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Functional Analysis of the Cytoplasmic Tail of Moloney Murine Leukemia Virus Envelope Protein

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The cytoplasmic tail of the immature Moloney murine leukemia virus (MoMuLV) envelope protein is approximately 32 amino acids long. During viral maturation, the viral protease cleaves this tail to release a 16-amino-acid R peptide, thereby rendering the envelope protein fusion competent. A series of truncations, deletions, and amino acid substitutions were constructed in this cytoplasmic tail to examine its role in fusion and viral transduction. Sequential truncation of the cytoplasmic tail revealed that removal of as few as 11 amino acids resulted in significant fusion when the envelope protein was expressed in NIH 3T3 cells, similar to that seen following expression of an R-less envelope (truncation of 16 amino acids). Further truncation of the cytoplasmic tail beyond the R-peptide cleavage site toward the membrane-spanning region had no additional effect on the level of fusion observed. In contrast, some deletions and nonconservative amino acid substitutions in the membrane-proximal region of the cytoplasmic tail (residues L602 to F605) reduced the amount of fusion observed in XC cell cocultivation assays, suggesting that this region influences the fusogenicity of full-length envelope protein. Expression of the mutant envelope proteins in a retroviral vector system revealed that decreased envelope-mediated cell-cell fusion correlated with a decrease in infectivity of the resulting virions. Additionally, some mutant envelope proteins which were capable of mediating cell-cell fusion were not efficiently incorporated into retroviral particles, resulting in defective virions. The cytoplasmic tail of MoMuLV envelope protein therefore influences both the fusogenicity of the envelope protein and its incorporation into virions.

The envelope protein of murine leukemia viruses (MuLV) is initially translated as an 85-kDa glycosylated protein (gp85). This glycoprotein is posttranslationally cleaved by a cellular protease to generate a 70-kDa extracellular glycoprotein (gp70 or SU) and a 15-kDa membrane-spanning protein (p15E or TM), which remain associated through noncovalent, and possibly disulfide, interactions (25). Oligomerization of the envelope protein occurs in the endoplasmic reticulum and is necessary for intravesicular transport to the membrane surface (24). Various properties of MuLV envelope protein have been identified with specific extracellular regions of the protein, including a host range-determining region (1, 14, 17, 20, 22), a fusion peptide (16), an NH_4Cl-sensitive region (18, 19, 22, 28), and a leucine zipper-like region (9, 30).

During the viral maturation process, the 15-kDa TM protein is further cleaved by a viral protease at a site 16 amino acids from the carboxy terminus to yield p12E and p2 (or R peptide) (13). Proteolytic cleavage of cytoplasmic tails during viral maturation has also been observed in Mason-Pfizer monkey virus (M-PMV) (35) and equine infectious anemia virus (32). Truncation of the cytoplasmic tail affects envelope fusion properties, as both our laboratory (29) and others (31) have previously demonstrated that expression of a Moloney MuLV (MoMuLV) envelope protein lacking the R peptide in NIH 3T3 cells results in massive cell-cell fusion. It was therefore proposed that the removal of the R peptide during viral maturation renders the envelope protein competent for fusion following an interaction with its cellular receptor. A similar increase in fusogenicity has also been reported for M-PMV (2), and the expression of truncated forms of both human and simian immunodeficiency virus (HIV and SIV) envelope proteins in CD4-positive cells causes increased syncytium formation (4, 8, 21, 33, 43). Interestingly, it has recently been shown that the addition of the MuLV R peptide to the cytoplasmic tail of a truncated SIV_{239} envelope protein prevents this increased fusogenicity (42), suggesting a role for the R peptide in the regulation of envelope protein-mediated fusion.

To examine the role of the cytoplasmic tail of the MoMuLV envelope protein in fusion and subsequent retroviral transduction, a series of truncations, deletions, and amino acid substitutions were constructed in this region. These mutant envelope proteins were analyzed for the ability to direct cell-cell fusion and viral transduction of target cells. As previously reported (29, 31), truncation of the R peptide results in an increase in cell-cell fusion when the envelope protein is expressed in NIH 3T3 cells. Our data are consistent with a model whereby the cytoplasmic tail of MuLV envelope contains a fusion-controlling structure which is naturally removed upon R-peptide cleavage. Additionally, although the complete removal of the cytoplasmic tail produces an envelope that is more fusogenic than the wild-type molecule, certain deletions and substitutions within the membrane-proximal region of the cytoplasmic tail (residues 602 to 605) inhibit fusion, suggesting a role for the hydrophobic residues in this region in the fusion process. Finally, we also observed that certain deletions and substitutions within the cytoplasmic tail prevent the incorporation of envelope protein into retroviral particles.

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MATERIALS AND METHODS

Cell lines. XC rat sarcoma cells were subcloned and selected for their propensity to fuse when exposed to MoMuLV envelope-expressing cells. They were maintained in basal medium Eagle with Earle's salts (Gibco/BRL, Grand Island, N.Y.) supplemented with 10% fetal calf serum (Intergen, Purchase, N.Y.) and 2 mM glutamine (Gibco/BRL). GPL cells are NIH 3T3 cells which constitutively express MoMuLV *gag* and *pol* genes and contain the LNL6 vector, which carries the neomycin phosphotransferase (Neo^R) gene (described in reference 17). 293T cells were obtained from the American Type Culture Collection. GPL, NIH 3T3, and 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 2 mM glutamine.

Plasmids and mutagenesis. Mutations were introduced in the wild-type envelope protein expression plasmid, pCEE+ (17), using either an oligonucleotide-directed mutagenesis kit (Amersham, Arlington Heights, Ill.) or recombinant PCR splice overlap extension (15). All mutations were confirmed by sequencing the final construct.

Plasmid DNA preparation and transfection. Plasmid DNA was isolated from bacterial cultures by using alkaline lysis and a Qiagen-tip 500 (Qiagen, Chatsworth, Calif.) according to the manufacturer's protocol. The DNA was further purified by using Chromaspin TE+400 columns (Clontech Laboratories, Inc., Palo Alto, Calif.) according to the manufacturer's instructions.

Transfections were carried out by the $CaPO_4$ precipitation method (10, 20, 39), using a calcium phosphate mammalian cell transfection kit (5Prime→3Prime, Inc., Boulder, Colo.). Approximately 16 to 20 h before transfection, cells were plated at 6×10^5/100-mm-diameter petri dish or as specified in the individual experiments. The medium was changed 2 to 4 h before addition of the $CaPO_4$-DNA precipitation mix. Approximately 16 to 18 h after transfection, the cells were washed with phosphate-buffered saline (PBS; Gibco/BRL), and fresh medium was added.

Viral titer determination. GPL cells were transfected with various envelope expression plasmids as described above. Approximately 72 h after transfection, the supernatant was removed and filtered through a 0.45-μm-pore-size filter (Millipore, Marlborough, Mass.), and the viral supernatants were either frozen at −70°C or analyzed immediately. Viral titer was determined as described previously (20). Essentially, NIH 3T3 cells were exposed to various dilutions of viral supernatant for 24 h and then selected in G418 (800 μg/ml; Gibco/BRL) for 8 to 10 days. Surviving G418-resistant colonies were stained with 1% methylene blue in methanol and counted.

NIH 3T3-XC cocultivation fusion assay. Approximately 2×10^5 NIH 3T3 cells were plated in a 60-mm-diameter petri dish containing 2-mm grids (Corning Glass Works, Corning, N.Y.) and transfected with 12 μg of envelope expression vector as described above. Approximately 36 to 40 h after transfection, 5×10^5 XC cells were added to the transfected NIH 3T3 cells and incubated for 24 h, after which the plates were washed with PBS and stained with 1% methylene blue in methanol, and syncytia (cells containing four or more nuclei) were counted as previously described (28).

NIH 3T3 cell fusion assay. Approximately 3×10^5 NIH 3T3 cells were plated in a 60-mm-diameter petri dish containing 2-mm grids and transfected with 12 μg of envelope expression vector. Forty-eight hours posttransfection, the cells were washed with PBS and stained with 1% methylene blue in methanol, and syncytia (cells containing four or more nuclei) were counted.

Analysis of envelope protein incorporation into virions. Retroviral particles were produced by the cotransfection of envelope expression plasmids into 293T cells, along with the *gag-pol* expression plasmid pHIT60, as described previously (36). Culture supernatants were pelleted through 25% sucrose at 25,000 rpm (SW41 rotor) for 4 h at 4°C. Viral proteins were resolved on precast 14% sodium dodecyl sulfate-polyacrylamide gels (Novex Experimental Technology, San Diego, Calif.) and electroblotted onto Immobilon-P membranes (Millipore). Membranes were blocked with 5% dried milk in Tris-buffered saline (10 mM Tris-HCl [pH 7.5], 30 mM NaCl, 0.25% Tween 20) for 20 min at room temperature and subsequently cut into two sections, upper (>46 kDa) and lower (<46 kDa). The upper section was incubated for 1 h at room temperature with a 1:1,000 dilution of a goat antiserum 805-019 (Quality Biotech, Camden, N.J.) that recognizes MoMuLV SU (gp70). The secondary antibody was horseradish peroxidase (HRP)-conjugated rabbit anti-goat immunoglobulin G $F(ab)_2$ (Pierce, Rockford, Ill.) used at a 1:10,000 dilution. The lower section was incubated for 1 h at room temperature with a 1:2,500 dilution of a rabbit antiserum that recognizes MoMuLV TM (p15E) and CA (p30) (a gift from Alan Rein, NCI-Frederick Cancer Research and Development Center, Frederick, Md.). The secondary antibody was HRP-conjugated goat anti-rabbit immunoglobulin G (Boehringer Mannheim Corp., Indianapolis, Ind.) at 1:10,000 dilution. Specific interactions were visualized by using the SuperSignal CL-HRP substrate system (Pierce) and exposure to Hyperfilm ECL (Amersham).

Cell surface expression of envelope protein. The level of cell surface envelope protein expression was measured by fluorescence-activated cell sorting (FACS) analysis of 293T cells which transiently expressed the wild-type or mutant envelope proteins. The 293T cells were transfected with 30 μg of envelope protein expression vector and 48 h after transfection were removed from the plates with trypsin-free cell dissociation buffer (Gibco/BRL). The cells were washed with 10% goat serum–PBS and then incubated for 1 h at 4°C with 250 μl of monoclonal antibody 83A25 (5). After incubation, the cells were washed with 10% goat serum–PBS, resuspended in 100 μl of 1:100-diluted fluorescence-activated cell sorting-labeled goat anti-rat antibody (Kirkegaard & Perry Laboratories, Gaithersburg, Md.), and incubated at 4°C for 1 h. The cells were then washed with 10% goat serum–PBS and resuspended in 4% paraformaldehyde–PBS. Samples were analyzed in a Becton Dickinson FACScan and standardized with Sphero Rainbow Particles (6.7-μm-diameter fluorescent beads; Spherotech, Inc., Libertyville, Ill.). The Sphero Rainbow Particles are latex spheres of known fluorescence intensities. Mean fluorescence values of the peaks were plotted against the known fluorescence values to generate a standard curve. This standard curve was used to calculate the absolute fluorescence values of the experimentally determined mean fluorescence values.

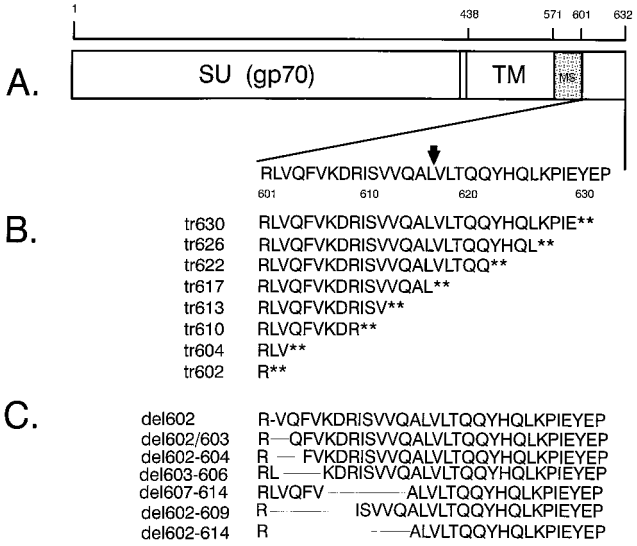

FIG. 1. (A) Schematic diagram of the MoMuLV envelope protein and the amino acid sequence of the cytoplasmic tail. SU, surface envelope glycoprotein (gp70); TM, transmembrane envelope glycoprotein (p15E); MS, membrane-spanning domain. The arrow indicates the R-peptide cleavage site. (B) Truncation mutations. Stop codons are indicated with asterisks. (C) Deletion mutations. Deleted residues are replaced by a line.

RESULTS

Construction and fusion analysis of deletion and truncation mutants. Ragheb and Anderson (29) and Rein et al. (31) have previously shown that expression of a MoMuLV envelope protein lacking the last 16 amino acids (the R peptide) results in increased receptor-dependent cell-cell fusion. To further analyze the role of the cytoplasmic tail in augmenting receptor-dependent fusion, we constructed a series of truncations extending from the C terminus of the MoMuLV envelope protein to the start of the membrane-spanning domain. In addition, we produced several envelope protein mutants with internal deletions in the region between the membrane-spanning domain and the R-peptide cleavage site (Fig. 1).

To determine whether the truncation or deletion mutations affected the fusion properties of the envelope protein, we transfected NIH 3T3 cells with a plasmid that coded for either wild-type envelope protein or a mutant envelope protein and then cocultivated the cells with XC cells approximately 36 to 40 h later. The XC rat sarcoma cell line is characterized by its ability to fuse when exposed to cells and/or virus containing ecotropic envelope proteins, so that cocultivation with envelope-expressing NIH 3T3 cells results in the formation of syncytia.

Analysis of the envelope truncation mutants in this assay demonstrated that truncation of three amino acids from the C terminus of the envelope protein (*tr*630) had no effect on NIH 3T3-XC fusion, with the number of syncytia observed being

TABLE 1. Effects of cytoplasmic tail truncations and deletions on envelope protein-mediated cell-cell fusion and retroviral transduction

Envelope construct	Relative % XC cell fusion[a]	NIH 3T3 syncytia counted/2 mm[b]	Relative % Neo^R titer[c]
Wild type	100	0	100
*tr*630	91	0	97
*tr*626	218	0	106
*tr*622	370	21	43
*tr*617	>500	106	8
*tr*613	>500	103	21
*tr*610	>500	102	16
*tr*604	>500	100	3
*tr*602	>500	78	1
*del*602	12	0	<1
*del*602-603	83	0	<1
*del*602-604	50	0	<1
*del*603-606	5	0	<1
*del*607-614	498	16	5
*del*602-609	62	0	<1
*del*602-614	48	0	<1

[a] Fusion of XC cells with NIH 3T3 cells which transiently expressed the indicated envelope protein. Values are relative to those for wild-type envelope protein.

[b] Fusion of NIH 3T3 cells which transiently expressed the indicated envelope protein. Results are presented as number of syncytia (cells with four or more nuclei per cell) counted per 2-mm grid; six grids were counted per transfected plate, and at least three plates were analyzed.

[c] Percentage Neo^R titer of virions containing the indicated mutant envelope protein relative to virions with wild-type envelope protein. Wild-type titers were in the range of 10^3 to 10^4; each value reported is the mean of three to five experiments.

similar to those produced by the wild-type envelope (Table 1). However, truncation of seven amino acids (*tr*626) caused a significant increase in syncytia compared to the wild type. The level of syncytia further increased to 3.7-fold above the wild-type level when 11 amino acids were truncated and continued to a maximal level (too numerous to count) for truncations of 16 to 31 amino acids. The levels of syncytium formation with *tr*617 and *tr*602 were similar to previously reported results of Ragheb and Anderson (29) for equivalent constructs CEETR and CEET, respectively.

We next examined the effect on cell-cell fusion of a series of internal deletion mutations constructed in the region between the membrane-spanning domain and the R-peptide cleavage site (Table 1). With the notable exception of mutant *del*607-614, which showed enhanced fusogenicity, all of the deletion mutants were less fusogenic than the wild-type envelope. The most severely affected mutants were the deletions L602 and V603 to V606, which reduced the number of syncytia to 12 and 5% of the wild-type level, respectively. These results suggest that certain alterations in the membrane-proximal region of the cytoplasmic tail inhibit fusion. In contrast, the construct with the deletion of residues more distal to the membrane-spanning domain, *del*607-614 (K607-Q614), resulted in an envelope protein which mediated extensive fusion of XC cells, at a level similar to that seen for an R-less envelope.

Fusion of NIH 3T3 cells. NIH 3T3 cells do not normally form syncytia when expressing full-length MoMuLV envelope, although certain *ras* mutations (41) or the addition of amphotericin B (26) will allow fusion. However, the expression of an R-less MoMuLV envelope protein results in extensive receptor-dependent syncytium formation in NIH 3T3 cells (29, 31). During the course of the NIH 3T3-XC cocultivation experiments, it was noted that when at least 11 residues were truncated from the C terminus of the envelope protein, the NIH 3T3 cells formed syncytia before the XC cells were added to the culture plate. This result is similar to the fusion phenotype observed when an R-less envelope is expressed in NIH 3T3 cells. The truncation and deletion mutants were therefore examined for the ability to promote the fusion of NIH 3T3 cells alone (Table 1). Significant fusion of NIH 3T3 cells was observed when 16 amino acids were removed (*tr*617), which corresponds to an R-less envelope, and for deletions of up to 29 amino acids extending further toward the membrane-spanning domain (*tr*613 to *tr*604). Truncation to the membrane-spanning domain (*tr*602) produced slightly less fusion than the R-less construct, which is consistent with previous results (29). The removal of only 11 amino acids (*tr*622) resulted in an envelope protein that was more fusogenic than the wild-type protein, indicating that removal of the entire R peptide was not necessary to achieve significant NIH 3T3 cell fusion.

We also analyzed the abilities of the internal deletion mutants to cause syncytia in NIH 3T3 cells. Fusion was only observed with the deletion of residues K607 to Q614, which resulted in a low level of activity (16% of the R-less level). Overall, the data from both sets of mutants are in good agreement with the fusion data obtained from the XC cell cocultivation assay.

Amino acid substitution analysis of the membrane-proximal region. The results from the analysis of the internal deletion mutants suggested that the membrane-proximal region of the cytoplasmic tail may play a role in the fusion process. To analyze this region further, a number of single and double amino acid substitutions of residues L602 and V606 were introduced into an envelope protein expression vector and tested for the ability to cause fusion in the NIH 3T3-XC cocultivation fusion assay.

Approximately half of the mutations that we made in this region significantly inhibited the fusion of XC cells (Table 2), in particular those which contained nonconservative substitutions of more than one residue. While single amino acid substitutions of Q604 had little effect on XC cell fusion, the double substitution of both Q604 and F605 in various combinations significantly inhibited fusion in this assay. Residue L602 also appears to be important for fusogenicity, as the substitution L602Y abolished fusion. In addition, although the substitution of L602S alone allowed 58% of the wild-type level of syncytium formation, the combination of L602S with V603L or V603R prevented fusion. The membrane-proximal hydrophobic residues L602, V603, and F605 therefore appear important for envelope fusogenicity.

Analysis of fusion properties of R-less envelope proteins. Full-length envelope protein expressed in the absence of the viral protease is not cleaved at the R-peptide cleavage site and is minimally fusogenic in NIH 3T3 cells, although highly fusogenic in XC cells. In contrast, envelope protein that has the R peptide removed will fuse NIH 3T3 cells. It is possible that mutations that have no apparent effect on fusion in the context of full-length envelope have a phenotype that is revealed when the envelope protein is expressed in the virally mature R-less form. Accordingly, we analyzed the effects of certain mutations on fusion in R-peptide truncated envelopes.

Several of the membrane-proximal mutants were made R-less by using overlapping PCR mutagenesis to introduce consecutive stop codons at residues 617 and 618. These R-less mutants were transiently expressed in NIH 3T3 cells, and syncytia were quantitated (Table 3). Most of the mutations which severely impaired the ability of the full-length envelope protein to mediate fusion of XC cells in a cocultivation assay (Table 1) also prevented fusion of NIH 3T3 cells in the R-less configuration. Similarly, those mutations that were not disruptive to

TABLE 2. Effects of amino acid substitutions on envelope protein-mediated cell-cell fusion and retroviral transduction

Envelope construct	Relative % XC cell fusion[a]	Relative % Neo^R titer[b]
L602S	58	14
V603Y	68	69
L602F/V603L	80	67
Q604C	90	54
Q604G	105	47
Q604P	95	78
Q604R	97	60
Q604V	82	71
Q604Y/F605W	98	98
V606C	90	34
V606T	95	76
L602W/V603K	89	3
Q604W/F605T	87	2
L602Y	<1	3
L602S/V603L	<1	<1
L602S/V603R	<1	<1
L602W/V603D	<1	<1
V603D/Q604P	3	<1
Q604D/F605E	5	<1
Q604K/F605Q	7	<1
Q604A/F605M	18	2
Q604L/F605S	5	<1
Q604N/F605S	6	<1
Q604S/F605S	13	<1
Q604G/F605G	20	<1
Q604S/F605V	3	3

[a] Fusion of XC cells with NIH 3T3 cells which transiently expressed the indicated envelope protein. Values are relative to those for the wild-type envelope protein. Syncytia are defined as cells with four or more nuclei. Twenty 2-mm grids were counted on at least three 60-mm tissue culture plates.

[b] Percentage Neo^R titer of virions containing the indicated mutant envelope protein relative to virions with wild-type envelope protein. Wild-type titers were approximately 10^3 CFU/ml.

XC cell fusion resulted in NIH 3T3 fusion at levels similar to that for the wild-type R-less envelope protein, *tr*617. Three exceptions were mutant envelopes *del*603-606, *del*602-604, and Q604W/F605T, which showed different results in the two fusion assays. It is possible that the presence or absence of the R peptide can influence the secondary structure of the cytoplasmic tail, making some mutants better tolerated in the mature or immature protein and therefore more or less permissive for fusion.

Transduction properties of mutants. To determine whether the mutations that we introduced into the cytoplasmic tail of envelope protein affected the ability of the virus to transduce target cells, GPL cells were transfected with the wild-type or a mutant envelope expression plasmid, and culture supernatants were harvested approximately 72 h later. The viral titer was assessed by the transfer of the Neo^R vector as demonstrated by colony formation in the presence of G418. As expected, the envelope mutations which inhibited cell fusion also significantly reduced the titer of the viral supernatant (Tables 1 and 2). Although we cannot exclude the possibility that these mutations affected retroviral transduction at a stage other than fusion, these data suggest a defect in virus-cell fusion.

Incorporation of mutant envelope proteins into retroviral particles. While many of the deletions and substitutions that we introduced into the cytoplasmic tail clearly had an effect on fusion, some of the mutations seemed to have a greater effect on titer than would have been predicted from the defect in fusion. Several of the deletion mutants (*del*602-603, *del*602-604, *del*602-609, and *del*602-614) reduced the titer to <1% of that of the wild-type envelope while retaining significant ability to mediate NIH 3T3-XC cell fusion in the cocultivation assay. One possible explanation for such discrepancies is that these mutant envelope proteins were inefficiently incorporated into virions. Accordingly, we analyzed the levels of SU (gp70) and TM (p15E) proteins present in virions purified from the supernatant of transfected cells.

As can be seen in Fig. 2, the wild-type TM protein in retroviral particles is present as both the full-length p15E protein and the R-peptide truncated p12E form. Analysis of the sequential truncation mutants (Fig. 2A) revealed that once the envelope protein was truncated beyond three residues, the levels of both gp70 and p15E present in the particles decreased. In addition, truncation beyond the R-peptide cleavage site produced a correspondingly smaller TM protein. The reduction in p15E incorporation was paralleled by a reduction in gp70 and appeared to correspond to the decrease in titer observed for the truncation mutants. For mutant *tr*602, which retains only a single arginine of the cytoplasmic tail, neither gp70 nor p15E was detected by Western blot analysis, although clearly some protein must be incorporated into virions to produce the low level of infectivity observed with this mutant.

The series of internal deletions in the membrane-proximal region of the cytoplasmic tail was also examined for the presence of the envelope proteins in virions (Fig. 2B). The hydrophobic residue L602, adjacent to the membrane-spanning domain, appears to be important for the efficient incorporation of envelope protein into particles. The deletion of L602 alone resulted in a slightly decreased amount of incorporation, while the deletion of residues L602 and V603 together resulted in no detectable protein. Other deletions removing L602 also reduced the level of envelope protein in the particles, including *del*602-609 and *del*602-614. Deletions in this region of the protein also appeared able to influence the processing of the R peptide. Mutants *del*602-609, *del*602-614, and *del*607-614 were unable to cleave the R peptide, although *del*607-614 was incorporated into virions more efficiently than *del*602-609 and *del*602-614.

The influence of the membrane-proximal residues on envelope protein incorporation was also demonstrated by the analysis of substitution mutants in this region (Fig. 2C). In particular, mutants L602S/V603L, L602S/V603R, and V603D/Q604P

TABLE 3. Effects of amino acid substitution and deletion mutations on the fusion properties of R-peptide truncated envelope proteins

Envelope construct	Relative % NIH 3T3 syncytia[a]
Wild type	0
R-less (*tr*617)	100
L602S	81
L602Y	0
Q604V	51
Q604A/F605M	1
Q604G/F605G	1
Q604K/F605Q	0
Q604L/F605S	3
Q604S/F605S	1
Q604S/F605V	12
Q604W/F605T	26
*del*602-604	0
*del*603-606	20

[a] Percentage of syncytia (cells with four or more nuclei) relative to the wild-type R-less envelope protein (*tr*617). At least six 2-mm grids were counted on each of three or more 60-mm tissue culture plates.

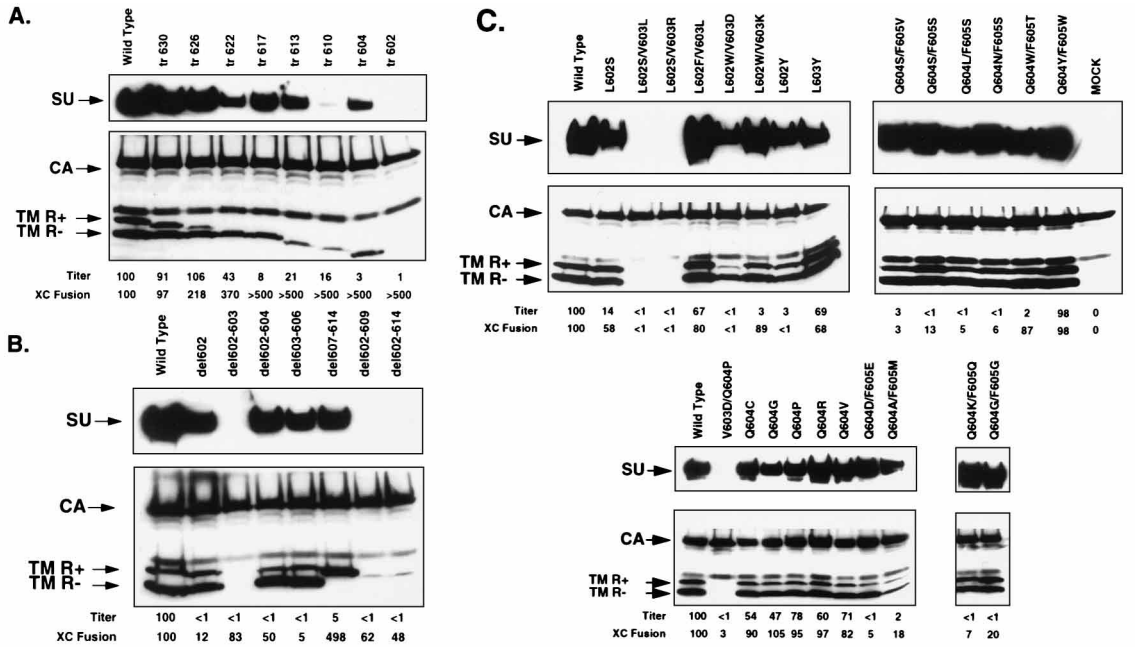

FIG. 2. Incorporation of the MoMuLV envelope protein into virions as determined by Western blotting. Retroviral supernatant was collected, centrifuged through a 25% sucrose cushion, and analyzed by Western blotting with a rabbit polyclonal serum which recognizes p30 (CA), p15E (TM R+), and p12E (TM R−) as well as a goat polyclonal serum which recognizes gp70 (SU). A viral protein band which migrates slightly slower than TM R+ was occasionally seen in the Western blots but has not been identified. The corresponding percent titers and XC fusion values relative to the wild type are indicated below each lane (values from Tables 1 and 2). (A) Truncation mutations; (B) deletion mutations; (C) amino acid substitution mutations. Mock, retroviral vector without envelope protein.

had no detectable envelope proteins in the virions. Interestingly, mutants L602W/V603K and Q604W/F605T, which had wild-type fusogenicity in the XC cell assays but low infectivity, had normal levels of p15E and gp70 present in virions.

Cell surface expression levels of envelope protein. Cytoplasmic tail mutations in Rous sarcoma virus and vesicular stomatitis virus G envelope glycoproteins have been shown to decrease the transport and cell surface expression of the mutant envelope proteins (34, 40). Several of the mutations which were constructed in the MoMuLV envelope protein resulted in decreased XC cell fusion as well as inefficient viral incorporation. One explanation for these observations may be that the mutations caused protein instability or defects in transport to the cell surface. We therefore measured the levels of envelope protein expressed onto the cell surface for certain mutants that had reduced levels of envelope protein in the retroviral particles. Wild-type and mutant envelope protein expression vectors were transfected into 293T cells and the level of cell surface expression of the transiently expressed proteins was measured by FACS analysis (Table 4).

In general, there was good correlation between the level of surface expression of a protein and its ability to be incorporated into virions. Those envelope proteins that were efficiently incorporated into retroviral particles (*del*603-606 and L602S) were also found to be efficiently expressed on the cell surface. Conversely, three mutant envelope proteins (L602S/V603L, L602S/V603R, and V603D/Q604P) that were not present in pelleted viral material were also not detected on the cell surface by this assay, suggesting that the primary defect of these mutants was protein stability or transport to the cell surface. A third group of mutants (*del*602, *del*602-609, *del*602-614, L602Y, and L602W/V603D) had an intermediate phenotype, with reduced levels of surface expression and reduced levels of incorporation into virions. Again, the primary defect in these virions is most likely at the level of protein stability and/or transport. A final group of mutants (*tr*602, *del*602-603, and Q604A/F605M) were found to be expressed on the cell surface but absent or decreased from virions. These data suggest a defect in the incorporation of envelope protein into viral particles and indicate that the cytoplasmic tail of MoMuLV envelope protein plays a role in this process.

DISCUSSION

Retroviral envelope proteins direct the entry of a virus into a host cell in a two-stage process, requiring an initial interaction of the envelope protein with its cognate cellular receptor

TABLE 4. Cell surface expression level of wild-type and mutant envelope proteins as measured by FACS analysis

Envelope construct	Relative % cell surface expression[a]
*tr*602	52
*del*602	24
*del*602/603	20
*del*602-609	11
*del*602-614	7
*del*603-606	71
L602S	47
L602Y	21
L602S/V603L	2
L602S/V603R	4
L602W/V603D	13
V603D/Q604P	8
Q604A/F605M	100
Q604G/F605G	21

[a] Calculated by comparing the mean channel fluorescence of each experimental sample to the wild-type mean channel fluorescence. Mean channel fluorescence was determined as described in Materials and Methods.

and a subsequent fusion between the viral and host cell membranes. While the receptor binding domain of the envelope protein resides in the SU component, membrane fusion requires the function of the TM protein (reviewed in reference 38). Several regions of TM, including a hydrophobic fusion peptide at the amino terminus (16) and a heptad repeat motif also present in the extracellular domain (30), have been implicated in the fusion process. In this study, we have identified the cytoplasmic tail of the MoMuLV envelope protein as important for both its incorporation into retroviral particles and its fusion properties.

A series of truncations in the cytoplasmic tail of MoMuLV TM protein were examined for their effects on the envelope protein's ability to mediate cell-cell fusion. In agreement with previous studies (29, 31), we have shown that deletion of the R peptide produces a protein that is highly fusogenic compared to the wild-type envelope, giving rise to syncytia when expressed in NIH 3T3 cells. In addition, we noted that the removal of only 11 of the 16 amino acids of the R peptide results in the formation of syncytia in NIH 3T3 cells. Subsequent deletions toward the transmembrane domain of p15E resulted in an envelope protein that retained maximal fusogenicity. Even the complete loss of the cytoplasmic tail produced an envelope that had 78% of the fusogenicity of an R-less construct.

In contrast to the cell-cell fusion results, the loss of the R peptide and sequences closer to the transmembrane region had a marked effect on viral infectivity in a retroviral vector assay. Truncation of the tail at the R-peptide cleavage site (*tr*617) resulted in retroviral vectors with titers that were 8% of the wild-type level, which is in agreement with the previous finding of Rein et al. (31), and truncation at the transmembrane domain (*tr*602) produced only 1% of the wild-type titer. It is possible that a reduction in the level of envelope protein present in the viral particles accounts for the decreased infectivity, as the sequential truncation of the cytoplasmic tail reduced the level of gp70 and p15E proteins detected in the virions by immunoblotting and the *tr*602 protein could not be detected at all by this analysis. However, it is also possible that the NIH 3T3-XC cell-cell fusion assay is less sensitive to changes in envelope protein function than the virus-cell fusion event that occurs during infection.

Although the membrane-proximal region of the cytoplasmic tail could be truncated without preventing envelope-mediated cell-cell fusion, this region of the tail was far less tolerant of internal deletions and point mutations. Internal deletions within this region resulted in variable amounts of reduced fusogenicity. For example, membrane-proximal mutant *del*603-606 produced only 5% of the wild-type level of syncytia in the XC cell cocultivation assay, despite being readily detectable on the surface of transfected cells. In contrast, deletion of residues more distal to the transmembrane domain, *del*607-614, enhanced fusogenicity.

The difference in phenotype between the membrane-proximal and membrane-distal deletion mutants suggests a role for residues L602 to V606 in the fusion process in the context of an R-peptide-containing tail. The deletion or substitution of these residues always produced an envelope protein with impaired fusogenicity. Examples of such mutations include the deletion of L602 and the substitution L602Y, which resulted in 12 and <1% of wild-type fusion, respectively. Three double substitutions of L602 and V603 and several other amino acid substitutions in the region from L602 to V606 also reduced the fusogenicity of the full-length envelope protein in XC cells. Furthermore, the defect in fusion in both *del*602-604 and L602Y could not be restored by removal of the R peptide. It is possible that some of these mutants were defective because a conformational structure formed in the cytoplasmic tail that is not compatible with the formation of a fusion-competent envelope protein. A similar situation has been observed for the HIV type 1 envelope protein, where sequential truncations of the cytoplasmic tail produced both functional and nonfunctional envelopes (7).

Certain deletions in the membrane-proximal region of the tail (*del*607-614, *del*602-609, and *del*602-614) also prevented cleavage of the R peptide by the viral protease. These observations suggest that alterations of the cytoplasmic tail's secondary structure can influence the recognition of the R-peptide cleavage site by the viral protease or distort the normal interaction of the envelope protein cytoplasmic tail with the retroviral particle. Since cleavage of the R peptide is thought to be required for viral infectivity (31), the lack of processing could account for the reduced infectivity of the nonprocessed mutants, despite their ability to form syncytia when coexpressed with XC cells.

All of the mutations in the cytoplasmic tail that decreased the ability of the envelope protein to mediate cell-cell fusion were also detrimental to retroviral transduction. However, the converse was not always true, with several of the mutants that allowed extensive cell-cell fusion producing virions with very low titers. This observation was especially clear in the case of truncations of the tail amino terminal to the R-peptide cleavage site. Since the NIH 3T3-based GPL producer cells were extensively fused by these truncated envelope proteins, it is possible that lower amounts of infectious virus were a direct result of this cytotoxicity. Another explanation for the discrepancy between the effects on fusogenicity and infectivity is that some of these cytoplasmic tail mutations diminished the incorporation of the envelope protein into budding virions. Finally, it is also possible that there are differences in cell-cell fusion as assayed on XC cells and virus-cell fusion as measured by infection of NIH 3T3 cells that account for these differences.

The importance of the cytoplasmic tail for the incorporation of envelope proteins into retroviral particles is suggested by genetic analysis of HIV type 1 (6, 7) and M-PMV (3), although Rous sarcoma virus appears to be able to incorporate envelope protein without a cytoplasmic tail (23). For MoMuLV, certain mutations in the tail have previously been reported to affect incorporation. They include an insertion of 12 amino acids at residues I610 and S611 (12) and the deletion of residues N600 to V606 (11). Such a phenotype is also suggested by some of our mutants, including the complete truncation of the cytoplasmic tail (*tr*602) and the deletion of residues L602 and V603. Interestingly, a further group of mutants, including L602W/V603K and Q604W/F605T, retained the ability to fuse XC cells, were incorporated into virions at wild-type levels, and yet had reduced infectivity. While these discrepancies between fusogenicity and infectivity in the XC cell fusion and NIH 3T3 cell transduction could be artifacts of the assay systems used, it is also possible that these changes in the cytoplasmic tail affect some other step of the life cycle. Further experiments are under way to address these issues.

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