The 19–27 Amino Acid Segment of gp51 Adopts an Amphiphilic Structure and Plays a Key Role in the Fusion Events Induced by Bovine Leukemia Virus*

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Previous results indicate that the external glycoprotein gp51 of bovine leukemia virus plays an important role in the process of cell fusion induced by bovine leukemia virus (Bruck, C., Mathot, S., Portetelle, D., Berte, C., Franssen, J. D., Herion, P., and Burny, A. (1982) Virology 122, 342–352; Vönèche, V., Portetelle, D., Kettmann, R., Willems, L., Limbach, K., Paoletti, E., Ruysschaert, J. M., Burny, A., and Brasseur, R. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3810–3814) and suggest that a region encompassing residues 23 and 25 of gp51 is involved in this process (Portetelle, D., Couez, D., Bruck, C., Kettmann, R., Mammerickx, M., Van der Maaten, M., Brasseur, R., and Burny, A. (1989) Virology 169, 27–33; Mamoun, R., Morisson, M., Rebeyrotte, N., Busseta, B., Couez, D., Kettmann, R., Hospital, M., and Guillemain, B. (1990) J. Virol. 64, 4180–4188). X-ray diffraction studies performed on envelope glycoproteins of influenza virus indicate that the NH2-terminal part of the external glycoprotein lies very close to the fusion peptide. The same overall structure seems to exist in human immunodeficiency virus as suggested by site-directed mutagenesis followed by syncytia induction assays.

Our theoretical studies indicate that a segment expanding between residues 19 and 27 of gp51 probably adopts an amphipathic β-strand structure. We hypothesize that the amphipathic 19–27 structure of gp51 plays an important role in the process of membrane fusion by interacting with the fusion peptide or with another region of gp30. Mutational analysis disrupting the amphipathy of the 19–27 region strongly altered the fusogenic capacity of the gp51-gp30 complex.

Membrane fusion induced by enveloped viruses, such as bovine leukemia virus (BLV), is a necessary step in the infection of host cells and transmission of the virus from cell to cell eventually leading to syncytia formation.

BLV is the etiological agent of enzootic bovine leukosis, the most frequent bovine neoplastic disease. The envelope glycoproteins of BLV are derived from a precursor, gp72, through endoproteolytic cleavage. gp51, the external glycosylated component, is responsible for the binding to a cell receptor determining the tropism of BLV, gp80, the transmembrane subunit, anchors the envelope glycoprotein complex into the plasma membrane of virions and infected cells (Burny et al., 1987).

The BLV glycoproteins play a central role in membrane fusion during infection of host cells and syncytia formation: polyclonal sera and some monoclonal antibodies directed against gp51 indeed inhibit syncytia formation (Graves et al., 1981; Bruck et al., 1982a, 1982b). On the other hand, mutational analyses indicate that 1) both gp51 and gp30 are necessary and sufficient for cell fusion, 2) the cleavage of the gp72 precursor into gp51 and gp30 is required for syncytia formation, 3) the hydrophobic NH2-terminal segment of gp30 plays a critical role in the fusion process, and 4) the oblique orientation of this segment in a lipid bilayer parallels the fusogenic capacity of the BLV envelope glycoprotein complex (Vönèche et al., 1992).

These results suggest that the process of membrane fusion depends on at least one region of BLV gp51. In order to identify that putative region of gp51, we compared the antigenicity and amino acid sequence of the envelope glycoproteins of seven BLV natural variants (Portetelle et al.; 1989; Mamoun et al., 1990). These variants differed by their reactivity to monoclonal antibodies directed against gp51, notably mono H, able to block syncytia formation by the prototype BLV. Two variants exhibited an H– phenotype. Their fusogenic capacity was comparable with that of the wild-type virus, but they were unreactive to the monoclonal antibody mono H. Their gp51 amino acid sequence differed from that of the wild-type protein by 1 amino acid residue located either at position 23 (Phe instead of Ser) or 25 (Ala instead of Ser). It is also of interest to note that, besides its capacity to inhibit syncytia formation, the monoclonal antibody mono H blocks BLV/VSV pseudotype infectivity (Bruck et al., 1982b). These data thus suggest that the region of gp51 encompassing resi—

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The abbreviations used are: BLV, bovine leukemia virus; HIV, human immunodeficiency virus; ELISA, enzyme-linked immunosorbent assay.
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**RESULTS**

**Theoretical Analysis of the Amino-terminal Part of gp51**

- Theoretical studies were performed on the NH2-terminal part of gp51 of a BLV variant characterized by a H+ phenotype (a variant recognized by the anti-gp51 monoclonal antibody mono H) in order to predict possible secondary structures adopted by the peptide region encompassing amino acid positions 23 and 25. A peptide stretch expanding between amino acids 19 and 27 could adopt a β-strand structure as indicated by the prediction methods of Gamier (1978) and Chou-Fasman (1978). The hydrophobic cluster analysis (HCA) method (Lemesle-Varloot et al., 1990) also predicts an amphipathic β-strand in this region but does not exclude other two-dimensional structures, considering that similar hydrophobic cluster analysis profiles correspond to helical regions of HA2 influenza hemagglutinin (Wiley and Skehel, 1987).

**Possible Role of the 19–27 Segment of gp51 in Cell Fusion**

- X-ray diffraction studies of the influenza hemagglutinin established that the NH2-terminal region of HA1 (the external envelope segment) lies very close to the fusogenic peptide, the NH2-terminal segment of HA2 (the transmembrane envelope subunit) (Wilson et al., 1981; Weis et al., 1988; Wiley and Skehel, 1987). Moreover, the ectodomain of HA generated by bromelaine digestion can be cleaved by trypsin into a soluble fragment and an aggregating complex consisting of residues 1–27 of HA1 and 1–175 of HA2 (Skehel et al., 1982; Weis et al., 1990). On the other hand, molecular modeling of the BLV envelope glycoproteins using HA1 and HA2 as reference structures shows that here also the NH2-terminal region of gp51 can fold in the vicinity of the fusion peptide (Busetta, 1989; Mamoun et al., 1990).

- The fusion peptide of BLV probably adopts a β-strand structure in solution as it was demonstrated by infrared spectroscopy for the fusion peptide of simian immunodeficiency virus (Martin et al., 1991) and is characterized by relative amphipathy as defined by the position of the most hydrophobic residues on one face and the hydrophilic and less hydrophobic residues on the other face. Fig. 1 illustrates two possible modes of interaction between two amphipathic β-strands such as the fusogenic peptide and the 19–27 segment of gp51; the peptides interact via their hydrophobic side only or via their hydrophobic faces and their hydrophilic faces. In both situations, the amphipathic structure of the β-strands is...
involved in the stability of the folding. We cannot, however, exclude a second hypothesis, suggesting interaction to occur between the 19–27 segment and other amphipathic structures of the external part of gp30.

**Mutagenesis of the 19–27 Segment of gp51**—In order to test the biological relevance of these hypotheses, single amino acid changes were introduced into the 19–27 region of gp51. Amino acids 22 (Phe), 24 (Ile), and 26 (Ile) were replaced by His, Gln, and Gln, respectively, in order to disrupt the amphipathy of this region. Disruption of the amphipathic character of the original structure could affect its possible interaction with the fusion peptide or with another region of gp30 involved in the interaction with gp51 and thereby alter the fusogenic activity of the gp51-gp30 complex.

Mutant and wild-type BLV envelope glycoproteins were expressed using vaccinia recombinants; recombinant ENV expressed wild-type gp51 and gp30 and recombinant MU6 expressed mutated gp51 and wild-type gp30.

**Analysis of Mutated gp51**—Level of expression and correct processing of the viral antigens were monitored by Western blot (Fig. 2). It appeared that cells infected with ENV or MU6 recombinant expressed gp51 and gp51-related products (precursor and degradation products) in comparable amounts, indicating that mutations introduced in the NH2-terminal region of gp51 did not affect expression and processing of BLV glycoproteins.

To determine whether gp51 was expressed at the cell surface, indirect immunofluorescence assays were performed using a mixture of anti-gp51 monoclonal antibodies (Fig. 3A). This analysis showed that cells infected by vaccinia recombinant ENV or MU6 expressed comparable amounts of viral antigens at the surface, suggesting that the intracellular transport of gp51 to the plasma membrane was not affected by the mutations introduced into the 19–27 region of gp51.

The antigenic reactivity of gp51 produced in cells infected with the ENV or MU6 recombinants was examined in an ELISA test using monoclonal antibodies directed against the biologically active and conformational epitopes F, G, and H (Fig. 4). The reactivity of the monoclonals, including mono H, was not altered.

The amounts of gp51 expressed by recombinants ENV and MU6 were quantitatively compared by ELISA assay using the same mono F, mono G, and mono H antibodies (Fig. 4) and found to be similar.

**Fusogenic Capacity of the Mutated Envelope Glycoproteins of BLV**—The MU6 recombinant was then tested for its fusogenic capacity in two cell culture systems (Fig. 3, B and C). Briefly, VERO cells were infected with recombinants ENV or MU6 expressing equal amounts of gp51 as estimated by ELISA and co-cultivated with C81 cells, indicators of syncytia formation. In the second system, CV1 cells were infected with the recombinants. Twenty-four hours after infection, syncytia were counted. These experiments revealed that the MU6 recombinant induced syncytia with a significantly lower efficiency (35%) as compared with the ENV recombinant (100%), indicating that the mutations introduced in the 19–27 region of gp51 indeed altered the fusogenic activity of the BLV envelope glycoproteins.

**DISCUSSION**

The molecular mechanisms involved in membrane fusion remain poorly understood. Theoretical and experimental studies underline the fusogenic activity of the hydrophobic NH2-terminal segment of the transmembrane glycoproteins of enveloped viruses (Scheid and Choppin, 1977; Richardson et al., 1980; White et al., 1983; Kowalski et al., 1987; Gallaher, 1987; Brasseur et al., 1988; Bosch et al., 1989; Freed et al., 1990; Horth et al., 1991; Vonèche et al., 1992).

Reports from several groups have emphasized the role of various regions of the external glycoprotein of enveloped viruses in the fusion events. In addition to binding domains necessary for the interaction with the CD4 receptor (Lasky et al., 1987), the glycoprotein gp120 of HIV-1 contains regions crucial for membrane fusion but not involved in CD4 binding (Kowalski et al., 1987; Skinner et al., 1988; Ho et al., 1988; Willey et al., 1988).

Here we investigated the possible role of the amino-terminal region of gp51 in the cell fusion induced by BLV. This region was supposed to be part of the neutralizable conformational epitope H as variants characterized by the H' phenotype contain point mutations at amino acid positions 23 and 25. Using various algorithms we concluded that the
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peptide segment between amino acids 19 and 27 most probably adopted an amphipathic β-sheet structure that could interact with the fusogenic peptide of BLV gp30 or other amphipathic structures.

In order to test this hypothesis, gp51 was mutated within the 19–27 segment and expressed using the vaccinia recombinant MU6. The mutated complex gp51-gp30 produced by MU6 displayed a strongly reduced fusogenic capacity relative to the wild-type complex produced by recombinant ENV, demonstrating the crucial role of the 19–27 region of gp51 in the fusogenic process.

The antigenic structure of gp51 produced by the recombinants ENV and MU6 was examined: the three monoclonal antibodies, mono F, mono G, and mono H, displayed similar reactivity with the corresponding epitopes present on mutant and wild-type gp51. This suggested that the amino acid substitutions introduced in the 19–27 peptide segment of gp51 did not affect the mono H reactivity, although this region was supposed to contain or be part of the H epitope.

Amino acid changes characterizing the natural H− variant affect the hydrophilic face of the amphipathic 19–27 structure of gp51 (Ser23 and Ser25 replaced by Phe and Ala, respectively (Mamoun et al., 1990)). These mutations prevent the recognition of gp51 by the monoclonal antibody, mono H (Portetelle et al., 1989), but do not alter the fusogenic capacity of the H− variant (data not shown). Here we introduced amino acid substitutions on the hydrophobic face of the amphipathic 19–27 segment (Phe22, Ile24, and Ile26 replaced by His, Glu, and Gln, respectively). These modifications diminished the fusogenic activity of the gp51-gp30 complex but did not affect the recognition of gp51 by mono H. These observations suggest that one face of the amphipathic 19–27 segment influences the recognition of gp51 by mono H, whereas the other face is involved in the process of cell fusion.

Although we may not completely exclude a possible role of this region in receptor binding, the reduced fusogenic capacity obtained for the mutated gp51-gp30 complex agrees with our hypothesis concerning the interaction of the NH2-terminal region of gp51 with the fusogenic peptide or with another region of gp30.

In a multimeric model, three or four fusogenic peptides could be surrounded by three or four amino-terminal domains of gp51, as a sword in a sheath, and thus be isolated from the aqueous environment. This is in agreement with the model proposed by White et al. (1983, 1987) for the post-binding events in the case of the hemagglutinin HA of influenza. After internalization of the virus in endocytotic vesicles, acidic pH induces conformational change in HA leading to fusion between viral and endosomal membrane; after modification of interaction between HA1 and HA2, the trimer of HA1 opens as a trilobated flower, drawing the fusogenic peptide (NH2-terminal segment of HA2) until it inserts into the endosomal membrane and induces fusion. Except for the drop in pH, the mechanistic model proposed for HA1-HA2 probably holds for gp51-gp30 (Burny et al., 1988).

Acknowledgments—V. V. is grateful to Dr. G. Meulemans for invaluable help in preparation of vaccinia recombinants and to M. Nuttink for technical assistance.

REFERENCES
Bosch, M. L., Earl, P. L., Fargnoli, K., Picciafuoco, S., Giombini, F., Wong-Staal, F., and Franchini, G. (1988) Science 244, 694–697
Brasseur, R., Vandenbergban, M., Cornet, B., Burny, A., and Ruysschaert, J.-M. (1980) Biochim. Biophys. Acta 1029, 267–273
Bruck, C., Mathot, S., Portetelle, D., Berte, C., Franssen, J. D., Herion, P., and
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Bruck, C., Portetelle, D., Burny, A., and Zavada, J. (1982b) Virology 122, 353-362
Burny, A., Lemaire, B., and van der Maaten, M. (1984) Virology 139, 20-31
Burny, A., Cleuter, Y., Mammerickx, M., Marbaix, G., Portetelle, D., Van Den Broeke, A., Willems, L., and Thomas, R. (1987) Cancer Surv. 6, 139-159
Burny, A., Bex, F., Brasseur, R., Khim, M. C., Delchambre, M., Horth, M., and Verdin, E. (1988) J. Acquired Immune Defic. Syndr. 1, 579-582
Bussete, B. (1989) Biochim. Biophys. Acta 998, 301-309
Callebaut, I., Burny, A., Krchnak, V., Gras-Masse, H., Wathelet, B., and Porte-Chou, P. Y., and Fasman, G. D. (1978) Biochemistry 13, 222-227
Fischinger, P. J., Blevins, C. S., and Nomura, S. (1974) J. Virol. 14, 177-179
Freed, E. O., Myers, D. J., and Risser, R. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 4650-4654
Gallaher, W. R. (1987) Cell 50, 327-328
Garnier, J., Osguthorpe, D. J., and Robson, B. (1978) J. Mol. Biol. 120, 97-120
Graves, D. C., and Jones, L. V. (1981) J. Virol. 38, 1055-1063
Hirstch, E., Osbeshvsky, U., Furman, C., and Sodroski, J. (1991) J. Virol. 65, 2119-2123
Ho, D. D., Kaplan, J. C., Rackauskas, I. E., and Gurney, M. E. (1988) Science 239, 1021-1023
Horth, M., Lambrecht, B., Chuah Lay Khim, M., Bex, F., Thiriart, C., Royshaert, J. M., Burny, A., and Brasseur, R. (1991) EMBO J. 10, 2747-2755
Kowalski, M., Potz, J., Basiripour, L., Dorfman, T., Goh, W. C., Terwilliger, E., Dayton, A., Rosen, C., Haseltine, W., and Sodroski, J. (1987) Science 237, 1351-1355
Lasky, L. A., Nakamura, G., Smith, D. H., Fennie, C., Shimassaki, C., Potter, E., Berman, P., Gregory, T., and Capon, D. J. (1987) Cell 50, 975-985
Leone, N., Honne, H., Gaboroud, C., Rissey, V., Mourg, A., and Morrow, J. P. (1990) Biochimie (Paris) 72, 555-574
Mamoun, R., Monnson, M., Rebeyrotte, N., Bussette, B., Couer, D., Kettmann, R., Hospital, M., and Guillemin, B. (1990) J. Virol. 64, 4180-4188
Martin, I., Defrise-Quertin, F., Mandieu, V., Nielsen, N. M., Seamen, T., Burny, A., Brasseur, R., Royshaert, J. M., and Vandenbranden, M. (1991) Biochem. Biophys. Res. Commun. 175, 572-579
Meulemans, G., Antoine, O., and Haten, P. (1978) Ann. Med. Vet. 122, 45-50
Richardson, C. D., Scheid, A., and Choppin, P. W. (1980) Virology 196, 205-222
Scheid, A., and Choppin, P. (1977) Virology 80, 54-66
Skehel, J. J., Bayley, P. M., Brown, E. B., Martin, S. R., Waterfield, M. D., White, J. M., Wilson, I. A., and Wiley, D. C. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 969-972
Skinner, M. A., Langlois, F. P., McDaniel, J. B., McDougal, J. S., Bolognesi, D. P., and Matthews, T. J. (1988) J. Virol. 62, 4198-4209
Vorobie, V., Portetelle, D., Kettmann, R., Willems, L., Limbach, K., Paulet, E., Royshaert, J. M., Burny, A., and Brasseur, R. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 3810-3814
Wess, W., Brown, J. H., Cusack, S., Paulson, J. C., Skehel, J. J., and Wiley, D. C. (1988) Nature 333, 426-431
Wess, W., Cusack, S. C., Brown, J. H., Daniels, R. S., Skehel, J. J., and Wiley, D. C. (1989) EMBO J. 8, 17-24
White, J. M., Kielian, M., and Helenius, A. Q. (1983) Rev. Biophys. 16, 151-195
Wiley, D. C., and Skehel, J. J. (1987) Annu. Rev. Biochem. 56, 365-394
Willey, R. L., Smith, D. H., Lasky, L. A., Theodore, T. S., Earl, P. L., Moss, B., Capon, D. J., and Martin, M. A. (1986) J. Virol. 60, 139-147
Wilson, I. A., Skehel, J. J., and Wiley, D. C. (1981) Nature 289, 366-373