

Conserved Cysteine Residues in the Human Immunodeficiency Virus Type 1 Transmembrane Envelope Protein Are Essential for Precursor Envelope Cleavage

DOUGLAS DEDERA, RUILI GU, AND LEE RATNER*

*Departments of Medicine and Molecular Microbiology, Washington University
School of Medicine, St. Louis, Missouri 63110*

Received 13 March 1991/Accepted 4 November 1991

The transmembrane (TM) protein of human immunodeficiency virus type 1 has been demonstrated to be involved in viral infectivity and syncytium formation. Two highly conserved cysteine residues in the extracellular region of the TM protein are shown to be essential for processing the 160-kDa envelope precursor into the active 120- and 41-kDa mature forms.

The human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of AIDS (1). The envelope glycoprotein is initially synthesized as a 160-kDa precursor (gp160), which is subsequently cleaved into a 120-kDa surface protein (gp120, designated SU), and a 41-kDa transmembrane protein (gp41, designated TM) (12). The SU protein of HIV is necessary for binding virus to CD4⁺ cells (9). The TM protein has been demonstrated to be involved in fusion of the viral and cellular membranes allowing virus entry into the cytoplasm and syncytium formation (2, 5; our unpublished observations). The extracellular portion of TM has also been shown to be necessary for attachment of SU to TM (5, 9; our unpublished observations). Additionally, the TM protein has been demonstrated to be necessary for the oligomerization of the envelope protein to form dimers, trimers, and tetramers (4, 15).

The determinants necessary for the processing of gp160 into the SU and TM proteins have previously been demonstrated to lie at the carboxy terminus of the SU protein (12). However, the regions of the TM protein necessary for cleavage of gp160 have not been previously identified. The results presented in the current study demonstrate the role of two conserved cysteine residues of TM (residues 598 and 604) in HIV infectivity and processing of gp160 precursor. Additionally, the cysteine residues are shown not to have a role in the oligomerization of the envelope protein.

Complete conservation of cysteine residues 598 and 604 was found in the TM proteins of HIV-1, HIV-2, and simian immunodeficiency virus strains (Fig. 1). Furthermore, cysteine residues are found at similar positions in the TM proteins of other retroviruses, including feline immunodeficiency virus, visna virus, and human T-cell lymphotropic virus type I (Fig. 1) (14). Cysteine residues in TM are also found at the same position in Rous sarcoma virus (8), numerous strains of murine leukemia viruses (13), Mason Pfizer monkey virus, and mouse mammary tumor virus (6).

To analyze the function of the cysteine residues in the TM protein, mutations were made in the parental proviral HIV-1 clone HXB2. Mutagenic oligonucleotides which replaced the cysteine residues at positions 598 and 604 with serine residues were synthesized and were designated DC3 and DC4, respectively (10). The DC3 mutation was created by chang-

ing nucleotides 7592 and 7593 (T and T) (16) to G and A, respectively, converting the cysteine to a serine codon and inserting a *SacI* site. The DC4 mutation was created by changing nucleotides 7611 and 7622 from T and T to A and A, respectively, again converting the cysteine codon to a serine codon and inserting a *PstI* site downstream. Plasmids were synthesized and used to make vaccinia virus recombinants to express the HXB2, DC3, and DC4 envelope proteins. The envelope genes of HXB2, DC3, and DC4 were inserted into the SC11.4 plasmid (a modified SC11 plasmid with a polylinker placed in the *SmaI* site) (2a, 11) and used to create the recombinant vaccinia viruses vvENV (HXB2 envelope gene expressor), vvDC3 (DC3 envelope gene expressor), and vvDC4 (DC4 envelope gene expressor), with the nonrecombinant vaccinia virus, designated vvWT.

The infectivity of HXB2, DC3, and DC4 was determined by transfecting COS-1 and COS-7 cells and then by cocultivation with CEM, MOLT 3, SUP T1, and MT4 T-cell lines. After cocultivation, the cells were maintained in culture for 4 weeks and examined periodically for reverse transcriptase or p24 antigen production. As expected, the HXB2 clone produced infectious virus, but DC3 and DC4 produced virus from COS cells that was noninfectious.

Vaccinia virus recombinants were used to ascertain the defect in the envelope proteins of DC3 and DC4 compared with those of HXB2. A pulse-chase analysis in which BSC-40 cells were infected with vvWT, vvENV, vvDC3, and vvDC4, labeled with [³⁵S]cysteine and [³⁵S]methionine (Translabel; ICN Pharmaceuticals Inc.) for 2 h, and chased for 0, 2, and 4 h was performed. The cells were lysed and then immunoprecipitated with pooled antisera collected from HIV-positive patients. Figure 2a shows the results of the pulse-chase analysis, revealing normal cleavage of gp160 of vvENV into the TM and SU proteins. However, vvDC3- and vvDC4-infected cells showed a major reduction in cleavage of the gp160 to active SU and TM. The lack of cleavage was further demonstrated when SUP T1 cells were infected with vvDC4 and vvENV, followed by surface iodination of the intact cells with Na¹²⁵I. Figure 2b shows substantial surface expression of SU by cells infected with vvENV, whereas the cells infected with vvDC4 showed very little SU expressed on the cell surface. These results would be expected since very little gp160 reaches the cell surface and about 85% is degraded in the lysosomes (17).

The data on surface envelope expression were substanti-

* Corresponding author.

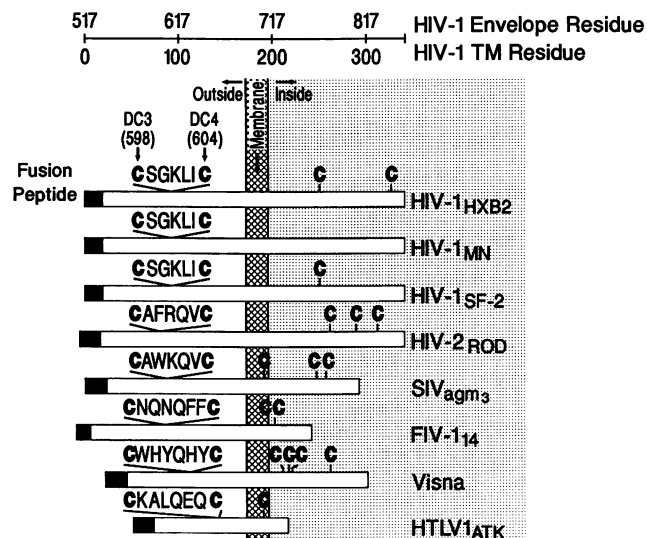


FIG. 1. Diagram of several retroviral transmembrane envelope proteins showing conservation of cysteine residues in the extracellular domain and their relative locations within that domain. SIV, simian immunodeficiency virus; FIV, feline immunodeficiency virus; HTLV, human T-cell lymphotropic virus.

ated by examining SU release into the conditioned culture medium (Fig. 2c). SU from vvENV-infected cells accumulated in the conditioned medium, but no detectable envelope proteins from vvDC3- and vvDC4-infected cells were found in the conditioned medium. These data suggest that the results with DC3 and DC4 envelope proteins are not due to weak association of SU and TM but rather to a lack of precursor protein cleavage.

To explain the defect in cleavage, possible defects in envelope folding in vaccinia virus recombinant-infected cells were examined. Two conformationally dependent properties, i.e., CD4 binding (Fig. 2d) and anti-TM monoclonal antibody binding (Fig. 2e), were examined. In both cases, no significant differences in reactivity with sCD4 or monoclonal antibody 50-69 compared with the differences in reactivity of the pooled HIV-positive antiserum with vvENV-infected cells and with vvDC3- and vvDC4-infected cells were found.

The TM protein has been suggested to be responsible for the oligomerization of the envelope protein into dimers, trimers, and tetramers (4, 15). Further, it has been shown that the extracellular domain of TM is necessary for oligomerization (4). Thus, labeled lysates from vaccinia virus-infected cells were sedimented into 10 to 35% sucrose gradients by using the procedure described by Earl and colleagues (4). Thirteen fractions were collected from each sucrose gradient and immunoprecipitated with pooled antiserum, as shown in Fig. 2f. With vvENV-infected cells, SU proteins in fractions 9 and 10 are consistent with the protein being monomeric in form. The gp160 protein of vvENV and

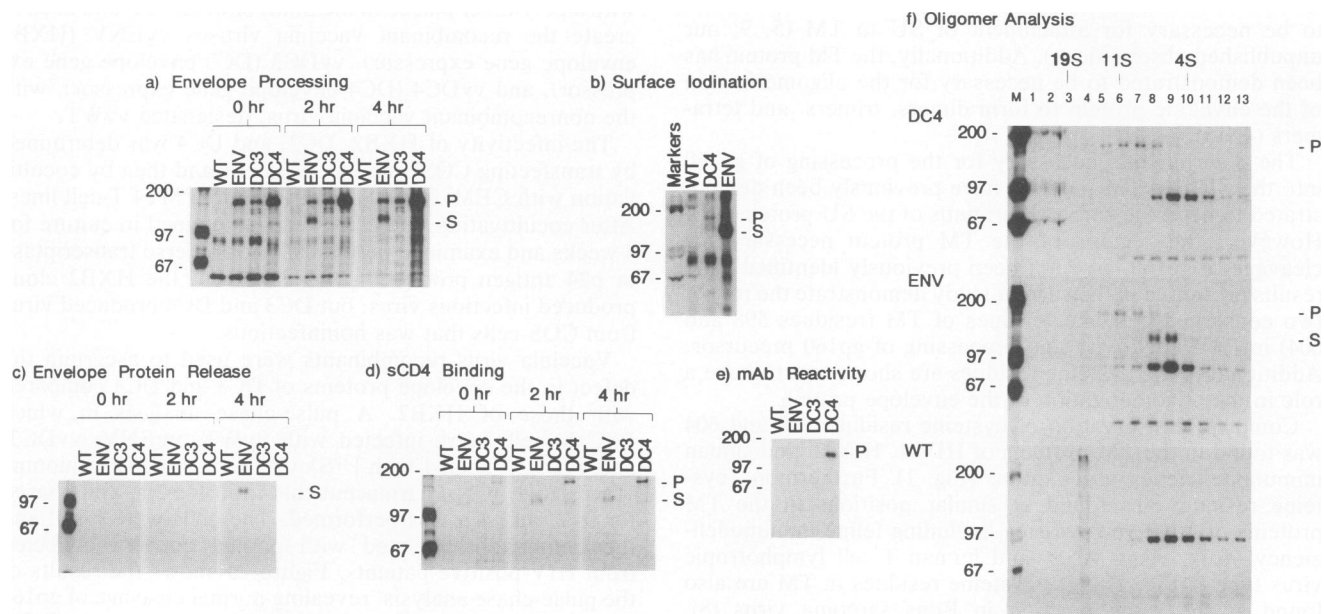


FIG. 2. Expression of envelope proteins in recombinant vaccinia viruses. (a) BSC-40 cells were infected with 2 PFU of vvWT, vvENV, vvDC3, or vvDC4 per cell and pulsed with [35 S]cysteine and [35 S]methionine for 2 h and then chased with label-free medium for 0, 2, and 4 h. Cell lysates were immunoprecipitated with a polyclonal human antiserum. (b) SUP T1 cells were infected with 10 PFU of vvWT, vvDC4, or vvENV per cell, washed, and cultured for 24 h. Then intact cells were labeled with [125 I] (7) and precipitated with the pooled antiserum from HIV-positive patients. (c) BSC-40 cells were infected and labeled as described for panel a, and conditioned medium was immunoprecipitated with the pooled human antiserum. (d) Lysates used for panel a were immunoprecipitated with a conformationally dependent monoclonal antibody to the HIV-1 TM protein 50-69. (e) Lysates used for panel a were immunoprecipitated with a conformationally dependent monoclonal antibody to the HIV-1 TM protein 50-69. (f) BSC-40 cells were infected with vvWT, vvDC4, and vvENV, pulse-labeled with [35 S]cysteine and [35 S]methionine for 4 h, lysed in 250 mM octylglucoside lysis buffer (4), and sedimented onto 10 to 35% sucrose gradients with an SW50.1 rotor (45,000 rpm for 12 h at 4°C). Fractions were precipitated with pooled HIV-positive antiserum. Gradient standards used were thyroglobulin (19S; 630 kDa), catalase (11S; 250 kDa), and bovine serum albumin (4S; 70 kDa). Numbers listed at the left are [14 C]-labeled molecular weight markers in thousands. P, gp160 precursor; S, surface envelope protein; T, transmembrane envelope protein.

vvDC4 was found in fractions 3 through 9. Fractions 6 to 8 are consistent with a monomeric form of the gp160 precursor. Fractions 4 to 6 are consistent with envelope precursor in a dimeric form. Lesser amounts of gp160 were found in fractions 2 to 4, indicating the presence of trimers and tetramers. The results presented in Fig. 2f show no differences in oligomerization between HXB2 and DC4 envelope precursors, suggesting that the cysteine residues are not responsible for TM oligomerization.

These results demonstrate the importance of the cysteine residues in the TM protein for processing of the gp160 precursor into SU and TM. The extremely high conservation of these residues in a wide range of retroviruses suggests an important function in retrovirus replication. Furthermore, these results demonstrate for the first time determinants in the TM protein necessary for processing of the gp160 precursor. The means by which the potential disulfide-bonded loop facilitates processing is not known. We speculate that the loop might be recognized by the cellular protease which cleaves the precursor envelope. Alternatively, loss of the disulfide loop may so disturb the conformation of the gp160 precursor that sequences necessary for binding a protease are no longer recognized. Though gross conformational alterations are unlikely in light of the CD4- and monoclonal antibody-binding data, more subtle changes cannot be excluded. One additional possibility is that the cysteine residues are critical for transport of the envelope precursor from the endoplasmic reticulum to the site of cleavage in the Golgi apparatus.

The lack of cleavage observed in DC3 and DC4 is likely the cause of the loss of infectivity observed in the virus produced by these mutants, since envelope cleavage is necessary for its activity. A similar loss of infectivity was observed with virus produced from *N*-butyl deoxynojirimycin-treated cells, which was also observed to have a reduction in cleavage of the envelope precursor (3).

We thank L. Westfield and E. Sadler (Howard Hughes Medical Institute) for the oligonucleotides.

The work was supported by PHS grant AI24745 and contract DAMD17-87C-7107. L.R. is an American Cancer Society Research Professor.

ADDENDUM IN PROOF

Similar findings were reported recently by Syu et al. (W.-J. Syu, W.-R. Lee, B. Du, Q.-C. Yu, M. Essex, and T.-H. Lee, *J. Virol.* **65**:6349-6352, 1991).

REFERENCES

- Blattner, W., R. C. Gallo, and H. M. Temin. 1988. HIV causes AIDS. *Science* **241**:515-516.
- Bosch, M. L., P. L. Earl, K. Fargnoli, S. Picciafuoco, F. Biombini, F. Wong-Staal, and G. Franchini. 1989. Identification of the fusion peptide of primate immunodeficiency viruses. *Science* **244**:694-697.
- Braciale, T. Personal communication.
- Dedera, D., N. Vander Heyden, and L. Ratner. 1990. Attenuation of HIV-1 infectivity by an inhibitor of oligosaccharide processing. *AIDS Res. Hum. Retroviruses* **6**:785-794.
- Earl, P. L., R. W. Doms, and B. Moss. 1990. Oligomeric structure of the human immunodeficiency virus type 1 envelope glycoprotein. *Proc. Natl. Acad. Sci. USA* **87**:648-652.
- Freed, E. O., D. J. Myers, and R. Risser. 1990. Characterization of the fusion domain of the human immunodeficiency virus type 1 envelope glycoprotein gp41. *Proc. Natl. Acad. Sci. USA* **87**:4650-4654.
- Gallaher, W. R., J. M. Ball, R. F. Garry, M. C. Griffin, and R. C. Montelaro. 1989. A general model for the transmembrane proteins of HIV and other retroviruses. *AIDS Res. Hum. Retroviruses* **5**:431-440.
- Harlow, E., and D. Lane. 1988. *Antibodies, a laboratory manual*, p. 335. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Hunter, E., E. Hill, M. Hardwick, A. Bhowan, D. E. Schwartz, and R. Tizard. 1983. Complete sequence of the Rous sarcoma virus *env* gene: identification of structural and functional regions of its product. *J. Virol.* **46**:920-936.
- Kowalski, M., J. Potz, L. Basiripour, T. Dorfman, W. C. Goh, E. Terwilliger, A. Dayton, C. Rosen, W. Haseltine, and J. Sodroski. 1987. Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. *Science* **237**:1351-1355.
- Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* **82**:488-492.
- Mackett, M., and G. L. Smith. 1986. Vaccinia virus expression vectors. *J. Gen. Virol.* **67**:2067-2082.
- McCune, J. M., L. B. Rabin, M. B. Feinberg, M. Lieberman, J. C. Kosek, G. R. Reyes, and I. L. Weissman. 1988. Endoproteolytic cleavage of gp160 is required for the activation of human immunodeficiency virus. *Cell* **53**:55-67.
- Merregaert, J., J. M. Nuyten, and M. Janowski. 1985. Nucleotide sequence of the envelope gene of radiation leukemia virus. *Virology* **144**:457-467.
- Myers, G., J. A. Berzofsky, A. B. Rabson, T. F. Smith, and F. Wong-Staal (ed.). 1990. *Human retroviruses and AIDS, 1990: HIV sequence data base*. Los Alamos National Laboratory, Los Alamos, N.Mex.
- Pinter, A., W. J. Honnen, S. A. Tilley, C. Bona, H. Zaghouni, M. K. Gorny, and S. Zolla-Pazner. 1989. Oligomeric structure of gp41, the transmembrane protein of human immunodeficiency virus type 1. *J. Virol.* **63**:2674-2679.
- Ratner, L., A. Fisher, L. L. Jagodzinski, H. Mitsuya, R. S. Liou, R. C. Gallo, and F. Wong-Staal. 1987. Complete nucleotide sequences of functional clones of the AIDS virus. *AIDS Res. Hum. Retroviruses* **3**:57-69.
- Willey, R. L., J. S. Bonfacino, B. J. Potts, M. A. Martin, and R. D. Klausner. 1988. Biosynthesis, cleavage, and degradation of the human immunodeficiency virus 1 envelope glycoprotein gp160. *Proc. Natl. Acad. Sci. USA* **85**:9580-9584.