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Analysis of the Murine Leukemia Virus R Peptide: Delineation of the Molecular Determinants Which Are Important for Its Fusion Inhibition Activity

CHINGLAI YANG AND RICHARD W. COMPANS*

Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322

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In previous studies, the C-terminal R peptide of the murine leukemia virus (MuLV) Env protein was shown to be a potent inhibitor of viral fusion activity. In the present study, we investigated the molecular determinants in the MuLV Env protein cytoplasmic tail which are important for the fusion inhibition activity of the R peptide. We constructed a series of mutant MuLV *env* genes which express Env proteins with serial truncations, internal deletions, or amino acid substitutions in the cytoplasmic tail. To analyze their cell fusion activity, we employed a quantitative fusion assay. We found that truncations of up to 7 amino acids from the C terminus of the cytoplasmic tail had no detectable effect on the lack of fusion activity of the full-length Env protein; however, further truncations resulted in a progressive increase in cell fusion activity. Studies of mutant proteins with amino acid substitutions in the cytoplasmic tail showed that Leu-627 plays an important role in fusion inhibition by the R peptide, while most of the other amino acids in the R peptide were not essential for fusion inhibition. Studies of mutant proteins with internal deletions upstream of the cleavage site in the cytoplasmic tail showed that this region is also involved in fusion inhibition by the R peptide, although only to a limited extent. The results are consistent with a model in which the MuLV R peptide exhibits its fusion inhibition activity through interaction with a cellular factor(s).

Enveloped viruses enter the cells through a membrane fusion process, in which the envelope glycoproteins play a critical role. Fusion induced by viral glycoproteins can be generally categorized into two types. One group of envelope glycoproteins induces membrane fusion only under low pH conditions and is represented by the envelope proteins of influenza virus (hemagglutinin [HA]) and vesicular stomatitis virus (G protein). Viruses of this type enter the cell by endocytosis, and subsequent fusion with endosome membranes is mediated by the envelope proteins (22, 38). A second group of envelope proteins can initiate membrane fusion under neutral pH conditions, and members of this group include the envelope glycoproteins of parainfluenza viruses and most retroviruses (21, 23). Viruses of this group enter the cell by direct fusion with the plasma membrane.

The envelope (Env) glycoproteins of retroviruses are synthesized as precursor proteins which are glycosylated and cleaved into surface (SU) and transmembrane (TM) subunits by a cellular protease (14, 18, 33, 40). The SU protein mediates binding to receptors and determines the host range of virus infection (3, 24, 27). The TM protein has three distinct domains: an extracellular domain, which interacts with the SU protein and which also contains a stretch of about 20 hydrophobic amino acids at its N terminus, designated the fusion peptide (11); a membrane-spanning domain, which anchors the Env protein in the cell or viral membrane; and the cytoplasmic tail.

Studies on the mechanism of the membrane fusion process induced by viral envelope proteins have been largely focused on the involvement of their ectodomains, while a possible role of the cytoplasmic tail in their fusion activities was not expected. However, several studies with retrovirus Env proteins have indicated that the cytoplasmic tails may also function to modulate fusion activity. Studies with simian immunodeficiency virus (SIV) have shown that, after passage in human cell lines, a virus containing a truncated TM protein is selected (7, 17). Further studies showed that the truncation of most of the Env protein cytoplasmic tail of SIVmac239 greatly increases its fusion activity (31, 43) and also induces a conformational change in its extracellular domain as detected by surface biotinylation (35). In murine leukemia virus (MuLV) and Mason-Pfizer monkey virus (MPMV), the TM protein cytoplasmic tail is further processed by the viral protease after virus particle assembly (12, 34). In MuLV, this processing of the TM protein produces the mature TM protein p12E and releases a fragment of 16 C-terminal amino acids, which has been designated as the R peptide (12, 15). Recent studies have shown that the processing of the cytoplasmic tail of the Env glycoproteins of MPMV and MuLV is important for their fusion activities. It has been shown that cleavage of the MuLV R peptide is critical for the activation of the cell fusion activity of the Env protein (28–30), and the fusion activity of the MPMV Env protein is also greatly increased by the cleavage of its cytoplasmic tail (4).

In recent studies, we have shown that the cytoplasmic tail of the MuLV Env protein also exerts a profound inhibitory effect on the fusion activity of SIV-MuLV chimeric Env proteins (41). We further showed that the inhibition of fusion is due to the presence of the MuLV R peptide. In the present study, we have delineated the molecular determinants of the MuLV R peptide which are important for its fusion inhibition activity by using mutant Env glycoproteins with serial truncations or amino acid substitutions in the cytoplasmic tail. We have determined that amino acids close to the R peptide cleavage site play a critical role in the fusion inhibition activity of the R peptide.

MATERIALS AND METHODS

Cells and viruses. HeLa T4 cells and XC cells were obtained from the American Type Culture Collection, Rockville, Md. They were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (GIBCO BRL). A recombinant vaccinia virus (vTF7-3) expressing the T7 polymerase was provided by B. Moss.

* Corresponding author.

Plasmid construction and mutagenesis. All restriction endonucleases and DNA modification enzymes used for plasmid construction were purchased from Boehringer Mannheim Biochemicals. Construction of plasmids MuL Venv and MuL VenvR$^-$ has been described elsewhere (41). The genes encoding mutant MuLV Env proteins with serial truncations in the cytoplasmic tail region were constructed as follows. A portion of the Friend MuLV *env* gene was amplified by PCR. The sequence of the 5′ primer was 5′(6801)-AAAACTCAAGAGTG CTGG-3′, while the sequences for the 3′ primers were 5′(7807)-TATCATGGCT CTTATTCTAGTGA-3′ (for Menv638Tr), 5′(7793)-TTCTAGTGGTTATA GCTGGTGGT-3′ (for Menv634Tr), 5′(7783)-TTTAGCTGGTGTTATTGT TGAGT-3′ (for Menv630Tr), and 5′(7715)-GTGGTATTGTTAAGTCAGG ACTA-3′ (for Menv628Tr). The PCR fragments were then cloned into plasmid pSP72 at the *Pvu*II site, and then the coding region for the extracellular domain and the TM domain of the MuLV Env protein was inserted at the *Eco*RV site of the plasmid vector pSP72 and the *Cla*I site in the *env* gene under the control of the T7 promoter. The resulting constructs, designated plasmids Menv638Tr, Menv634Tr, Menv630Tr, and Menv628Tr, express mutant MuLV Env proteins with truncations after amino acids 638, 634, 630, and 628, respectively.

The Menv634Tr gene was subjected to further site-directed PCR mutagenesis. The resulting constructs encode mutant MuLV Env proteins which are designated Menv634Tr/L627P, Menv634Tr/L627A, Menv634Tr/T628A, Menv634Tr/Q629,630A, Menv634Tr/Q633A, Menv634Tr/H632A, Menv634Tr/L634H, and Menv634Tr/H632A,L634H. The primers used for these constructs were 5′(7788)-GTGGTTATAGCTGGTGGTATTGTTGAGTCGCCACTAAA GCG-3′, 5′(7788)-GTGGTTATAGCTGGTGGTATTGTTGAGTCGGGACT AAAGC-3′, 5′(7788)-GTGGTTATAGCTGGTGGTATTGTTGAGCCAGGA C-3′, 5′(7788)-GTGGTTATAGCTGGTGGTATGCTGCAGTCAG-3′, 5′ (7788)-GTGGTTATAGCGCGTGGTATTG-3′, 5′(7786)-GGTTATAGCTGG GCGTATTGTTGAG-3′, 5′(7786)-GGTTATAGCTGGTGGTATTGTTGAG-3′, and 5′(7786)-GGTTAATGCTGGGCGTATTGTTGAG-3′, respectively. Mutant MuLV *env* genes with mutation of the codon for Leu-627 to codons for proline or alanine were constructed by site-directed mutagenesis using single-stranded DNA as described previously (41). Briefly, the *Pst*I fragment of the Friend MuLV *env* gene was cloned into plasmid M13RFmp18, and single-stranded DNA was generated and used for mutagenesis. The primers used were 5′(7779)-GCTGGTGGTATTGTTGAGTCGCCACTAAA-3′ and 5′(7779)-GCTGGTGGTATTGTTGAGTCGGGACTAAA-3′ for MenvL627A and MenvL627P, respectively. After mutagenesis, the *Pst*I fragment was cloned back to the MuLV *env* gene plasmid, and the resulting plasmids were designated MenvL627A and MenvL627P, respectively.

Mutant MuLV *env* genes with internal deletions were constructed by the following procedure. The coding region for the cytoplasmic tail of the MuLV Env protein was amplified by PCR, blunt ended by Klenow fill-in reaction, and then linked to the MuLV *env* gene construct which had been digested with *Cla*I and blunt ended by Klenow fill-in reaction. The resulting constructs, encoding mutant MuLV Env proteins with deletions from amino acid 609 to 614 and from amino acid 609 to 625, were designated Menv/d609-614 and Menv/d609-625, respectively, and the primers used for these constructs were 5′(7708)-GATT AGTTCAATTGTTTAAAGACAGGA-3′ and 5′(7743)-GTCCAGGCTTTAA ACCTGACTCAACAA-3′, respectively. By site-directed PCR mutagenesis using primer 5′(7755)-CTAAAGCCTAGACTACTGAG, a stop codon was introduced into plasmid Menv/d609-614 to generate plasmid MenvR$^-$/d609-614, which encodes a mutant MuLV Env protein with deletion of amino acids 609 to 614 and truncation of the R peptide. All mutations were confirmed by DNA sequencing (32).

Plasmid G4-βGal, which encodes the β-galactosidase gene under the control of the T7 promoter, was constructed by excising the *Xho*I fragment of plasmid pSV-βGal (which contains the β-galactosidase gene) and inserting it into plasmid pGEM4.

Protein expression, metabolic labeling, and immunoprecipitation. Protein expression was carried out with the vaccinia virus T7 system (10). HeLa T4 cells were seeded in 35-mm dishes. Twenty-four hours later, they were infected with vTF7-3, a recombinant vaccinia virus expressing T7 polymerase, at a multiplicity of infection of 10 for 90 min. After infection, the cells were washed once with DMEM, and then transfected by using Lipofectin (GIBCO-BRL) in DMEM by plasmid DNA constructs described in the figure legends. At 12 h after infection and transfection, the cells were labeled with 100 μCi of [^{35}S]Met-Cys in 600 μl of Eagle's medium deficient in methionine and cysteine for 45 min, then washed once with DMEM plus 10% bovine calf serum, and then chased in DMEM plus 10% bovine calf serum. After 4 h of chase, cells were washed once with DMEM and then lysed with lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate [pH 7.5]) and immunoprecipitated with appropriate antibodies and protein A-agarose (Pierce) at 4°C overnight. Samples were prepared with 2× sample buffer (125 mM Tris-HCl [pH 7.5], 4% sodium dodecyl sulfate [SDS], and 20% glycerol, plus 10% β-mercaptoethanol for 2× reducing sample buffer) and heated at 95°C for 5 min before they were loaded onto gels for SDS-polyacrylamide gel electrophoresis (PAGE) (20).

Biotinylation of the surface proteins. Surface expression of each construct was assayed by a surface biotinylation assay (35). HeLa T4 cells were seeded, infected, and transfected as described above. At 12 h postinfection, cells were starved with Eagle's medium deficient in methionine and cysteine for 45 min and then pulse-labeled with 150 μCi of [^{35}S]methionine and cysteine (Du Pont, NEN) in 800 μl of methionine- and cysteine-deficient DMEM for 45 min. After labeling, cells were washed once with DMEM plus 10% bovine calf serum and then chased in DMEM plus 10% bovine calf serum. After 4 h of chase, the cells were washed three times with phosphate-buffered saline (PBS), biotinylated at 4°C for 30 min with 0.5 mg of NHS-SS-Biotin (Pierce)/ml in PBS, lysed with lysis buffer, and then immunoprecipitated with appropriate antibodies and protein A-agarose at 4°C overnight. Samples were washed twice in lysis buffer plus 0.4% SDS and then split into two equal portions. One portion was mixed with 15 μl of reducing sample buffer and used as the control for total cellular expression. The other portion was mixed with 20 μl of 10% SDS and heated at 95°C for 15 min in order to dissociate the antigen–antibody–protein A-agarose complex. Dissociated proteins were then dissolved in 700 μl of lysis buffer and incubated with 10 μl of streptavidin-agarose (Pierce) for 5 h at 4°C. Biotinylated samples were washed three times with lysis buffer, mixed with 15 μl of reducing sample buffer, and heated at 95°C for 5 min before they were loaded onto SDS-PAGE gels.

Fusion assay of wild-type and mutant MuLV Env proteins. The fusion activities of MuLV *env* constructs were determined by the following procedure. HeLa T4 cells, which were seeded into 24-well plates the previous day and grown to 80% confluence, were infected with vTF7-3 and transfected with plasmid constructs containing wild-type or mutant MuLV *env* genes by using Lipofectin (GIBCO BRL) as described above for protein expression. At 12 h postinfection, they were overlaid with XC cells, a transformed rat cell line which is sensitive to fusion by the MuLV Env proteins. Cell fusion was monitored 4 h later, and cells were photographed with a phase-contrast microscope.

Quantitation of the fusion activities of the wild-type and mutant MuLV Env proteins was performed according to the following procedure developed by Nussbaum et al. (26). Briefly, HeLa T4 cells seeded into 24-well plates and grown to 80% confluence were infected with vTF7-3 and transfected with plasmid constructs containing genes encoding the wild-type or mutant MuLV Env proteins by using Lipofectin (GIBCO BRL) as described above for protein expression. At the same time, XC cells grown to confluence were transfected with plasmid G4-βGal by using Lipofectin. At 12 h postinfection, transfected XC cells were detached from plates by treatment with 2 mM EDTA (in Ca^{2+}- and Mg^{2+}-deficient PBS) and suspended in DMEM with 10% bovine calf serum. They were then overlaid onto the infected HeLa T4 cells in the 24-well plate. Cell fusion was monitored and quantitated by assay for β-galactosidase activity using colorimetry.

RESULTS

The effect of serial truncations in the cytoplasmic tail on the fusion activity of the MuLV Env protein. As has been shown in previous studies, truncation of the R peptide of the MuLV Env protein greatly increased its cell fusion activity (28–30, 41). To further define the molecular determinants in the R peptide of the MuLV Env protein which are important for its fusion inhibition activity, we first generated mutant MuLV Env proteins with serial truncations in the R peptide. The amino acid sequences of the truncated MuLV Env proteins are shown schematically in Fig. 1. As shown in Fig. 2, these Env proteins were found to be expressed and transported to the cell surface at levels similar to those of the wild-type Env proteins. Truncations in the cytoplasmic tail resulted in faster migration of the TM proteins in SDS-PAGE; however, the processing of the Env proteins was not affected. To determine the effect of truncations in the R peptide region on its fusion inhibition activity, we analyzed the cell fusion activities of these mutant proteins. As shown in Fig. 3, quantitation of the fusion activities of these proteins revealed that Menv628Tr and Menv630Tr exhibited about 75 and 50%, respectively, of the fusion activity of MenvR$^-$, in which the R peptide is completely removed. Menv634Tr and Menv638Tr, as well as the full-length wild-type Env protein, Menv, did not induce any significant fusion above the background level. These results indicate that successive truncations of the R peptide result in a progressive increase of the fusion activity of the Env protein.

Mutations of Gln-629, Gln-630, Gln-633, His-632, or Leu-634 in the R peptide did not affect its fusion inhibition activity. The results of the above studies also showed that the C-terminal 7 amino acids are not required for the fusion inhibition by the MuLV cytoplasmic tail, indicating that the molecular determinant in the R peptide which is important for its fusion inhibition activity probably resides in the region from amino acid 626 to 634. Based on these results, we generated site-directed mutants with amino acid substitutions in the cytoplas-

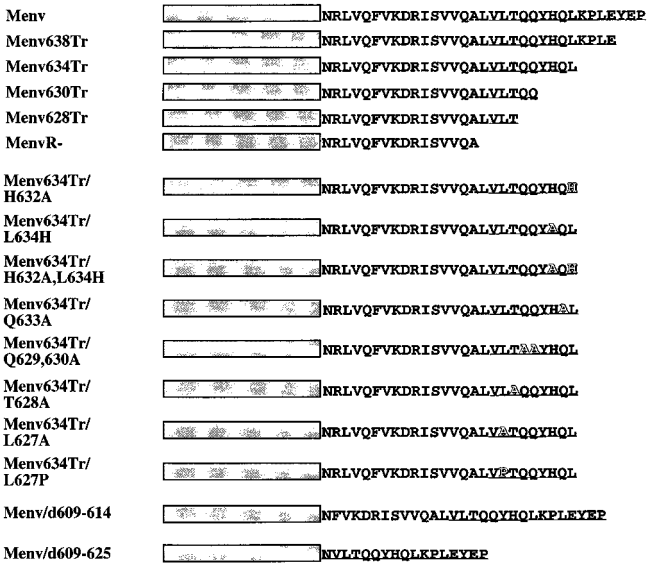

FIG. 1. Schematic diagram of mutant and wild-type Env proteins. Construction of genes encoding these mutant Env proteins is described in Materials and Methods. The amino acid sequence of the cytoplasmic tail is shown. The designation of each Env protein is given on the left. Amino acids belonging to the R peptide are underlined. Mutated amino acids are shown with shadowed letters.

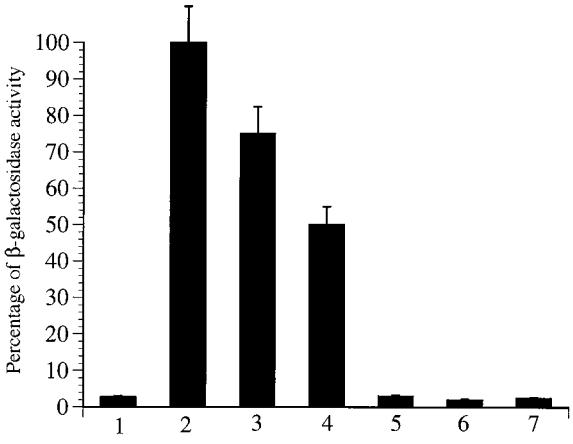

FIG. 3. Fusion of XC cells by MuLV Env proteins. Cell fusion by mutant Env proteins with truncations in the R peptide was analyzed as described in Materials and Methods. Briefly, HeLa T4 cells were infected with vTF7-3 and transfected as described for protein expression. At 12 h posttransfection, they were overlaid with XC cells. The β-galactosidase activity produced by each mutant Env protein was then converted into the percentage of that produced by MenvR$^-$ (which is taken to be 100%). Error bars, standard deviations. Bars: 1, control; 2, MenvR$^-$; 3, Menv628Tr; 4, Menv630Tr; 5, Menv634Tr; 6, Menv638Tr; 7, Menv.

mic tail of the Menv634Tr mutant, which retained full fusion inhibition activity.

Previous studies with MPMV have shown that the processing of the cytoplasmic tail of the MPMV Env protein by the viral protease also enhances its fusion activity (4). A sequence alignment of the cytoplasmic tails of the Env proteins of different strains of MuLV and MPMV is shown in Fig. 4. His-632 and Leu-634 of the MuLV Env protein are conserved at similar positions in the MPMV Env protein. As shown above, truncation at amino acid 630 resulted in a drastic increase in the fusion activity of the MuLV Env protein. To determine whether these histidine or leucine residues constitute an essential feature for the fusion inhibition activity, we constructed mutant MuLV *env* genes in which the codon for His-632 was changed into that for Ala (Menv634Tr/H632A), the codon for Leu-634 was changed into that for His (Menv634Tr/L634H), or both (Menv634Tr/H632A,L634H). Glutamine 633 was also mutated into an alanine residue (Menv634Tr/Q633A). As shown by the results with the truncation mutants, further truncation of the two glutamine residues (Gln-629 and Gln-630) resulted in a higher fusion activity (comparing Menv628Tr to Menv630Tr). Interestingly, the natural truncation site in the cytoplasmic tail of the SIVmac239 Env protein is also located between two consecutive glutamine residues. To investigate whether these glutamine residues may also be involved in the fusion inhibition activity of the R peptide, we also generated mutant MuLV Env proteins in which Gln-629 and Gln-630 were replaced with alanine residues. The amino acid sequences of the cytoplasmic tails of these mutant Env proteins are shown schematically in Fig. 1.

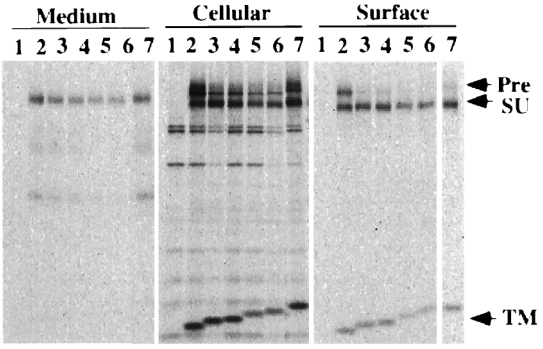

FIG. 2. Expression of wild-type and truncated MuLV Env proteins. HeLa T4 cells were infected with vTF7-3 and then transfected with plasmids containing genes encoding wild-type or truncated MuLV Env protein. At 12 h postinfection, cells were labeled with [^{35}S]Met-Cys as described in Materials and Methods. After labeling, cells were biotinylated and then immunoprecipitated with antibodies against MuLV plus protein A-agarose beads at 4°C overnight. The samples were prepared with reducing sample buffer, heated at 95°C for 5 min, then loaded onto an SDS-PAGE gel. Lanes: 1, control; 2, MenvR$^-$; 3, Menv628Tr; 4, Menv630Tr; 5, Menv634Tr; 6, Menv638Tr; 7, Menv.

The expression, processing, and cell surface expression levels of the mutant Env proteins were found to be similar to those of the wild-type (Menv) and the R-peptide-truncated (MenvR$^-$) Env proteins (Fig. 5). When analyzed for cell fusion activities, these mutant Env proteins did not induce any syncytium formation above the background level. Results from the quantitative assay showed that these constructs exhibited very little or no fusion activity, similar to the full-length wild-type Env protein (Fig. 6). These results, together with results of cell fusion by MuLV Env proteins with serial truncations or internal deletions in the cytoplasmic tail, indicated that although a certain length of flanking amino acids is necessary for fusion inhibition, there is no specific amino acid requirement in this segment (from amino acid 629 to 634) for the fusion inhibition activity of the R peptide.

The effect of the amino acids in the MuLV cytoplasmic tail upstream of the R-peptide cleavage site on fusion inhibition activity. To determine whether the amino acid sequences in the MuLV Env protein cytoplasmic tail upstream of the R peptide may also be involved in the fusion inhibition by the MuLV R peptide, we constructed mutant MuLV Env proteins with internal deletions in the cytoplasmic tail. Menv/d609-614 was constructed to delete amino acids from 609 to 614 in the cytoplasmic tail, and Menv/d609-625 was constructed to delete amino acids from 609 to 625, which encompasses the entire MuLV cytoplasmic tail upstream of the R peptide. As shown in Fig. 7, the transport and

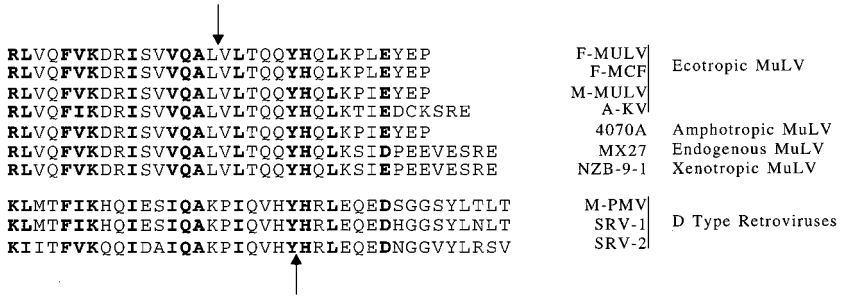

FIG. 4. Sequence comparison of the cytoplasmic tails of type C and type D retrovirus Env proteins. The amino acid sequences of the cytoplasmic tails of the Env proteins of different strains of MuLV and MPMV were aligned. The name of each virus is given on the right. Conserved amino acids which are aligned at the same positions are shown in bold. Arrows indicate the cleavage sites in the cytoplasmic tails of the MuLV and MPMV Env proteins.

processing of these mutant Env proteins, compared to those of the wild-type Env protein, were not affected by the deletions; however, their cellular and surface expression levels were substantially lower than was found with other mutant Env proteins (Fig. 7; compare lanes 6 and 7 to other lanes). When they were analyzed for their cell fusion activities, as shown by the quantitative fusion assay (Fig. 8), Menv/d609-625 exhibited about 20% of the fusion activity of MenvR$^-$ when they were expressed at a similar level (by reducing the expression level of MenvR$^-$), while Menv/d609-614 exhibited no fusion activity. These results indicate that the region upstream of the R peptide also plays a role in its fusion inhibition activity.

Leu-627 plays a critical role in the fusion inhibition activity of the MuLV R peptide. The results of the above studies showed that mutation or deletion of most of the C-terminal 13 amino acids in the R peptide does not affect its fusion inhibition activity, indicating that they are not critical for the fusion inhibition function of the R peptide. We have reported that the attachment of the MuLV R peptide to the C-terminal end of a highly fusogenic truncated SIV Env protein also greatly reduced its fusion activity (41). It has also been reported that mutation of Val-626 into an arginine residue prevented the cleavage of the R peptide and inhibited the fusion activity of the MuLV Env protein (30). We therefore generated further mutant Env proteins in which Leu-627 or Thr-628 was changed into alanine. We also generated a mutant MuLV Env protein in which Leu-627 was mutated into proline. The amino acid sequences of the cytoplasmic tails of these mutant Env proteins are shown in Fig. 1. As shown in Fig. 7, these mutant Env proteins were found to be expressed and successfully transported to the cell surface. These mutant proteins were then analyzed for their cell fusion activity. As shown in Fig. 8, mutation of Thr-628 into alanine did not affect the cell fusion activity of the Env protein. However, mutation of Leu-627 into either alanine or proline activated the cell fusion activity of the Env protein to about 60 or 80% (respectively) of that of the R-peptide-truncated Env protein, MenvR$^-$.

These results suggested that Leu-627 plays a critical role in the fusion inhibition activity of the MuLV R peptide. However, these mutants were generated on the basis of the truncation mutant Menv634Tr, which remains nonfusogenic. To investigate whether the release from the fusion inhibition activity is due to the combined effects of the mutation of Leu-627 and truncation of the C-terminal 7 amino acids in the Menv634Tr mutant, we introduced Leu-627 mutations in the context of the full-length MuLV Env protein (MenvL627A and MenvL627P). As shown in Fig. 9A, the mutant Env proteins were expressed at levels similar to those of the full-length or R-peptide-truncated MuLV envelope proteins (compare lanes 3 and 4 to lanes 2 and 6). When these mutant Env proteins were analyzed for their fusion activities, as shown in Fig. 9B, the two Leu-627 mutants exhibited about 70 to 80% of the fusion activity of MenvR$^-$, which is similar to the fusion activities of their Menv634Tr counterparts. These results

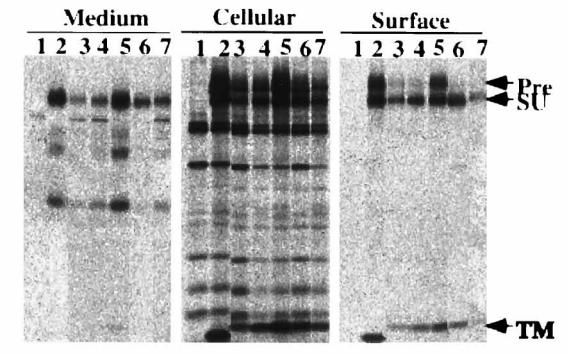

FIG. 5. Expression of mutant Env proteins with amino acid substitutions in the R peptide from amino acid 629 to 634. Protein expression was carried out as described in Materials and Methods and in the legend to Fig. 2. Lanes: 1, control; 2, MenvR$^-$; 3, Menv634Tr/H632A; 4, Menv634Tr/L634H; 5, Menv634Tr/H632A,L634H; 6, Menv634Tr/Q633A; 7, Menv634Tr/Q629,630A.

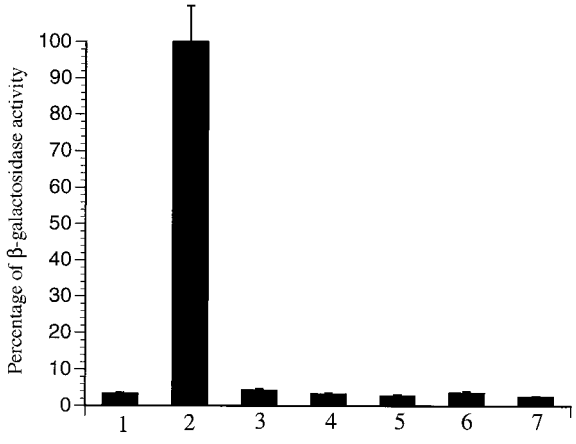

FIG. 6. Fusion activities of mutant Env proteins with amino acid substitutions in the R peptide from amino acid 629 to 634. Fusion activities of these constructs were quantitated as described in Materials and Methods. The fusion activity of each mutant was expressed as a percentage of the β-galactosidase activity produced by MenvR$^-$. Error bars, standard deviations. Bars: 1, control; 2, MenvR$^-$; 3, Menv634Tr/H632A; 4, Menv634Tr/L634H; 5, Menv634Tr/H632A,L634H; 6, Menv634Tr/Q633A; 7, Menv634Tr/Q629,630A.

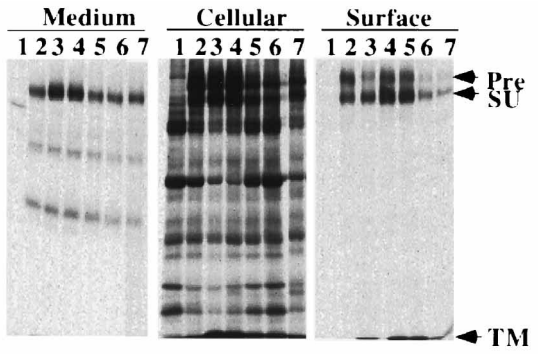

FIG. 7. Expression of mutant Env proteins with amino acid substitutions of Leu-627 and Thr-628 and internal deletions upstream of the R peptide. Protein expression was carried out as described in Materials and Methods and in the legend to Fig. 2. Lanes: 1, control; 2, MenvR$^-$; 3, Menv634Tr/L627A; 4, Menv634Tr/L627P; 5, Menv634Tr/T628A; 6, Menv634Tr/d609-614; 7, Menv634Tr/d609-625.

further confirmed that Leu-627 plays a critical role in fusion inhibition by the MuLV R peptide. To determine whether the lack of fusion activity of Menv/d609-614 is because deletion of amino acids 609 to 614 removed some molecular determinants important for the fusion activity of the MuLV envelope protein, we also constructed a mutant MuLV *env* gene which expresses an Env protein with deletion of amino acids 609 to 614 and contains a truncation of the R peptide (MenvR$^-$/d609-614). MenvR$^-$/d609-614 was expressed at a level similar to that of MenvR$^-$ (Fig. 9A, lane 5) and also exhibited a fusion activity comparable to that of MenvR$^-$ (Fig. 9B), indicating that this segment (amino acids 609 to 614) does not play a significant role in the fusion activity of the MuLV envelope protein.

DISCUSSION

The cytoplasmic tail of the MuLV Env protein is processed by the viral protease during virus assembly, releasing a fragment containing 16 C-terminal amino acids, which is designated the R peptide (12, 15). It has been reported that cleavage of the R peptide is important for the activation of the fusion activity of the Env protein, and mutant MuLV Env proteins with the R peptide truncated exhibited enhanced cell fusion activity (28–30). In our previous studies, we have shown that the MuLV R peptide could also exert a profound inhibitory effect on the fusion activities of SIV-MuLV chimeric proteins (41). In the present study, we have constructed mutant MuLV Env proteins with serial truncations or amino acid substitutions in the R-peptide region, and we have defined the molecular features which are important for the fusion inhibition activity of the MuLV R peptide. All these mutant Env proteins were found to be properly processed and to be transported to the cell surface as efficiently as the wild-type Env protein, as shown by results from pulse-chase labeling and surface biotinylation studies. We also used a sensitive fusion assay which enabled us to quantitatively assess the effect of each mutation on the fusion activity of the Env protein. Our results show that Leu-627 plays a critical role in the fusion inhibition by the R peptide, while most of the other amino acids in the cytoplasmic tail are not essential for this function. Results from studies on the truncation and internal deletion mutants show that regions flanking Leu-627 are also important for its fusion inhibition effect, although they are tolerant to multiple amino acid replacements. However, amino acids 609 to 614 are not required for the fusion inhibition by the R peptide, as indicated by lack of fusion activity by Menv/d609-614 and the loss of fusion inhibition by its R-peptide-truncated counterpart MenvR$^-$/d609-614. The presence or absence of the 7 C-terminal amino acids in the R-peptide area also did not affect the fusion activity of the Env protein, as indicated by the serial truncation mutants.

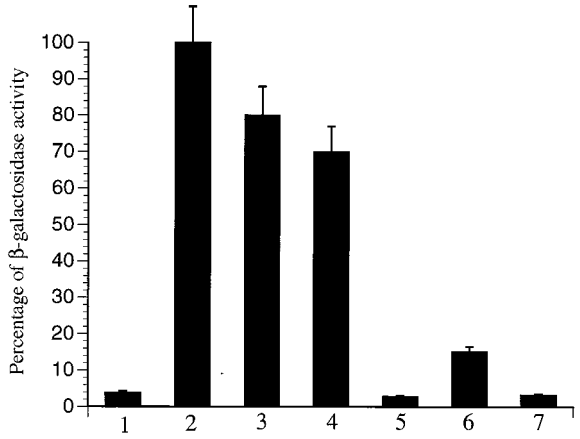

FIG. 8. Cell fusion activities of Leu-627 mutants, a Thr-628 mutant, and internal deletion mutants. Quantitation of fusion by these constructs was carried out as described in Materials and Methods. The fusion activity of each mutant was expressed as a percentage of the β-galactosidase activity produced by MenvR$^-$. Error bars, standard deviations. Bars: 1, control; 2, MenvR$^-$; 3, Menv634Tr/L627A; 4, Menv634Tr/L627P; 5, Menv634Tr/T628A; 6, Menv/d609-625; 7, Menv/d609-614.

Studies on the mechanism of fusion by viral envelope proteins have shown that the affinity for the cellular receptor and the hydrophobic property of the fusion peptide, which are largely determined by properties of the extracellular domain of the Env proteins, are critical for their fusion activity. The mechanism of fusion by viral envelope proteins is best illustrated by studies of the influenza HA protein. It has been demonstrated that low pH conditions trigger a conformational change in the extracellular domain of the HA protein. This conformational change results in the exposure of the highly hydrophobic fusion peptide, which then is thought to insert into the target membrane (6, 36, 37). However, a possible role of the cytoplasmic tail in the fusion activity of viral envelope proteins was not anticipated from these studies. In fact, only recently has it become evident that changes in the cytoplasmic tails of viral envelope proteins can affect their cell fusion activity. It has been reported that truncations in the cytoplasmic tail of the human immunodeficiency virus envelope protein can affect its cell fusion activity (8, 9, 25, 39). Studies with SIV Env proteins have shown that Env proteins with most of the cytoplasmic tail truncated exhibited increased cell fusion activity in established human T-cell lines (31, 43). Studies with MuLV envelope proteins have shown that the cleavage of the R peptide is important to activate its fusion activity (29, 30). Furthermore, our previous studies have shown that the R peptide can also inhibit the fusion of SIV-MuLV chimeric envelope proteins, which utilize a different receptor. Studies with the SIV envelope proteins have shown that truncation of the SIV Env cytoplasmic tail induced a conformational change in the extracellular domain of the Env protein, as detected by a surface biotinylation assay (35), and it was postulated that this conformational change may render the SIV Env protein more fusogenic. As shown in the present study, as well as our previous studies, no significant difference in surface biotinylation between most of the mutant Env proteins and the wild-type Env protein was observed. However, it is possible that changes in the cytoplasmic tail of the MuLV Env

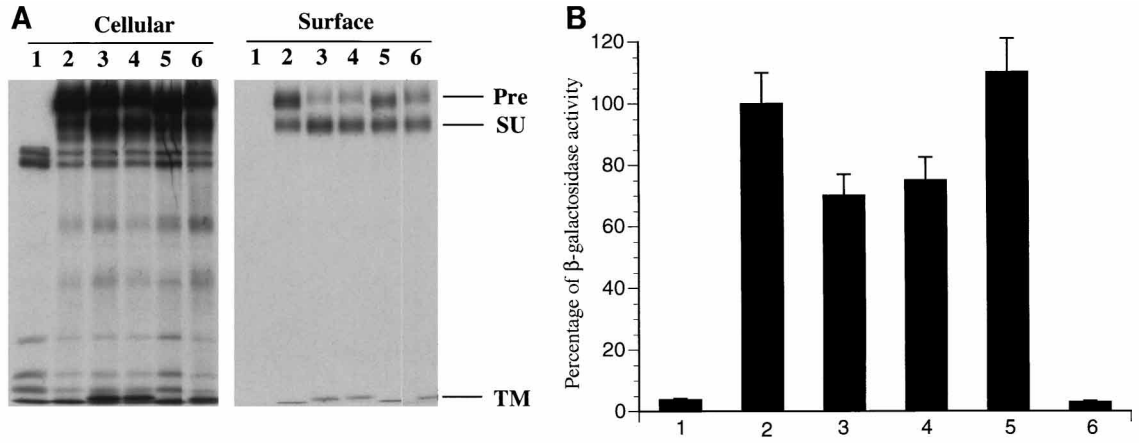

FIG. 9. Effect of Leu-627 mutation on cell fusion activity of the full-length MuLV envelope glycoprotein. (A) Protein expression was carried out as described in Materials and Methods and the legend to Fig. 2. Lanes: 1, control; 2, MenvR$^-$; 3, MenvL627A; 4, MenvL627P; 5, MenvR$^-$/d609-614; 6, Menv. (B) Quantitation of fusion by these constructs was carried out as described in Materials and Methods. The fusion activity of each mutant was expressed as a percentage of the β-galactosidase activity produced by MenvR$^-$. Error bars, standard deviations. Lanes: 1, control; 2, MenvR$^-$; 3, MenvL627A; 4, MenvL627P; 5, MenvR$^-$/d609-614; 6, Menv.

protein induce conformational changes that cannot be detected by this method. It is interesting that it was reported that fusion with XC cells was not significantly affected by the truncation of the R peptide (17a, 30). This difference probably reflects the different cell lines or expression system used in our studies. As shown by Jones and Risser, the MuLV envelope protein exhibited differences in apparent molecular weight when it was expressed in different cell lines (17a).

The cytoplasmic tails of viral envelope proteins may also affect their stability on the cell surface. Studies with the SIV Env protein have shown that mutation of a tyrosine residue (Tyr-723) in its cytoplasmic tail resulted in increased surface expression (19), whereas in parainfluenza virus type 3, truncation of the cytoplasmic tail of the F protein impaired its transport to the surface (42). Interestingly, there is a conserved tyrosine residue (Y613) present in the R peptide. However, in our previous studies, we have shown that mutation of Tyr-631 into a cysteine residue did not affect the cell fusion activity of the MuLV Env protein or its processing or surface expression (41a). Most of the mutations introduced into the cytoplasmic tail of the MuLV Env protein were not found to affect its processing or surface expression. To ensure that the observed differences in fusion activity were not caused by the differences of the level of surface expression, we analyzed the expression of each mutant protein in parallel. Our results showed that changes in surface expression levels had only a minimal effect on the fusion activity of the wild-type and mutant MuLV Env proteins (data not shown).

One possible mechanism of inhibition of fusion by the MuLV R peptide may involve a cellular factor(s) which participates in the fusion process. Several studies have suggested that fusion by viral envelope proteins involves cellular actin microfilaments (1, 5) and cell surface integrins (2, 13, 16). However, the cellular machinery which promotes the fusion of membranes is still not well characterized. The cytoplasmic tail of the MuLV Env protein could interact with cellular proteins and thus affect cell fusion activity. The R peptide could inhibit fusion activity either by binding to inhibitory cellular proteins or by preventing the Env proteins from interacting with cellular proteins that promote cell fusion. Such an interaction could be centered around the critical leucine residue in the R peptide (Leu-627). Although the presence of other specific amino acids is not critical, as indicated by the lack of effect of other amino acid substitutions in the cytoplasmic tail, the presence of adjacent residues may be necessary for providing correct positioning of Leu-627 for interaction with a cellular factor(s). The progressive increase in fusion activity observed for mutants with serial truncations or internal deletions in the cytoplasmic tail could result from a quantitative effect on the affinity of the R peptide with a cellular factor(s). A mutant Env protein in which Leu-627 was replaced with a proline residue also showed higher fusion activity than the mutant Env protein in which Leu-627 was replaced with an alanine residue. Since the R peptide also inhibits cell fusion activity of SIV-MuLV chimeric Env proteins and MuLV in different cell types, any cellular factor(s) involved in this process must exist in cell types of different origins. Brody et al. (4) reported that cleavage of the MPMV Env protein cytoplasmic tail also increased its fusion activity. As shown by sequence comparison (Fig. 4), Leu-627 of the MuLV R peptide is aligned with an isoleucine residue in the cytoplasmic tail of the MPMV Env protein. It will be interesting to know whether inhibition of fusion by the cytoplasmic tails of these two Env proteins involves a common mechanism or a similar cellular factor(s). A comprehensive understanding of the function of the cytoplasmic tail in the fusion activities of viral envelope proteins will lead us to a better understanding of the fusion process and to delineation of the cellular machinery involved in membrane fusion.

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REFERENCES

1. **Anderson, K. B.** 1995. Characterization of retrovirus-induced SC-1 cell fusion. Virus Res. **37:**177–198.
2. **Aroeti, B., and Y. I. Henji.** 1988. Effects of fusion temperature on the lateral mobility of Sendai virus glycoproteins in erythrocyte membranes and on cell fusion indicate that glycoprotein immobilization is required for cell fusion. Biochemistry **265:**62–72.
3. **Battini, J.-L., J. M. Heard, and O. Danos.** 1992. Receptor choice determinants in the envelope glycoproteins of amphotropic, xenotropic, and polytropic murine leukemia viruses. J. Virol. **66:**1458–1475.
4. **Brody, B. A., S. S. Rhee, and E. Hunter.** 1994. Postassembly cleavage of a retroviral glycoprotein cytoplasmic domain removes a necessary incorporation signal and activates fusion activity. J. Virol. **68:**4620–4627.

5. **Busso, M., J. Thornthwaite, and L. Resnick.** 1991. HIV-induced syncytium formation requires the formation of conjugates between virus-infected and uninfected T-cells in vitro. AIDS **5:**1425–1432.
6. **Carr, C. M., and P. S. Kim.** 1993. A spring-loaded mechanism for the conformational change of influenza hemagglutinin. Cell **73:**823–832.
7. **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.
8. **Dubay, J. W., S. J. Roberts, B. H. Hahn, and E. Hunter.** 1992. Truncation of the human immunodeficiency virus type 1 transmembrane glycoprotein cytoplasmic domain blocks virus infectivity. J. Virol. **66:**6616–6625.
9. **Earl, P. L., S. Koenig, and B. Moss.** 1991. Biological and immunological properties of human immunodeficiency virus type 1 envelope glycoprotein: analysis of proteins with truncations and deletions expressed by recombinant vaccinia viruses. J. Virol. **65:**31–41.
10. **Fuerst, T. R., P. L. Earl, and B. Moss.** 1987. Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. Mol. Cell. Biol. **7:**2538–2544.
11. **Gallaher, W. R.** 1987. Detection of a fusion peptide sequence in the transmembrane protein of the human immunodeficiency virus. Cell **50:**327–328.
12. **Green, N., T. M. Shinnick, O. Witte, A. 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.
13. **Gruber, M. F., D. S. Webb, T. L. Gerrard, H. S. Mostowski, L. Vujcic, and H. Golding.** 1991. Reevaluation of the involvement of the adhesion molecules ICAM-1/LFA-1 in syncytia formation of HIV-1 infected subclones of a CEM T-cell leukemic line. AIDS Res. Hum. Retroviruses **7:**45–53.
14. **Hallenberger, S., V. Bosch, H. Angliker, E. Shaw, H.-D. Klenk, and W. Garten.** 1992. Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160. Nature (London) **360:**358–361.
15. **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.
16. **Hildreth, J. E. K., and R. J. Orentas.** 1989. Involvement of a leukocyte adhesion receptor (LFA-1) in HIV-induced syncytium formation. Science **244:**1075–1079.
17. **Hirsch, V. M., P. Edmondson, M. Murphey-Corb, B. Arbeille, P. R. Johnson, and J. I. Mullins.** 1989. SIV adaption to human cells. Nature (London) **341:**573–574.

17a. **Jones, J. S., and R. Risser.** 1993. Cell fusion induced by the murine leukemia virus envelope glycoprotein. J. Virol. **67:**67–74.

18. **Kozarsky, K., M. Penman, L. Basiripour, W. Haseltine, J. Sodroski, and M. Krieger.** 1989. Glycosylation and processing of the human immunodeficiency virus type 1 envelope protein. J. Acquired Immune Defic. Syndr. **2:**163–169.
19. **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.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
21. **Lamb, R. A.** 1993. Paramyxovirus fusion: a hypothesis for changes. Virology **197:**1–11.
22. **Marsh, M., and A. Helenius.** 1989. Viral entry into animal cells. Adv. Virus Res. **36:**107–151.
23. **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.
24. **McDougal, J., J. Nicholson, G. Cross, S. Cort, M. Kennedy, and A. Mawle.** 1986. Binding of the human retrovirus HTLV/LAV/ARV/HIV to the CD4 (T4) molecule: conformation dependence, epitope mapping, antibody inhibition and potential for idiotypic mimicry. J. Immunol. **137:**2937–2944.
25. **Mulligan, M. J., G. V. Yamshchikov, G. D. J. 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.
26. **Nussbaum, O., C. C. Broder, and E. A. Berger.** 1994. Fusogenic mechanisms of enveloped-virus glycoproteins analyzed by a novel recombinant vaccinia virus-based assay quantitating cell fusion-dependent reporter gene activation. J. Virol. **68:**5411–5422.
27. **Ott, D., and A. Rein.** 1992. Basis for receptor specificity of nonecotropic murine leukemia virus surface glycoprotein $gp70^{SU}$. J. Virol. **66:**4632–4638.
28. **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.
29. **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.
30. **Rein, A., J. Mirro, J. Gordon Haynes, S. M. Ernst, and K. Nagashima.** 1994. Function of the cytoplasmic domain of a retroviral transmembrane protein: p15E-p2E cleavage activates the membrane fusion capability of the murine leukemia virus Env protein. J. Virol. **68:**1773–1781.
31. **Ritter, G. D., M. J. Mulligan, S. L. Lydy, and R. W. Compans.** 1993. Cell fusion activity of the simian immunodeficiency virus envelope protein is modulated by the intracytoplasmic domain. Virology **197:**255–264.
32. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
33. **Schultz, A., and A. Rein.** 1985. Maturation of murine leukemia virus env proteins in the absence of other viral proteins. Virology **145:**335–339.
34. **Sommerfelt, M. A., S. R. Petteway, Jr., G. B. Dreyer, and E. Hunter.** 1992. Effect of retroviral proteinase inhibitors on Mason-Pfizer monkey virus maturation and transmembrane glycoprotein cleavage. J. Virol. **66:**4220–4227.
35. **Spies, C. P., G. D. Ritter, Jr., M. J. Mulligan, and R. W. Compans.** 1994. Truncation of the cytoplasmic domain of the simian immunodeficiency virus envelope glycoprotein alters conformation of the external domain. J. Virol. **68:**585–591.
36. **Stegmann, T., R. W. Doms, and A. Helenius.** 1989. Protein-mediated membrane fusion. Annu. Rev. Biophys. Biophys. Chem. **18:**187–211.
37. **White, J. M.** 1992. Membrane fusion. Science **258:**917–924.
38. **White, J. M.** 1990. Viral and cellular membrane fusion proteins. Annu. Rev. Physiol. **52:**675–697.
39. **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.
40. **Willey, R. L., D. H. Smith, L. Laskey, T. Theodore, P. Earl, B. Moss, D. Capon, and M. Martin.** 1988. In vitro mutagenesis identifies a region within the envelope gene of the human immunodeficiency virus that is crucial for infectivity. J. Virol. **62:**139–147.
41. **Yang, C., and R. W. Compans.** 1996. Analysis of the cell fusion activities of chimeric simian immunodeficiency virus-murine leukemia virus envelope proteins: inhibitory effects of the R peptide. J. Virol. **70:**248–254.

41a. **Yang, C., and R. W. Compans.** Palmitoylation of the murine leukemia virus envelope glycoprotein transmembrane subunits. Virology **221:**87–97.

42. **Yao, Q., and R. W. Compans.** 1995. Differences in the role of the cytoplasmic domain of human parainfluenza virus fusion proteins. J. Virol. **69:**7045–7053.
43. **Zingler, K., and D. R. 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.