into the lipid bilayer in an oblique orientation. The fusogenic capacity of this recombinant, however, is very low (23%). This result is surprising and suggests that, in addition to being inserted into the lipid bilayer in an oblique orientation, the angle of insertion of the fusion peptide is important. [The angle of insertion of the MU2 fusion peptide (31°) is considerably lower than the angle of insertion of the wild-type fusion peptide (60°).]

The different fusogenic capacities of recombinants MU0, MU1, MU2, MU3, and MU4 support our hypothesis that insertion of the BLV fusion peptide in the lipid bilayer at an oblique angle is necessary, but not sufficient, for membrane fusion. Similar results were also obtained with the fusion peptide of the SIV transmembrane glycoprotein gp32 (30).

A VV recombinant was also constructed in which the BLV fusion peptide was replaced by its SIV counterpart. The fusogenic capacity of this recombinant, MU5, is 100%. Therefore, the presence of the first 12 amino acids of SIV gp32 at the N terminus of BLV gp30 did not affect the fusogenic activity of the BLV envelope glycoprotein, even though the amino acid sequence of these two fusion peptides is totally different. This result is consistent with the observation that very different amino acid sequences can adopt similar structures in a lipid environment (the angle of insertion of MU5 fusion peptide is 60°, as compared with 53° for SIV fusion peptide) and can exhibit similar fusogenic activities (5, 25, 26).

In conclusion, our results provide insight into the fusion process of BLV by showing the importance of the orientation in the lipid bilayer of the N-terminal peptide of gp30.

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