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Cytoplasmic Tail of Moloney Murine Leukemia Virus Envelope Protein Influences the Conformation of the Extracellular Domain: Implications for Mechanism of Action of the R Peptide

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The envelope (Env) protein of Moloney murine leukemia virus (MoMuLV) is a homotrimeric complex whose monomers consist of linked surface (SU) and transmembrane (TM) proteins cleaved from a precursor protein by a cellular protease. In addition, a significant fraction of virion-associated TM is further processed by the viral protease to remove the C-terminal 16 amino acids of the cytoplasmic domain, the R peptide. This cleavage greatly enhances the fusogenicity of the protein and is necessary for the formation of a fully functional Env protein complex. We have previously proposed that R peptide cleavage enhances fusogenicity by altering the conformation of the ectodomain of the protein (Y. Zhao et al., J. Virol. 72:5392-5398, 1998). Using a series of truncation and point mutants of MoMuLV Env, we now provide direct biochemical and immunological evidence that the cytoplasmic tail and the membrane-spanning region of Env can influence the overall structure of the ectodomain of the protein and alter the strength of the SU-TM interaction. The R-peptide-truncated form of the protein, in particular, exhibits a markedly different conformation than the full-length protein.

Retroviral envelope (Env) glycoproteins are expressed on the surface of infected cells and are incorporated into the viral lipid envelope during budding of a virion from a cell. Following binding to a specific cell surface receptor, Env promotes fusion between the viral and host cell membranes and thereby initiates a new infection. For the murine leukemia viruses (MuLVs), Env is initially translated as a precursor protein, Pr85, which oligomerizes in the endoplasmic reticulum, probably as a homotrimer (17). This precursor is cleaved by a cellular protease into two subunits, the surface (SU) protein gp70 and the transmembrane (TM) protein p15E, which remain associated in a labile interaction which may include a disulfide bond (48, 51).

MuLV Env also undergoes a second cleavage event in the cytoplasmic tail of TM that is essential for protein function. The C-terminal 16 amino acids of the cytoplasmic tail, the R peptide, is removed from a subset of the virion TM proteins by the viral protease (22, 27, 60). This results in an Env protein that is inherently more fusogenic than the full-length protein, causing, for example, cell-cell fusion to occur when it is expressed in NIH 3T3 cells (29, 53, 54). It has been suggested that the virus has adopted this strategy of regulating Env fusogenicity in order to limit the expression of a potentially cytotoxic molecule on the cell surface (53, 54). Similar processing of Env cytoplasmic tails has been observed in the Mason-Pfizer monkey virus (M-PMV), the gibbon ape leukemia virus (GaLV), and the equine infectious anemia virus (4, 7, 56); in addition, truncated M-PMV and GaLV Env proteins are more fusogenic than their full-length counterparts (3, 7). Truncations of the long cytoplasmic domains of lentiviral Env proteins occur under certain culture conditions (5, 31, 32, 59), and an increase in Env fusogenicity has been reported for truncated versions of simian immunodeficiency virus (SIV), human immunodeficiency virus type 1 (HIV-1), and HIV-2 Envs (18, 45, 63, 64, 68, 80).

The current model of retroviral fusion is based largely on data obtained from structural and functional studies of the class I fusion proteins, influenza hemagglutinin (HA) and HIV-1 Env (reviewed in references 13 and 61). A central feature of the model is that the proteins are first primed to a metastable state, which is then ready to be activated to a fusion-promoting state following exposure to an appropriate trigger. HA consists of two covalently linked subunits, HA_1 and HA_2, organized in a trimeric complex. Cleavage of these proteins from the HA_0 precursor causes structural rearrangements to occur that are necessary to achieve the metastable conformation and which insert the fusion peptide at the N terminus of HA_2 into a charged pocket. Similarly, precursor processing is an essential event for the maturation of fusion proteins from retroviruses, orthomyxoviruses, and paramyxoviruses (12, 19, 30, 35, 41, 57), although it appears to be dispensable for the activity of the Ebola virus glycoprotein (69).

The trigger that allows a fusion protein to adopt a fusogenic conformation is typically either exposure to a low-pH environment (pH-dependent viruses) or the result of an interaction with its cell-surface receptor at neutral pH (pH-independent viruses). For the prototype influenza HA, exposure of the virus

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to low pH following internalization into an endosome causes major structural rearrangements to occur, including the partial displacement of HA_1, the creation of a six-helix bundle in HA_2, and the translocation of the fusion peptide towards the target cell membrane. Both the fusion peptide and the six-helix bundle are believed to be common features of class I fusion proteins, and structural studies have indicated that six-helix bundles can be formed in the transmembrane proteins of retroviruses, paramyxoviruses, and filoviruses (13). The adoption of a fusogenic formation can also be a more complex event, as the HIV and SIV Envs require sequential interactions with both CD4 and a coreceptor (33, 71), avian leukosis virus uses both receptor interaction and a subsequent low-pH step (44), and certain paramyxoviruses exhibit interactions between the HN and F proteins (10, 26). For the majority of retroviral Env proteins, the trigger is believed to be binding to a cell surface receptor at neutral pH (40). Alterations in Env proteins following interactions with their receptors have been detected for avian leukosis virus (20) and MoMuLV (28), and the dissociation of SU from TM (shedding) has been observed following the addition of soluble CD4 to HIV-1 virions (2, 43).

The mechanism whereby R peptide cleavage enhances fusogenicity is not well understood, but several mechanisms can be envisioned. Since the R peptide contains a putative Y-X-X-Hy internalization signal (where X is any amino acid and Hy is a bulky hydrophobic residue), its removal may simply serve to stabilize Env on the cell surface and thereby allow a greater number of membrane fusion events to occur. Increased steady-state levels of Env have been reported following the mutation of similar tyrosine motifs in the cytoplasmic tails of several retroviruses (15, 21, 34, 38, 46, 67), and the increased fusion seen for certain point and truncation mutants of retroviral Envs have previously been attributed to effects on cell surface levels. While a correlation between the surface density and the extent of cell-cell fusion has been documented for the HIV-1 Env (37), mutation of a tyrosine in the HIV-1 tail has also been shown to give rise to a protein with inherently different fusion properties (67).

It is also possible that the R peptide regulates Env function by interacting with other viral or cellular proteins or with the membrane itself. Such interactions could restrain or localize the protein in such a way as to interfere with the formation or widening of a fusion pore (6). In support of such a role, the R peptide has been shown to be palmitoylated (47), which could promote an association between the full-length cytoplasmic tail and the plasma membrane. In addition, the R peptide suppression of fusion appears to be transferable between certain Env proteins, which suggests an interaction with a common factor. Even though there is only 33% homology between the R peptides of MuLV and GaLV, we have found that their R peptides are interchangeable (7). More intriguingly, the suppression of fusion conferred by the MuLV R peptide may be transferable to less closely related proteins, as its addition to a truncated, hyperfusogenic SIV Env reduced fusogenicity (72).

In an alternative scenario, the presence of the R peptide may function to mask a fusion-promoting structure located in the more upstream region of the cytoplasmic tail. In support of this hypothesis, it is known that MoMuLV Env with no cytoplasmic tail is less fusogenic in cell-cell syncytium assays than the form lacking the R peptide (29, 42, 52), and a deletion of residues 603 to 606 in this upstream region can inhibit the fusion activity of both full-length and R-peptide-truncated proteins (29, 79). In addition, both insertions in this region (36) and point mutations (M. M. Januszeski and P.M. Cannon, unpublished data) can enhance the fusogenicity of a full-length Env, possibly by preventing an interaction with the R peptide. However in vitro experiments to analyze fusion pore formation do not support the model of a fusion-enhancing structure in the truncated tail, as a tailless Env was found to be just as fusogenic as a protein lacking the R peptide (42).

Whatever the initial consequence of R peptide removal for the cytoplasmic tail of MuLV Env, we have previously suggested that the increase in fusogenicity is a direct result of changes in the ectodomain of the protein and that R peptide cleavage is required for Env to achieve a metastable state (79). Our model is based on the finding that although an Env lacking the R peptide can stimulate fusion in *trans* within a mixed MuLV Env oligomer (containing both full-length proteins and proteins lacking the R peptide) (55, 78, 79), the enhancement of fusogenicity requires certain sequences to be present in the truncated protein. Specifically, mutations in either the upstream region of the tail or two separate regions in the ectodomain of TM prevent this effect when present on the truncated monomer but are tolerated when present on the full-length partner (79). These findings led us to propose that R peptide cleavage removes a conformational constraint on the cytoplasmic tail of the protein, with the resulting change in this domain being transmitted through to the ectodomain of the protein. Intriguingly, the two mutations in the ectodomain of TM that we mapped as being necessary for the transmission of this signal lie in two putative helical domains (equivalent to HR1 and HR2 in the HIV-1 Env) that are presumed to form the six-helix bundle that is characteristic of the fusion-competent forms of class I proteins.

A prediction of our model is that full-length Env proteins and those lacking the R peptide would have different overall structures. We now report direct biochemical and immunological evidence that the cytoplasmic tail and the membrane-spanning regions of MoMuLV Env can affect both the conformation of the ectodomain of the protein and the strength of the SU-TM interactions. This suggests that an event in the cytoplasmic tail, namely, R peptide processing, has long-range effects on the ectodomain of the protein which ultimately regulate the ability of the protein to be triggered to a fusogenic state following receptor interaction.

MATERIALS AND METHODS

Cell lines. NIH 3T3 cells and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Cell Culture Core Facility, University of Southern California) supplemented with 10% fetal calf serum (HyClone, Logan, Utah) and 2 mM glutamine (Gibco-BRL, Gaithersburg, Md.). XC cells were maintained in basal medium Eagle (Gibco-BRL) supplemented with 10% fetal calf serum and 2 mM glutamine.

Env constructs. The cytomegalovirus promoter expression plasmid for the wild-type MoMuLV Env protein, CEE, was previously described (39). The truncation mutants CEETR, CEET, and GLA15E were derived from CEE as described elsewhere (52, 53). The cytoplasmic tail point mutants, L618A and Y622A, were derived from CEE by PCR mutagenesis. Plasmid CEEΔEnv is the backbone expression plasmid for CEE, obtained by deleting the MoMuLV Env gene from plasmid CEE by *Eco*RI digestion and religation. The Env constructs used are shown in Fig. 1.

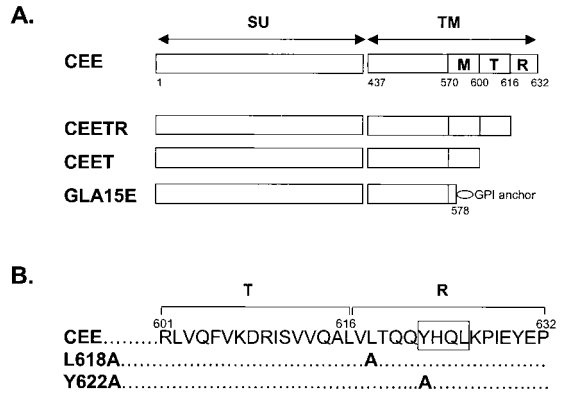


FIG. 1. Schematic of Env proteins. (A) The 632-amino-acid MoMuLV Env protein comprises SU and TM polypeptides. The membrane-spanning region of the TM protein is shown (M), while the cytoplasmic tail consists of the R peptide (R) and the mature tail (T) remaining after R peptide removal. Construct GLA15E retains eight amino acids of the membrane-spanning region linked to a GPI anchor. (B) Amino acid sequence of the cytoplasmic tail of MoMuLV Env, showing the substitutions made in the indicated mutants. A putative endocytosis motif in the R peptide is boxed.

Cell surface expression (CSE). 293T cells plated in 60-mm tissue culture dishes were transfected with up to 15 μg of an Env plasmid by calcium phosphate transfection. When the amount of Env plasmid DNA was less than 15 μg, plasmid CEEΔEnv was used to bring the total amount of DNA to 15 μg. Forty-eight hours posttransfection, the cells were harvested with enzyme-free cell dissociation solution and washed with phosphate-buffered saline (PBS) (Sigma, St. Louis, Mo.). Cells (5×10^5) were incubated with 250 μl of either undiluted anti-SU rat hybridoma supernatant 83A25 (16) or undiluted anti-SU mouse hybridoma supernatant 273 (National Institutes of Health AIDS Research and Reference Reagent Program) (77) for 1 h at 4°C. The cells were washed in PBS plus 10% goat serum (PBSG) and then incubated at 4°C for 1 h in a 1:100 dilution of fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin G (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) diluted in PBSG. Following a final wash with PBSG, the cells were resuspended in 4% paraformaldehyde in PBS. Samples were analyzed by flow cytometry by using a Becton Dickinson FACScan, and absolute fluorescence values were obtained by using latex beads of known fluorescence intensities (Sphero Rainbow Particles 6.7-μm-diameter beads; Spherotech, Inc., Libertyville, Ill.) as described previously (29). The absolute fluorescence value of each individual sample was normalized to the value obtained for the wild-type MoMuLV Env protein within the same experiment.

Cell-cell fusion assay. 293T cells (2×10^6) were plated in a 60-mm tissue culture dish marked with 2- by 2-mm grids (Corning Glass Works, Corning, N.Y.) and transfected with various amounts of Env expression plasmids. In all cases, the total amount of plasmid DNA used in the transfection was made to total 15 μg by using plasmid CEEΔEnv. Twenty-four hours after transfection, 6×10^5 XC cells were added to the 293T cell cultures. After incubation for an additional 18 h, the cells were fixed and stained with 1% methylene blue in methanol. The number of nuclei recruited into syncytia (fused cell masses containing four or more nuclei) in a 20-mm^2 area was counted under a light microscope.

Retroviral vector production and titer determination. Retroviral vectors were produced by transient transfection of 293T cells by calcium phosphate precipitation, essentially as described previously (23, 62). The plasmids used were the MoMuLV gag-pol expression plasmid pCgp (24), the retroviral vector pCnBg (23), which expresses *lacZ* and *neo*, and an Env expression plasmid. Vector supernatants were collected 48 h posttransfection and filtered through a 0.45-μm filter. Titers were determined by β-galactosidase expression as previously described (39).

Western blot analysis. Retroviral vectors were harvested from the supernatants of transfected 293T cells by centrifugation through 4 ml of 20% (mass/vol) sucrose at 4°C for 2 h at 30,000 rpm by using an SW41 rotor (Beckman Instruments, Inc., Palo Alto, Calif.). In addition, transfected 293T cells in a 60-mm dish were washed with PBS, dissociated by using enzyme-free cell dissociation buffer (Gibco-BRL), and lysed in 100 μl of lysis buffer (20 mM Tris-HCl [pH 7.5], 1% Triton X-100, 0.05% sodium dodecyl sulfate [SDS], 5 mg of sodium deoxycholate/ml, 150 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride [Sigma]) at 4°C for 10 min. Following centrifugation in an Eppendorf microfuge at 16,000 × *g* at 4°C for 10 min, the cleared supernatants were collected. Viral pellets and cell lysates were deglycosylated by incubation with 500 U of *N*-glycosidase F (NEB, Beverly, Mass.) for 1.5 h at 37°C. All samples were diluted 1:1 in 2× SDS gel loading buffer (Novex, San Diego, Calif.) plus 10% 2-mercaptoethanol, boiled for 5 min, and electrophoresed in 14% polyacrylamide gels (Novex). The proteins were transferred to an Immobilon P polyvinylidene fluoride transfer membrane (Millipore Corp., Bedford, Mass.) and blocked overnight at 4°C with blocking buffer (5% dried milk in PBST [0.01 M PBS {pH 7.4}, 0.25% Tween 20]).

For the detection of specific proteins, the membranes were cut into three strips that separately contained the SU, capsid (CA), or TM proteins and were subjected to the following detection protocols. All incubations were performed at room temperature by using antibodies or protein G at the stated dilutions in blocking buffer. Each wash step comprised three separate 10-min washes in PBST, although the wash temperatures varied as described. For the detection of SU, the relevant membrane strips were incubated for 3 h in a 1:400 dilution of goat anti-gp70 antibody (lot 79S656; Quality Biotech, Camden, N.J.), washed at 37°C, incubated for 1 h with 0.125 μCi of ^{125}I-labeled protein G/ml (NEN Life Science Products, Inc., Boston, Mass.), and then washed again at 37°C. CA proteins were labeled by a 3-h incubation in a 1:2,000 dilution of goat anti-p30 antibody (lot 78S221; Quality Biotech), washed at room temperature, incubated for 1 h with 2 μCi of ^{125}I-labeled protein G/ml, and then washed again at room temperature. TM proteins were labeled by a 1.5-h incubation in a 1:2,000 dilution of rat monoclonal anti-p15E antibody (50), washed at 4°C, incubated for 1 h in a 1:250 dilution of goat anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.), washed at 4°C, incubated in 0.5 μCi of ^{125}I-labeled protein G/ml for 1 h, and finally washed again at 4°C. The membranes were then exposed overnight to a PhosphorImager screen, and specific bands were detected by using a PhosphorImager 445 SI (Molecular Dynamics, Sunnyvale, Calif.).

Protein biotinylation and immunoprecipitation. Fifty-percent-confluent 293T cells plated in 100-mm tissue culture dishes were transfected with 30 μg of an Env expression plasmid as described above. Forty-eight hours later, the cells were harvested with enzyme-free cell dissociation solution, washed three times with ice-cold PBS (pH 8.0), and resuspended at a concentration of 2.5×10^7 cells/ml in PBS. The cells were then incubated with 0.5 mg of sulfo–*N*-hydroxysuccinimide–biotin/ml (Pierce, Rockford, Ill.) at 4°C for 1 h, followed by washing three times with ice-cold PBS. The cells were then lysed with 1 ml of immunoprecipitation buffer (10 mM $NaPO_4$ [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and 0.2 U of aprotinin/ml) on ice for 20 min and centrifuged for 10 min at 4°C in an Eppendorf microcentrifuge at 16,000 × *g*. The supernatants were collected and incubated with 4 μl of goat anti-gp70 antiserum (lot79S656; Quality Biotech) together with 20 μl of protein G-Sepharose (Sigma) in a total volume of 4 ml of immunoprecipitation buffer. The samples were incubated overnight at 4°C and the Sepharose beads were pelleted by a brief centrifugation and washed once with wash buffer 1 (100 mM Tris-Cl, [pH 7.5], 150 mM NaCl, 2 mM EDTA [pH 8.0], and 0.2% NP-40) and once with wash buffer 2 (100 mM Tris-Cl [pH 7.5], 500 mM NaCl, 2 mM EDTA [pH 8.0], and 0.2% NP-40). The samples were then resuspended in 2× SDS gel-loading buffer with 10% 2-mercaptoethanol, boiled for 5 min, and briefly recentrifuged, and the supernatants were electrophoresed on 14% polyacrylamide gels. Resolved proteins were transferred to Immobilon P membranes and blocked as described above. The membranes were then incubated at room temperature for 1 h with ^{125}I-conjugated streptavidin at a 1:200 dilution (Amersham Pharmacia Biotech, Piscataway, N.J.) and washed with PBST. The labeled proteins were quantitated by using a PhosphorImager 445 SI and ImageQuant software (Molecular Dynamics) following overnight exposure.

^{35}S-labeling and immunoprecipitation. For the analysis of cellular proteins, 50%-confluent 293T cells plated in 100-mm tissue culture dishes were transfected with 30 μg of an Env expression plasmid, as described above. Fourteen to eighteen hours later, the cells were washed and fresh medium was added, supplemented with 10 mM sodium butyrate. Eight to twelve hours later, the cells were incubated for 30 min with fresh DMEM without methionine or cysteine, followed by the addition of 100 μCi each of [^{35}S]methionine and [^{35}S]cysteine (Amersham Pharmacia Biotech). Twelve to eighteen hours later, the cells were harvested with enzyme-free cell dissociation solution, washed with ice-cold PBS, lysed, and immunoprecipitated with goat anti-SU antibody (lot 79S656; Quality Biotech), as described above.

Calculation of shedding indexes. Fifty-percent-confluent 293T cells in a 100-mm dish were transfected with 20 μg of an Env expression plasmid and 10 μg of plasmid pCgp and labeled with ^{35}S as described above. The culture

TABLE 1. Properties of Env proteins

Env protein	CSE[a]	293T-XC fusion[b]		Titer[c]
		0.5 μg	13 μg	
CEE	100 ± 10	399 ± 124	684 ± 149	100 ± 21
CEETR	189 ± 34	4,219 ± 406	3,604 ± 440	41 ± 21
CEET	92 ± 10	2,231 ± 450	3,332 ± 324	3 ± 2
GLA15E	366 ± 12	0 ± 0	0 ± 0	0 ± 0
L618A	57 ± 6	2,001 ± 258	2,429 ± 46	34 ± 16
Y622A	320 ± 17	855 ± 388	871 ± 162	92 ± 15

[a] CSE values of Env were derived by transfecting 60-mm plates of 293T cells with 13 μg of Env plasmid DNA and performing FACS analysis by using monoclonal antibody 273. Absolute fluorescence values were calculated and normalized to the CEE value within each experiment, ± standard deviations ($n = 4$).

[b] 293T cells in a 60-mm plate were transfected with either 0.5 or 13 μg of Env plasmid DNA, and XC cells were added 24 h later. The average number of nuclei recruited into syncytia in a 20-mm^2 area was calculated, ± standard deviations ($n = 3$).

[c] Average titers of retroviral vectors on NIH 3T3 cells, normalized to the CEE value, ± standard deviations ($n = 4$). CEE gave absolute titers of $1.8 \times 10^6 \pm 0.6$ CFU/ml.

supernatants were collected 2 days after transfection, and a 1.5-ml aliquot was stored at 4°C (total supernatant), while an additional 1.5-ml aliquot was diluted with 4 ml of DMEM, layered on top of 4 ml of 20% (mass/vol) sucrose, and centrifuged at 30,000 rpm in an SW41 rotor for 2 h at 4°C. Following centrifugation, the supernatant remaining on top of the 20% sucrose was collected (cleared supernatant), while the pellet was resuspended in 1 ml of immunoprecipitation buffer (viral pellet). All three samples were immunoprecipitated with goat anti-SU antibody as described above, and the proteins were resolved on 14% polyacrylamide gels. Specific bands were detected and quantitated with a PhosphorImager 445 SI and ImageQuant software following overnight exposure. Shedding indexes were calculated as the ratio of the SU signals immunoprecipitated from the cleared supernatants to those immunoprecipitated from the total supernatants.

RESULTS

Properties of MoMuLV Env proteins. We have previously described several truncation and point mutants of the MoMuLV Env (29, 52, 53). The Env constructs used in the present study are shown in Fig. 1. The truncation mutants include an R peptide-truncated form of Env (CEETR), a protein with the whole of the cytoplasmic tail deleted (CEET), and a construct comprising the ectodomain of the protein linked to the membrane by a glycophopsholipid (GPI) anchor (GLA15E). We also included in the present study mutant L618A, which is equivalent to mutant MenvL627A, previously reported to exhibit enhanced fusogenicity (74). Finally, we constructed mutant Y622A, which disrupts a putative endocytosis motif in the R peptide (Fig. 1B). Various characteristics of these Env constructs have previously been reported, and we include a summary of their properties from this work in Table 1.

A major consequence of the removal of the R peptide for the MuLV Env is enhanced fusogenicity. Forms of MoMuLV Env lacking the R peptide display greater ability to induce syncytia in cell-cell fusion assays than does the full-length protein, and this has been observed in various different cell types (29, 52, 53, 54, 66, 72, 74, 75, 79; our unpublished observations). For our present studies, we analyzed the ability of the Env proteins to induce fusion when transfected into 293T cells and cocultivated with receptor-expressing XC cells. No other components of the retrovirus were expressed in the cells to avoid complications due to R peptide processing by the viral protease. We chose the 293T-XC cocultivation system because even the wild-type protein, CEE, induces some syncytia, and we used two different values of input DNA in the transfections in order to observe any differences over a range of protein expression (Table 1).

In agreement with previous reports, we observed that artificially removing the R peptide produced a highly fusogenic protein, with construct CEETR inducing 5 to 10 times the number of syncytia as the full-length protein, depending on the amount of DNA transfected. Similarly, mutants CEET and L618A were also found to be more fusogenic than the wild-type protein for both input amounts of DNA. Although mutant Y622A exhibited a small enhancement of fusion at the lower amount of input DNA, it was not significantly different from the wild-type Env at the higher amount of DNA. Finally, the GPI-linked ectodomain construct, GLA15E, was always observed to be nonfusogenic, despite high levels of CSE in the transfected 293T cells. These results confirm that the membrane-spanning region of TM is essential for Env-mediated fusion (52, 65). The ability of retroviral vectors carrying the various Env proteins to transduce NIH 3T3 cells was also measured, and our results are in good agreement with previous reports (29, 52, 53).

MoMuLV Env proteins lacking the R peptide or the cytoplasmic tail are inherently more fusogenic than the full-length protein at equivalent levels of CSE. Since the R peptide contains a putative endocytosis motif, Y_{622}HQL, it is possible that removal of the R peptide enhances the cell-cell fusion activity of MoMuLV Env simply by increasing its steady-state levels on the cell surface. Indeed, our data for the Y622A mutant showing enhanced fusogenicity compared to the wild-type protein at low levels of transfected DNA suggested that this may be the case. To address this possibility more rigorously, we assessed whether the Y622A substitution resulted in increased CSE levels, and we examined whether this correlated with an increased ability to induce syncytia.

To precisely compare the relationship between CSE and fusogenicity, we first measured the steady-state surface levels of the different Env proteins when expressed in 293T cells as we varied the amount of Env expression plasmids used in the transfections. CSE was measured by fluorescence-activated cell sorter (FACS) analysis by using an antibody specific for the N terminus of the SU subunit (77). We believe that this antibody gives the most accurate relative measurements of CSE when different Env proteins are compared (see below). Our use of 293T cells for these measurements was necessary because these cells do not express the ecotropic receptor, thereby eliminating any artifacts caused by secreted or dissociated SU rebinding to the cell, as we have previously described (76).

For all constructs tested, the level of CSE increased as we used greater amounts of DNA in the transfections (Fig. 2A). However, for the same amount of input DNA, mutant Y622A was always expressed at the highest level, followed by CEETR, while both CEE and CEET gave lower levels. Since protein CEET displays significantly lower processing and/or transport rates than the other proteins (as shown in Fig. 4), it is not possible to draw any firm conclusions about the effect of the loss of a putative endocytosis motif in this construct. However, the data for CEE, CEETR, and Y622A are consistent with the hypothesis that the Y_{622}HQL motif in the R peptide is an

TABLE 2. Antibody recognition of cell surface SU

Env protein	Antibody recognition[a]		Relative recognition (N/C)[b]
	273 (N)	83A25 (C)	
CEE	100 ± 10	100 ± 9	1
CEETR	189 ± 34	89 ± 19	2.2 ± 0.35 ($P = 0.0003$)
CEET	92 ± 10	71 ± 14	1.3 ± 0.11 ($P = 0.02$)
GLA15E	366 ± 12	284 ± 36	1.3 ± 0.12 ($P = 0.04$)
L618A	57 ± 6	36 ± 4	1.6 ± 0.20 ($P = 0.009$)
Y622A	320 ± 17	229 ± 20	1.4 ± 0.04 ($P = 0.02$)

[a] Average absolute fluorescence values, normalized to CEE, ± standard deviation ($n = 4$), were obtained by using the indicated antibodies. Antibody 273 recognizes an epitope in the N terminus of SU, while antibody 83A25 recognizes an epitope in the C terminus of SU.

[b] The N/C ratio was calculated for each individual experiment and normalized to the CEE ratio in the same experiment. The mean and standard deviation of the N/C ratios for each construct were calculated ($n = 4$), and P values were calculated for observed ratio differences from CEE.

endocytosis signal and that the higher cell surface levels seen for both Y622A and CEETR result from the loss of this motif.

We next measured the fusogenicity of the Env proteins when present at different levels on the cell surface. A plot of CSE versus fusogenicity revealed that all of the Env proteins reached a plateau of fusogenicity at relatively low levels of CSE (Fig. 2B). When fusion levels were compared at equivalent levels of CSE, mutant Y622A exhibited a fusogenicity similar to that of the wild-type protein, while mutants CEETR and CEET were seen to be much more fusogenic. This result confirms that the proteins lacking the R peptide or the whole cytoplasmic tail are inherently more fusogenic than either the wild-type protein or the Y622A mutant. In addition, even though we were able to achieve much higher CSE levels for the full-length Y622A protein than for the wild-type Env, we were unable to increase its fusogenicity beyond the maximum level observed with the CEE protein. Fusion activity is therefore limited by the presence of the R peptide, and this limitation cannot be overcome simply by increasing the amount of Env on the surface of the cell.

Env truncations affect antibody recognition of the SU subunit. We have previously proposed that removal of the R peptide may enhance Env fusogenicity by promoting a conformational change in the ectodomain of the protein (79). In order to test this hypothesis further, we used antibody recognition profiles to look for evidence of gross differences in the structures of cell surface-expressed Env between the wild-type and mutant Env proteins.

Antibody binding to SU has previously been used to probe for conformational differences between receptor-bound and unbound SU (28). Two different monoclonal antibodies are available that recognize the C-terminal half of the SU protein from the endogenous defective MuLV, Fv-4^r. Although both antibodies recognize soluble SU bound to its receptor at 4°C, warming to 37°C promotes dissociation of only one antibody, which is suggestive of a receptor-induced conformational change in the bound SU (28). In a similar approach, we measured the ability of two different anti-SU antibodies to recognize the panel of truncation mutants expressed on the surface of transfected 293T cells. Antibody 83A25 is specific for the C terminus of SU (16), while antibody 273 recognizes the N terminus of SU (77).

Transfected 293T cells were reacted with each of the antibodies at 4°C, and binding was analyzed by FACS. Absolute fluorescence values were obtained for each antibody and normalized to the value obtained with the wild-type protein. Examination of the results revealed discrepancies between the normalized values obtained with the N- and C-terminal-specific antibodies for each of the mutants, and these differences are highlighted in the N/C relative recognition ratios shown in Table 2. Notably, the R peptide-truncated protein, CEETR, had the greatest discrepancy relative to the full-length protein ($P = 0.0003$); although CEETR recognition by the N-terminal antibody was increased by almost twofold over that of the full-length protein, recognition by the C-terminal antibody was actually slightly lower. This observation is consistent with the form of the protein that lacks the R peptide having an arrange-

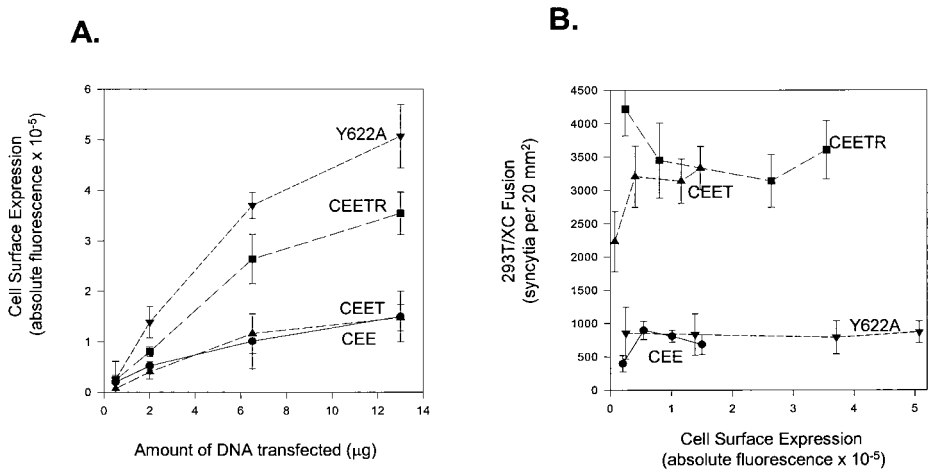


FIG. 2. Relationship between cell surface levels of Env and cell-cell fusion activities for different Env constructs. (A) Effect on CSE of increasing the amount of plasmid DNA transfected into 10-cm plates of 293T cells. CSE was measured by FACS analysis by using antibody 273. (B) Relationship between CSE levels in 293T cells and cell-cell fusion activity in the 293T-XC cocultivation system. Fusion activity was expressed as the number of nuclei recruited into syncytia in a 20-mm^2 area. All data points are the averages of at least three independent experiments.

ment of its SU components that is markedly different from that of the full-length protein.

Mutant L618A, which exhibits high fusogenic activity despite the presence of a full-length cytoplasmic tail, also resulted in a ratio that was highly significant when compared to the value for CEE ($P = 0.009$). Interestingly, the difference between the ratios for L618A and CEETR was also highly significant ($P = 0.006$), suggesting that the underlying causes of the increased fusogenicities of CEETR and L618A may be different. It is noteworthy that CEETR is always a more fusogenic protein than L618A (Table 1 and data not shown).

Although greater N/C ratios could result from either increased exposure of N-terminal epitope(s) or reduced availability of C-terminal epitopes, we favor the latter explanation because the C terminus is the region of SU involved in interactions with TM (51), so it is more likely that conformational changes triggered by alterations in TM would affect this domain. This possibility is consistent with a model whereby R peptide cleavage alters the conformation of the overall Env protein complex and thereby alters the accessibility of different SU epitopes.

Env truncations alter biotinylation of the ectodomain. We next examined whether we could detect gross differences in the structures of the ectodomains of the mutant Env proteins by using direct biochemical labeling with the membrane-impermeable biotinylation reagent sulfo–*N*-hydroxysuccinimide–biotin. This chemical reacts with free primary amine groups in lysines or arginines exposed at the surface of a protein, and the degree of labeling can be quantitated by using secondary detection with ^{125}I-conjugated streptavidin. We immunoprecipitated the labeled proteins by using a polyclonal anti-SU antibody that has previously been shown to immunoprecipitate both SU and the associated TM subunit (78).

In order to perform such an analysis, we needed to control for several variables that would affect the strength of the biotinylation signals obtained for the different Env mutants. These included differences in the processing and transport rates and overall stabilities of the truncated proteins, which could reduce their steady-state levels on the cell surface and therefore give a lower biotinylation signal. Similarly, changes in the ability of the TM subunit to remain associated with SU during the coimmunoprecipitation procedure would specifically lower the TM biotinylation signal. To control for these possibilities, we also labeled total cellular proteins with ^{35}S and immunoprecipitated both SU and the associated TM proteins from lysates by using the same anti-SU antibody (Fig. 3). The immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), and the biotinylated samples were further detected by using ^{125}I-conjugated streptavidin. The amount of radioactivity (^{35}S or ^{125}I) in each band was obtained by PhosphorImager analysis and made relative to the CEE values. From these data, we were able to calculate the biotinylation efficiency for each SU and TM subunit and, furthermore, to compare the ratio of biotinylation of the SU and TM subunits for each mutant (Table 3).

We hypothesized that any changes that occurred in the structure of the ectodomain of Env could alter the degree of labeling of either the SU or TM subunits, and therefore produce an altered SU:TM biotinylation ratio. For a protein that behaved exactly like the wild type, even if its overall surface levels were different, or if it had a greater tendency to shed the SU subunit, the inclusion of the ^{35}S-labeled proteins as correction factors would ensure that the SU:TM biotinylation ratio remained approximately 1. However, any structural changes in a mutant that led to the exposure or occlusion of potential biotinylation sites would distort this ratio. (A caveat of our approach is that compensatory alterations in both SU and TM could cancel out any such effect on the ratio and would be missed in this analysis.) Examination of the data obtained with the set of truncation mutants revealed that the CEETR ratio was once again the most distant from the wild-type value, due primarily to marked hypobiotinylation of the TM subunit relative to its ^{35}S signal (Table 3). Interestingly, the hyperfusogenic mutant, L618A, was not significantly different from the wild-type protein.

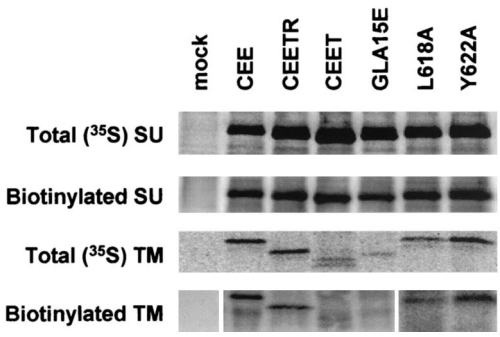


FIG. 3. Biotinylation of SU and TM from truncated Env mutants. Env proteins were expressed in 293T cells by transient transfection. Total cellular proteins were radiolabeled with ^{35}S, or surface-expressed proteins were biotinylated with a membrane-impermeable reagent. Both SU and TM subunits were immunoprecipitated by using a polyclonal anti-SU antiserum and subjected to SDS-PAGE. Biotinylated proteins were detected by using ^{125}I-streptavidin.

During these analyses, we observed that the SU protein from CEET ran faster on gels than the other SU proteins (Fig. 3). In order to investigate whether this was due to differences in N glycosylation, we ran gels of deglycosylated cell extracts and virions (Fig. 4), which confirmed that this was indeed the case. Alterations in the N glycosylation state of a protein are frequent correlates of aberrant processing and transport, and these gels also highlighted the fact that CEET was less efficiently processed from the precursor Env to SU. Taken together, these findings indicate that the complete loss of the cytoplasmic tail results in defects in transport, processing, and glycosylation for the MoMuLV Env.

The gels of deglycosylated proteins also revealed that GLA15E had a strong tendency to shed the SU subunit. Much of this protein was lost from the virion fraction, despite the retention of a strong TM signal (Fig. 4). Such a phenotype would be expected to reduce the ability of TM to stay associated with SU, which is in agreement with the low overall levels of TM that we were able to coimmunoprecipitate with SU for either the ^{35}S- or biotin-labeled proteins (Table 3).

An additional feature that was noted was that the radiolabeled TM proteins ran as doublets (Fig. 3). Since the assays were performed in the absence of the viral protease and the doublets were observed for both full-length and truncated constructs, the size difference could not be due to the removal of

TABLE 3. Biotinylation of cell surface SU and TM

Env protein	Relative signals				Biotinylation efficiency		SU:TM biotinylation ratio (C × B/A × D)
	Total SU[a] (A)	Total TM[a] (B)	Biotin SU[b] (C)	Biotin TM[b] (D)	SU (C/A)	TM (D/B)	
CEE	100 ± 32	100 ± 16	100 ± 19	100 ± 26	1	1	1
CEETR	125 ± 35	128 ± 38	92 ± 18	57 ± 9	0.74	0.45	1.7 ($P = 0.03$)
CEET	239 ± 36	55 ± 17	101 ± 29	21 ± 6	0.42	0.38	1.1 ($P = 0.4$)
GLA15E	100 ± 21	28 ± 7[c]	79 ± 30	17 ± 11	0.79	0.60	1.3 ($P = 0.2$)
L618A	141 ± 25	61 ± 16	125 ± 42	59 ± 5	0.89	0.97	0.9 ($P = 0.3$)
Y622A	197 ± 51	137 ± 41	151 ± 33	117 ± 32	0.77	0.85	0.9 ($P = 0.3$)

[a] Total SU and TM values were obtained by ^{35}S labeling, followed by immunoprecipitation with anti-SU antibody. Radioactivity was quantitated by using a PhosphorImager, and all values were made relative to CEE. Values shown are the means of results of four independent transfections, ± standard deviations.

[b] Surface SU and TM was biotinylated by using a membrane-impermeable reagent, followed by immunoprecipitation with anti-SU antibody. The biotin signal was quantitated by using ^{125}I-labeled streptavidin and PhosphorImager analysis. Values shown are the means of results of four independent transfections, ± standard deviations.

[c] Actual GLA15E ^{35}S TM signal was multiplied by 1.5 to control for the loss of three out of the nine Cys/Met residues in TM that resulted from this truncation.

the R peptide. The doublet was especially pronounced for mutant CEET, where the two forms of radiolabeled TM were equally represented, and a doublet was also seen with the biotin-labeled protein. The two different forms of TM could result from posttranslational modifications, such as glycosylation or palmitoylation, or it could be caused by the presence of an additional protease recognition site. However, we consider it unlikely that differences in glycosylation were the cause of the doublet pattern in TM. Although one consensus sequence for an N-linked glycosylation site exists in TM (N_{569}RSP), it is located right at the boundary of the membrane-spanning domain, which stretches from S_{571} to N_{600} (49), and the presence of the C-terminal proline is predicted to block utilization of the motif (1). Also, when the proteins were deglycosylated, although the lower band virtually disappeared from the gels, no shift in size was observed for the upper, more prominent band following this treatment (Fig. 4 and data not shown).

Instead, we consider it more probable that the two bands represent palmitoylated and nonpalmitoylated forms of TM. A palmitoylated cysteine has been identified in the membrane-spanning region of the MoMuLV TM (73). Since CEET is truncated only 4 amino acids beyond this residue, and since it is known that the removal of sequences downstream of palmitoylation sites can reduce the efficiency of this modification (25), it seems likely that the more pronounced lower band observed for CEET represents a nonpalmitoylated and therefore faster-running form of the protein.

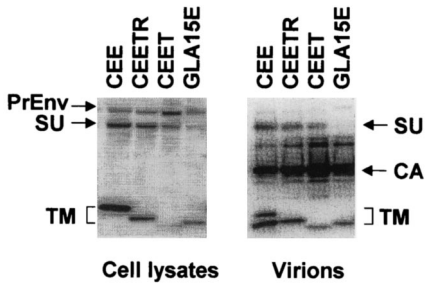
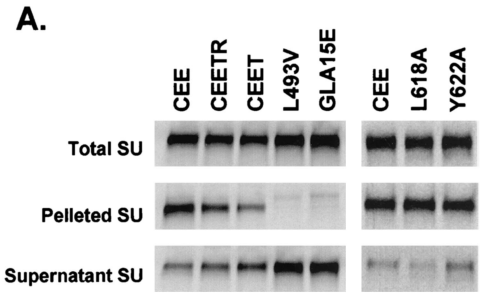


FIG. 4. Deglycosylated cell lysates and virions. 293T cells were transfected with MuLV Gag-Pol plasmids and the indicated Env constructs. Both cell lysates and pelleted viral supernatants were deglycosylated and subjected to SDS-PAGE. Specific proteins were detected by using appropriate antibodies. The various TM proteins run at different sizes due to the truncations they contain. In addition, the wild-type protein, CEE, is present in virions in both full-length and R- peptide-truncated forms. PrEnv is the uncleaved precursor Env protein, Pr85.

Env truncations weaken the association of the SU and TM subunits. An alteration in the interactions between the SU and TM subunits that facilitates exposure of the fusion protein and subsequent virus-cell fusion may occur following binding of retroviral Env proteins to their receptors. It has previously been shown that treating HIV-1 Env with soluble CD4 receptor induces the loss of SU (58). By analogy, we hypothesized that the changes we detected in the Env ectodomain following R peptide cleavage could also alter the strength of the SU-TM interaction, and these alterations could account for the greater fusogenicity of the protein lacking the R peptide. We examined this possibility by assessing the tendency of SU to be shed from virions in response to sheer stress.

Radiolabeled virions were harvested, and equal fractions were either untreated or pelleted by ultracentrifugation through 20% sucrose. SU was immunoprecipitated from the untreated supernatants, the pelleted (virion) fractions, and the cleared supernatants remaining after centrifugation that are expected to contain any dissociated SU. A shedding index was calculated as the ratio of SU immunoprecipitated from the cleared supernatants to that immunoprecipitated from the untreated supernatants. The TM subunit could be coimmunoprecipitated with SU only from total supernatants or pelleted fractions (data not shown), confirming that these fractions alone contained viral particles, while the cleared supernatant contained only dissociated SU. As a control, we included the TM point mutant L493V, which has previously been demonstrated to result in extensive shedding of SU (79).

This analysis revealed that sequential deletion of the cytoplasmic tail and the membrane-spanning region of TM greatly increased the tendency of SU to shed from virions (Fig. 5). In particular, removal of the membrane-spanning domain in construct GLA15E resulted in excessive SU dissociation, as suggested by Fig. 3 and 4. However, a simple increase in the shedding tendency of a protein did not correlate with enhanced Env fusogenicity, which is in agreement with previous observations (70, 79). Instead, these data suggest that an optimal level of SU-TM association must be retained for maximum fusogenicity and Env function.



B.

Env	Shedding Index
CEE	1 +/- 0.2
CEETR	2.4 +/- 1.0 (p = 0.04)
CEET	4.1 +/- 1.7 (p = 0.02)
GLA15E	6.3 +/- 2.5 (p = 0.01)
L493V	6.0 +/- 2.1 (p = 0.01)
L618A	0.9 +/- 0.2 (p = 0.30)
Y622A	1.7 +/- 0.4 (p = 0.02)

FIG. 5. Truncation of TM enhances shedding of SU from virions. ^{35}S-labeled viral supernatants were harvested from transiently transfected 293T cells. Aliquots were retained (total SU) or centrifuged through 20% sucrose to give pelleted and supernatant fractions. SU was immunoprecipitated from each fraction by using an anti-SU antibody, and the extent of radioactivity in each fraction was determined by SDS-PAGE and PhosphorImager analysis. Shedding indexes were calculated as the ratio of the amount of SU in the cleared supernatant to that in the total SU fraction; the data are shown as the averages plus or minus standard deviations of results of three independent experiments, with *P* values calculated for each mutant relative to CEE by using a *t* test. Mutant L493V contains a substitution in the heptad repeat region of TM and has previously been characterized as a shedding mutant (79).

DISCUSSION

Class I viral fusion proteins include the retroviral Env and influenza virus HA proteins. They are typically organized as homotrimers, with the monomers consisting of two linked subunits cleaved from a precursor protein and held together by noncovalent or disulfide linkages. Cleavage of the precursor protein is considered necessary to attain a metastable form of the protein, characterized by internal sequestration of the hydrophobic fusion peptide. The subsequent conversion to a fusion-active state is triggered by receptor binding and/or low pH and is believed to involve gross conformational changes in the membrane-anchored subunit in particular; these changes include the formation of a six-helix bundle and the exposure of the fusion peptide.

The gamma retroviruses, such as MuLV, additionally require removal of the C-terminal R peptide in the cytoplasmic tail of Env in order to achieve full fusogenicity (29, 53, 54). However, it is unclear how this event in the cytoplasmic domain regulates the fusogenic potential of the whole of the Env protein complex. It has previously been noted that the length of the cytoplasmic tail can affect the fusogenicity of retroviral Env proteins. Like MuLV, the cytoplasmic tails of the Env proteins from M-PMV and GaLV are processed by the viral protease to produce proteins that are more fusogenic than the full-length forms (3, 7). In addition, hyperfusogenic mutants resulting from tail truncations have been described for the SIV, HIV-1, and HIV-2 Envs (18, 45, 63, 68, 80). Interestingly, two studies of truncated variants of SIV and HIV-1 Env have provided evidence for corresponding alterations in the ectodomains of the proteins (14, 64).

The MuLV R peptide contains two features that may be important for its ability to regulate Env function, namely, a tyrosine-based endocytosis motif (Y_{622}QHL) and an unmapped palmitoylation site (47). As the levels of a fusion protein on the surface of a cell can influence the efficiency of cell-cell fusion (8, 37), we first asked whether R peptide removal and the accompanying loss of an endocytosis motif enhanced fusogenicity simply by increasing the steady-state levels of cell surface Env. We observed that although the mutation Y622A did indeed increase the levels of Env, the mutant retained a fusion profile similar to that of the wild-type protein when compared at equivalent levels of CSE above a threshold level. Furthermore, after a plateau of syncytium formation had been reached, we were unable to increase fusion further simply by increasing the amount of Env. Our data therefore suggest that R peptide removal alters the intrinsic fusogenicity of the Env protein.

Using a series of truncation and point mutants of MoMuLV Env, we further demonstrated that the cytoplasmic tail and membrane-spanning region of TM can change the conformation of the protein's ectodomain. These differences were observed as altered patterns of recognition of the SU subunit by antibodies and differential labeling of the TM subunit by biotinylation reagents. The changes were most pronounced for protein CEETR, which is truncated at the natural R peptide cleavage site and which is also the most fusogenic protein (summarized in Table 4).

In addition, we observed that sequential truncations of the tail and membrane-spanning domain weakened the intersubunit interactions between SU and TM, with the GPI-linked construct, GLA15E, having an extremely labile bond. However, no correlation was observed between shedding tendency and Env-mediated fusogenicity. While these observations are in agreement with our hypothesis that the cytoplasmic tail and membrane-spanning region of TM can influence the overall

TABLE 4. Summary of properties of Env mutants

Env protein	Cell surface Env			Viral Env shedding index
	293T-XC fusion[a]	N/C antibody ratio	SU:TM biotinylation ratio	
CEE	1	1	1	1
CEETR	10.6	2.2**[b]	1.7*	2.4*
CEET	5.6	1.3*	1.1	4.1*
GLA15E	0	1.3*	1.3	6.3**
L618A	5.0	1.6**	0.9	0.9
Y622A	2.1	1.4*	0.9	1.7*

[a] 0.5 μg of DNA transfected per 60-mm plate of 293T cells.
[b] *, significantly different from CEE value; **, highly significantly different from CEE value.

structure and subunit interactions of the ectodomain of Env, they also suggest that conformational changes can be induced by mutations that destabilize the subunit interactions in the protein without being part of the normal pathway that leads to a fusogenic form of Env. Alternatively, these destabilized Env proteins may reflect normal Env conformational states that have been achieved prematurely (e.g., prior to receptor binding).

Interestingly, although the substitution L618A enhanced the fusogenicity of a full-length protein, we observed no significant alterations in the ectodomain properties of this mutant in two out of the three assays that we used to detect conformational changes (Table 4). Furthermore, although the difference between this mutant and the wild-type protein in the N/C antibody ratio assay was highly significant, the L618A value was also highly significantly different compared to the CEETR value. Overall, this suggests that this mutation may be increasing Env fusogenicity by a different mechanism than that used by the R peptide-truncated protein. An alternative hypothesis that we cannot rule out at this stage is that any conformational changes resulting from R peptide truncation are incidental to its ability to enhance fusion.

The question remains as to how R peptide removal could lead to alterations in the conformation of the Env ectodomain. It has previously been suggested that the R peptide could interact with a cellular factor (72), with such an interaction influencing the mobility of Env in the membrane, either before or after receptor binding. This idea is supported by the finding that the fusion-suppressing properties of the R peptide can be transferred to a truncated SIV Env protein (72). However, this phenomenon was observed only when the coreceptor used by the SIV Env was Gpr15 and not CCR5 (75), and the R peptide does not reduce fusogenicity when present in chimeric HIV-1 (75) or human T-cell leukemia virus type 1 Env proteins (9). Furthermore, although variations have been seen in the overall susceptibility of cells to MoMuLV-induced syncytium formation, removal of the R peptide always enhances fusion to some extent (29, 52, 53, 54, 66, 72, 74, 79; our unpublished observations), and we have been unable to identify a cell line that could be defective for such a cellular factor (our unpublished observations).

An alternative explanation is that R peptide removal first alters the conformation of the cytoplasmic domain of Env and that this change is then transmitted through to the ectodomain of the protein. Two possible mechanisms could allow such an action. First, the R peptide in the full-length tail could interact with and mask a functional domain present in the more upstream region of the tail. For example, an amphiphilic helix has been predicted to form in this region (72, 79), and R peptide removal could allow the hydrophobic face of such a structure to associate with either the cell membrane or other hydrophobic protein domains. Liberation of this interface could thereby promote interactions between adjacent Env tails, either within a single oligomer or between neighboring complexes. Evidence that the two halves of the tail may interact in a full-length Env is provided by the findings that mutations in both regions can independently increase the fusogenicity of a full-length protein (29, 36, 74; M. M. Januszeski and P. M. Cannon, unpublished data). A second, related mechanism has been proposed by Olsen and Andersen (47), who reported that the R peptide can be palmitoylated. They have suggested that this modification promotes an association of the full-length Env with the membrane, while R peptide removal frees Env from this constraint and allows it to adopt a different conformation as a consequence of these changes in the cytoplasmic tail.

It is noteworthy that the ability of the R peptide to suppress fusion within an Env oligomer is not *trans* dominant, while the fusion-enhancing properties of an Env monomer lacking the R peptide do function in *trans* (55, 78, 79). Furthermore, transmission of the R peptide cleavage signal within an Env oligomer can be blocked by mutations in the upstream region of the tail (79). We believe that these observations support a model whereby removal of the R peptide reveals a structure in the cytoplasmic tail whose adoption leads to changes in this domain that are then transmitted through to the ectodomain of the protein. Transmission of a conformational change in this way can be considered the opposite of the events that occur in cell signaling following ligand-receptor docking. A high affinity of the truncated tail for its interacting interface could explain why the R-peptide-truncated phenotype dominates in mixed populations and why complete R peptide cleavage is neither necessary for Env function nor is it observed in virions (26).

It is intriguing that the ability of the R peptide to suppress fusion by a truncated SIV Env is sensitive to coreceptor usage (75). Recently, it has been reported that the sensitivity of the HIV-1 Env to HR2-based peptide inhibitors that interfere with six-helix bundle formation is also influenced by the choice of coreceptor (11). It has been previously demonstrated that the equivalent HR1 and HR2 regions in the MoMuLV TM are essential for the activation of Env by R peptide cleavage (79). Taken together, these findings suggest that long-range interactions occur within the retroviral Env complex, involving the receptor- or coreceptor-binding domain in SU, the HR1-HR2 region in TM, and the cytoplasmic tail of the protein. We speculate that for the MuLV Env, the HR1-HR2 region plays a central role in both the priming of Env to the metastable state following R peptide cleavage and the conversion to a fusion-active state following receptor interaction.

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REFERENCES

1. **Bause, E.** 1983. Structural requirements of N-glycosylation of proteins. Biochem. J. **209**:331–336.
2. **Berger, E. A., J. D. Lifson, and L. E. Eiden.** 1991. Stimulation of glycoprotein gp120 dissociation from the envelope glycoprotein complex of human immunodeficiency virus type 1 by soluble CD4 and CD4 peptide derivatives: implications for the role of the complementarity-determining region 3-like region in membrane fusion. Proc. Natl. Acad. Sci. USA **88:**8082–8086.
3. **Brody, B. A., E. Rhee, and E. Hunter.** 1994. Post-assembly cleavage of a retroviral glycoprotein cytoplasmic domain removes a necessary incorporation signal and activates fusion activity. J. Virol. **68:**4620–4627.
4. **Brody, B. A., S. S. Rhee, M. A. Sommerfelt, and E. Hunter.** 1992. A viral protease-mediated cleavage of the transmembrane glycoprotein of Mason-Pfizer monkey virus can be suppressed by mutations within the matrix protein. Proc. Natl. Acad. Sci. USA **89:**3443–3447.
5. **Chakrabarti, L., M. Emerman, P. Tiollais, and P. Sonigo.** 1989. The cytoplasmic domain of simian immunodeficiency virus transmembrane protein modulates infectivity. J. Virol. **63:**4395–4403.
6. **Chernomordick, L. V., V. A. Frolov, E. Leikina, P. Bronk, and J. Zimmer-**

berg. 1998. The pathway of membrane fusion catalyzed by influenza hemagglutinin: restriction of lipids, hemifusion, and lipidic fusion pore formation. J. Cell Biol. **140:**1369–1382.
7. **Christodoulopoulos, I., and P. M. Cannon.** 2001. Sequences in the cytoplasmic tail of the gibbon ape leukemia virus envelope protein that prevent its incorporation into lentiviral vectors. J. Virol. **75:**4129–4138.
8. **Clague, M. J., C. Schoch, and R. Blumenthal.** 1991. Delay time for influenza virus hemagglutinin-induced membrane fusion depends on hemagglutinin surface density. J. Virol. **65:**2402–2407.
9. **Denesvre, C., P. Sonigo, A. Corbin, H. Ellerbrok, and M. Sitbon.** 1995. Influence of transmembrane domains on the fusogenic abilities of human and murine leukemia retrovirus envelopes. J. Virol. **69:**4149–4157.
10. **Deng, R., Z. Wang, A. M. Mirza, and R. M. Iorio.** 1995. Localization of a domain on the paramyxovirus attachment protein required for the promotion of cellular fusion by its homologous fusion protein spike. Virology **209:**457–469.
11. **Derdeyn, C. A., J. M. Decker, J. N. Sfakianos, X. Wu, W. A. O'Brien, L. Ratner, J. C. Kappes, G. M. Shaw, and E. Hunter.** 2000. Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. J. Virol. **74:**8358–8367.
12. **Dong, J. Y., J. W. Dubay, L. G. Perez, and E. Hunter.** 1992. Mutations within the proteolytic cleavage site of the Rous sarcoma virus glycoprotein define a requirement for dibasic residues for intracellular cleavage. J. Virol. **66:**865–874.
13. **Eckert, D. M., and P. S. Kim.** 2001. Mechanisms of viral membrane fusion and its inhibition. Annu. Rev. Biochem. **70:**777–810.
14. **Edwards, T. G., S. Wyss, J. D. Reeves, S. Zolla-Pazner, J. A. Hoxie, R. W. Doms, and F. Baribaud.** 2002. Truncation of the cytoplasmic domain induces exposure of conserved regions in the ectodomain of human immunodeficiency virus type 1 envelope protein. J. Virol. **76:**2683–2691.
15. **Egan, M. A., L. M. Carruth, J. F. Rowell, X. Yu, and R. F. Siliciano.** 1996. Human immunodeficiency virus type 1 envelope protein endocytosis mediated by a highly conserved intrinsic internalization signal in the cytoplasmic domain of gp41 is suppressed in the presence of the Pr55gag precursor protein. J. Virol. **70:**6547–6556.
16. **Evans, L. H., R. P. Morrison, F. G. Malik, J. Portis, and W. J. Britt.** 1990. A neutralizable epitope common to the envelope glycoproteins of ecotropic, polytropic, xenotropic, and amphotropic murine leukemia viruses. J. Virol. **64:**6176–6183.
17. **Fass, D., S. C. Harrison, and P. S. Kim.** 1996. Retrovirus envelope domain at 1.7A resolution. Nature Struct. Biol. **5:**465–469.
18. **Freed, E. O., and M. A. Martin.** 1996. Domains of the human immunodeficiency virus type 1 matrix and gp41 cytoplasmic tail required for envelope incorporation into virions. J. Virol. **70:**341–351.
19. **Freed, E. O., D. J. Myers, and R. Risser.** 1989. Mutational analysis of the cleavage sequence of the human immunodeficiency virus type 1 envelope glycoprotein precursor gp160. J. Virol. **63:**4670–4675.
20. **Gilbert, J. M., L. D. Hernandez, J. W. Balliet, P. Bates, and J. M. White.** 1995. Receptor-induced conformational changes in the subgroup A avian leukosis and sarcoma virus envelope glycoprotein. J. Virol. **69:**7410–7415.
21. **Grange, M.-P., V. Blot, L. Delamarre, I. Bouchaert, A. Rocca, A. Dautry-Varsat, and M.-C. Dokhelar.** 2000. Identification of two intracellular mechanisms leading to reduced expression of oncoretrovirus envelope glycoproteins at the cell surface. J. Virol. **74:**11734–11743.
22. **Green, N., T. M. Shinnick, O. Witte, S. Ponticelli, J. G. Sutcliffe, and R. A. Lerner.** 1981. Sequence-specific antibodies show that maturation of Moloney leukemia virus envelope polyprotein involves removal of a COOH-terminal peptide. Proc. Natl. Acad. Sci. USA **78:**6023–6027.
23. **Han, J.-Y., P. M. Cannon, K.-M. Lai, Y. Zhao, M. V. Eiden, and W. F. Anderson.** 1997. Identification of envelope protein residues required for the expanded host range of 10A1 murine leukemia virus. J. Virol. **71:**8103–8108.
24. **Han, J.-Y., Y. Zhao, W. F. Anderson, and P. M. Cannon.** 1998. Role of variable regions A and B in receptor binding domain of amphotropic murine leukemia virus envelope protein. J. Virol. **72:**9101–9108.
25. **Hansen, S. G., D. W. Grosenbach, and D. E. Hruby.** 1999. Analysis of the site occupancy constraints of primary amino acid sequences in the motif directing palmitylation of the vaccinia virus 37-kDa envelope protein. Virology **254:**124–137.
26. **Heminway, B. R., Y. Yu, and M. S. Galinski.** 1994. Paramyxovirus mediated cell fusion requires co-expression of both the fusion and hemagglutinin-neuraminidase glycoproteins. Virus Res. **31:**1–16.
27. **Henderson, L. E., R. Sowder, T. D. Copeland, G. Smythers, and S. Oroszlan.** 1984. Quantitative separation of murine leukemia virus proteins by reversed-phase high-pressure liquid chromatography reveals newly described Gag and Env cleavage products. J. Virol. **52:**492–500.
28. **Ikeda, H., K. Kato, T. Suzuki, H. Kitani, Y. Matsubara, S. Takase-Yoden, R. Watanabe, M. Kitagawa, and S. Aizawa.** 2000. Properties of the naturally occurring soluble surface glycoprotein of ecotropic murine leukemia virus: binding specificity and possible conformational change after binding to receptor. J. Virol. **74:**1815–1826.
29. **Januszeski, M. M., P. M. Cannon, D. Chen, Y. Rozenberg, and W. F. Anderson.** 1997. Functional analysis of the cytoplasmic tail of Moloney murine leukemia virus envelope protein. J. Virol. **71:**3613–3619.
30. **Klenk, H. D., R. Rott, M. Orlich, and J. Blodorn.** 1975. Activation of influenza A viruses by trypsin treatment. Virology **68:**426–439.
31. **Kodama, T., D. P. W. Burns, H. W. Kestler III, M. D. Daniel, and R. C. Desrosiers.** 1990. Molecular changes associated with replication of simian immunodeficiency virus in human cells. J. Med. Primatol. **19:**431–437.
32. **Kodama, T., D. P. Wooley, Y. M. Naidu, H. W. Kestler III, M. D. Daniel, Y. Li, and R. C. Desrosiers.** 1989. Significance of premature stop codons in *env* of simian immunodeficiency virus. J. Virol. **63:**4709–4714.
33. **Kwong, P. D., R. Wyatt, J. Robinson, R. W. Sweet, J. Sodroski, and W. A. Hendrickson.** 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature **393:**648–659.
34. **LaBranche, C. C., M. M. Sauter, B. S. Haggarty, P. J. Vance, J. Romano, T. K. Hart, P. J. Bugelski, M. Marsh, and J. A. Hoxie.** 1995. A single amino acid change in the cytoplasmic domain of the simian immunodeficiency virus transmembrane molecule increases envelope glycoprotein expression on infected cells. J. Virol. **69:**5217–5227.
35. **Lazarowitz, S. G., and P. W. Choppin.** 1975. Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. Virology **68:**440–454.
36. **Li, M., C. Yang, and R. W. Compans.** 2001. Mutations in the cytoplasmic tail of murine leukemia virus envelope protein suppress fusion inhibition by R peptide. J. Virol. **75:**2337–2344.
37. **Lineberger, J. E., R. Danzeisen, D. J. Hazuda, A. J. Simon, and M. D. Miller.** 2002. Altering expression levels of human immunodeficiency virus type 1 gp120-gp41 affects efficiency but not kinetics of cell-cell fusion. J. Virol. **76:**3522–3533.
38. **Lodge, R., L. Delamarre, J.-P. Lalonde, J. Alvarado, D. A. Sanders, M.-C. Dokhelar, E. A. Cohen, and G. Lemay.** 1997. Two distinct oncornaviruses harbor an intracytoplasmic tyrosine-based basolateral targeting signal in their viral envelope glycoprotein. J. Virol. **71:**5696–5702.
39. **Mackrell, J. A., N. W. Soong, M. C. Curtis, and W. F. Anderson.** 1996. Identification of a subdomain in the Moloney murine leukemia virus envelope protein involved in receptor binding. J. Virol. **70:**1768–1774.
40. **McClure, M. O., M. A. Sommerfelt, M. Marsh, and R. A. Weiss.** 1990. The pH independence of mammalian retrovirus infection. J. Gen. Virol. **71:**767–773.
41. **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.
42. **Melikyan, G., R. M. Markosyan, S. A. Brener, Y. Rozenberg, and F. S. Cohen.** 2000. Role of the cytoplasmic tail of ecotropic Moloney murine leukemia virus Env protein in fusion pore formation. J. Virol. **74:**447–455.
43. **Moore, J. P., J. A. McKeating, R. A. Weiss, and Q. J. Sattentau.** 1990. Dissociation of gp120 from HIV-1 virions induced by soluble CD4. Science **250:**1139–1142.
44. **Mothes, W., A. L. Boerger, S. Narayan, J. M. Cunningham, and J. A. T. Young.** 2000. Retroviral entry mediated by receptor priming and low pH triggering of an envelope glycoprotein. Cell **103:**679–689.
45. **Mulligan, M. J., G. V. Yamshchikov, G. D. Ritter, Jr., F. Gao, M. J. Jin, C. D. Nail, C. P. Spies, B. H. Hahn, and R. W. Compans.** 1992. Cytoplasmic domain truncation enhances fusion activity by the exterior glycoprotein complex of human immunodeficiency virus type 2 in selected cell types. J. Virol. **66:**3971–3975.
46. **Ochsenbauer, C., S. R. Dubay, and E. Hunter.** 2000. The Rous sarcoma virus Env glycoprotein contains a highly conserved motif homologous to tyrosine-based endocytosis signals and displays an unusual internalization phenotype. Mol. Cell. Biol. **20:**249–260.
47. **Olsen, K. E. P., and K. B. Andersen.** 1999. Palmitoylation of the intracytoplasmic R peptide of the transmembrane envelope protein in Moloney murine leukemia virus. J. Virol. **73**: 8975–8981.
48. **Opstelten, D.-J. E., M. Wallin, and H. Garoff.** 1998. Moloney murine leukemia virus envelope subunits, gp70 and Pr15E, form a stable disulfide-linked complex. J. Virol. **72:**6537–6545.
49. **Pinter, A., and W. J. Honnen.** 1983. Topography of murine leukemia virus envelope proteins: characterization of transmembrane components. J. Virol. **46:**1056–1060.
50. **Pinter, A., W. J. Honnen, J.-S. Tung, P. V. O'Donnell, and U. Hammerling.** 1982. Structural domains of endogenous murine leukemia virus gp70s containing specific antigenic determinants defined by monoclonal antibodies. Virology **116:**499–516.
51. **Pinter, A., R. Kopelman, Z. Li, S. C. Kayman, and D. A. Sanders.** 1997. Localization of the labile disulfide bond between SU and TM of the murine leukemia virus envelope protein complex to a highly conserved CWLC motif in SU that resembles the active-site sequence of thiol-disulfide exchange enzymes. J. Virol. **71:**8073–8077.
52. **Ragheb, J. A., and W. F. Anderson.** 1994. Uncoupled expression of Moloney murine leukemia virus envelope polypeptides SU and TM: a functional analysis of the role of TM domains in viral entry. J. Virol. **68:**3207–3219.
53. **Ragheb, J. A., and W. F. Anderson.** 1994. pH-independent murine leukemia

virus ecotropic envelope-mediated cell fusion: implications for the role of the R peptide and p12E TM in viral entry. J. Virol. **68:**3220–3231.
54. **Rein, A., J. Mirro, J. G. Haynes, S. M. Ernst, and K. Nagashima.** 1994. Function of the cytoplasmic domain of retroviral transmembrane protein: p15E-p2E cleavage activates the membrane fusion capability of the murine leukemia virus Env protein. J. Virol. **68:**1773–1781.
55. **Rein, A., C. Yang, J. A. Haynes, J. Mirro, and R. W. Compans.** 1998. Evidence for cooperation between murine leukemia virus Env molecules in mixed oligomers. J. Virol. **72:**3432–3435.
56. **Rice, N. R., L. E. Henderson, R. C. Sowder, T. D. Copeland, S. Oroszlan, and J. F. Edwards.** 1990. Synthesis and processing of the transmembrane envelope protein of equine infectious anemia virus. J. Virol. **64:**3770–3778.
57. **Samson, A. C., and C. F. Fox.** 1973. Precursor protein for Newcastle disease virus. J. Virol. **12:**579–587.
58. **Sattentau, Q. J., and J. P. Moore.** 1991. Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. J. Exp. Med. **174:**407–415.
59. **Shimizu, H., F. Hasebe, H. Tsuchie, S. Morikawa, H. Ushijima, and T. Kitamura.** 1992. Analysis of a human immunodeficiency virus type 1 isolate carrying a truncated transmembrane glycoprotein. Virology **189:**534–546.
60. **Shultz, A., and A. Rein.** 1985. Maturation of murine leukemia virus env proteins in the absence of other viral proteins. Virology **145:**335–339.
61. **Skehel, J. J., and D. C. Wiley.** 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu. Rev. Biochem. **69:**531–569.
62. **Soneoka, Y., P. M. Cannon, E. E. Ramsdale, J. C. Griffiths, G. Romano, S. M. Kingsman, and A. J. Kingsman.** 1995. A transient three-plasmid expression system for the production of high titre retroviral vectors. Nucleic Acids Res. **23:**628–633.
63. **Spies, C. P., and R. W. Compans.** 1994. Effects of cytoplasmic domain length on cell surface expression and syncytium-forming capacity of the simian immunodeficiency virus envelope glycoprotein. Virology **203:**8–19.
64. **Spies, C. P., D. G. Ritter, M. J. Mulligan, and R. W. Compans.** 1994. Truncation of the cytoplasmic domain of the simian immunodeficiency virus envelope glycoprotein alters the conformation of the external domain. J. Virol. **68:**585–591.
65. **Taylor, G. M., and D. A. Sanders.** 1999. The role of the membrane-spanning domain sequence in glycoprotein-mediated membrane fusion. Mol. Biol. Cell **10:**2803–2815.
66. **Thomas, A., K. D. Gray, and M. J. Roth.** 1997. Analysis of mutations within the cytoplasmic domain of the Moloney murine leukemia virus transmembrane protein. Virology **227:**305–313.
67. **West, J. T., S. K. Weldon, S. Wyss, X. Lin, Q. Yu, M. Thali, and E. Hunter.** 2002. Mutations of the dominant endocytosis motif in human immunodeficiency virus type 1 gp41 can complement matrix mutations without increasing Env incorporation. J. Virol. **76:**3338–3349.
68. **Wilk, T., T. Pfeiffer, and V. Bosch.** 1992. Retained *in vitro* infectivity and cytopathogenicity of HIV-1 despite truncation of the C-terminal tail of the env gene product. Virology **189:**167–177.
69. **Wool-Lewis, R. J., and P. Bates.** 1999. Endoproteolytic processing of the Ebola virus envelope glycoprotein: cleavage is not required for function. J. Virol. **73:**1419–1426.
70. **Wu, B. W., P. M. Cannon, E. Gordon, F. Hall, and W. F. Anderson.** 1998. Characterization of the proline-rich region of murine leukemia virus envelope protein. J. Virol. **72:**5383–5391.
71. **Wu, L., N. P. Gerard, R. Wyatt, H. Choe, C. Parolin, N. Ruffing, A. Borsetti, A. A. Cardoso, E. Desjardin, W. Newman, C. Gerard, and J. Sodroski.** 1996. CD4-induced interaction of primary HIV-1 gp120 with the chemokine receptor CCR-5. Nature **384:**179–183.
72. **Yang, C., and R. W. Compans.** 1996. Analysis of the cell fusion activities of chimeric simian immunodeficiency virus-murine leukemia virus envelope proteins: inhibitory effect of the R peptide. J. Virol. **70:**248–254.
73. **Yang, C., and R. W. Compans.** 1996. Palmitoylation of the murine leukemia virus envelope glycoprotein transmembrane subunits. Virology **221:**87–97.
74. **Yang, C., and R. W. Compans.** 1997. Analysis of the murine leukemia virus R peptide: delineation of the molecular determinants which are important for its fusion inhibition activity. J. Virol. **71:**8490–8496.
75. **Yang, C., Q. Yang, and R. W. Compans.** 2000. Coreceptor-dependent inhibition of the cell fusion activity of simian immunodeficiency virus Env proteins. J. Virol. **74:**6217–6222.
76. **Yu, H., C. Empig, J. Xia, and W. F. Anderson.** 1998. Quantitation of MoMuLV envelope protein on the cell surface. Virology **243:**415–422.
77. **Yu, H., N. Soong, and W. F. Anderson.** 1995. Binding kinetics of ecotropic (Moloney) murine leukemia retrovirus with NIH 3T3 cells. J. Virol. **69:**6557–6562.
78. **Zhao, Y., S. Lee, and W. F. Anderson.** 1997. Functional interactions between monomers of the retroviral envelope protein complex. J. Virol. **71:**6967–6972.
79. **Zhao, Y., L. Zhu, C. A. Benedict, D. Chen, W. F. Anderson, and P. M. Cannon.** 1998. Functional domains in the retroviral transmembrane protein. J. Virol. **72:**5392–5398.
80. **Zingler, K., and D. Littman.** 1993. Truncation of the cytoplasmic domain of the simian immunodeficiency virus envelope glycoprotein increases Env incorporation into particles and fusogenicity and infectivity. J. Virol. **67:**2824–2831.