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The ''Putative'' Leucine Zipper Region of Murine Leukemia Virus Transmembrane Protein (P15e) Is Essential for Viral Infectivity

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In order to determine the role of the putative leucine zipper region of murine leukemia virus (MLV) transmembrane protein p15E, nine mutations in this region were introduced by site-directed mutagenesis. None of these mutations affected the expression or transport of the envelope protein or incorporation into virions. The mutants were analyzed for their ability to infect NIH3T3 cells and to induce cell fusion in a rat XC cell fusion assay. Mutations removing the charge of the hydrophilic residues reduced infectivity in NIH3T3 cells but had either no effect or a minor effect on envelope-induced XC cell fusion. Six mutations of hydrophobic residues of the putative leucine zipper region were constructed; four completely abolished the ability to infect NIH3T3 cells and these mutant envelopes were also unable to induce cell fusion in the XC cell fusion assay. These data demonstrate the absolute requirement for the putative leucine zipper region for both fusion and infection of MLV.

INTRODUCTION

The envelope protein of murine leukemia virus (MLV) consists of two polypeptides, an external glycosylated surface (SU) protein and a transmembrane anchoring protein (TM). SU is a 70-kDa glycoprotein, gp70, and TM is a 15-kDa polypeptide, p15E. The envelope gene product is synthesized as a precursor, gp80, which is cleaved by a host protease during its transport to the cell surface (Dickinson *et al.,* 1984; Witte *et al.,* 1977; Witte and Wirth, 1979). p15E is then further cleaved by the virally encoded protease to form p12E and a 16-amino-acid R-peptide (Green *et al.,* 1981). This cleavage occurs during and after virus release and serves to transform the TM protein from a fusion-incompetent protein to the competent form (Ragheb and Andersen, 1994; Rein *et al.,* 1994).

The envelope proteins oligomerize in the endoplasmic reticulum prior to transport to the cell surface and the gp70 and p15E proteins form a functional complex by noncovalent interactions and possibly a disulfide bond (Pinter and Flessner, 1979). Viral tropism is determined by a specific interaction between the SU protein and the viral receptor on the target cell. The ability of TM to oligomerize is thought to be essential for the stabilization and transport of the envelope proteins and for their transport to the cell surface. This has been demonstrated for other retroviruses (Earl *et al.,* 1990; Einfeld and Hunter, 1994; Hurtley and Helenius, 1989; Kreis and Lodish, 1986).

The TM protein has a number of functional regions. The first 35 amino acids form a fusion peptide and point mutations in this region have been shown to affect MLV-induced XC cell fusion (Jones and Risser, 1993). In p15E, the fusion peptide is adjacent to a putative leucine zipper motif which has been proposed to be involved in retroviral envelope oligomerization (Delwart *et al.,* 1990). Leucine zippers are characterized by an ability to form amphipathic alpha-helices displaying a pattern of hydrophobic and hydrophilic residues repeated every 7 positions (Cohen and Parry, 1986). They are present in a number of DNA binding proteins and have been shown to be essential for the dimerization of these proteins (Kouzarides and Ziff, 1988; Landschultz *et al.,* 1988). Putative leucine zippers are found next to the fusion peptides of a number of different viral fusion glycoproteins, including those of paramyxoviruses, influenza viruses, coronaviruses, and retroviruses (Chambers *et al.,* 1990). This suggests that these proteins may use similar strategies for viral fusion and entry.

The fusion protein of influenza virus (HA2) has been most extensively characterized and the three-dimensional structure is known (Wilson *et al.,* 1981). The HA2 molecule has a coiled coil backbone of three leucine zippers with the fusion peptides embedded within the hydrophobic core of the three helices. Upon a reduction in pH, a conformational change occurs which extends the length of the leucine zipper alpha-helix, resulting in the release of the fusion peptide and the adoption of a fusogenic state (Bollough *et al.,* 1994; Carr and Kim, 1993). In this system, therefore, the leucine zipper is central to the mechanism of fusion peptide sequestration and release. In HIV, a leucine zipper motif, in the N-

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terminal region of gp41, has been shown to be essential for infectivity. It seems that this region is not required for oligomerization but is required for some aspect of entry (Chen, 1994; Chen *et al.,* 1993; Soneoka *et al.,* 1995).

Here we describe the genetic analysis of the putative leucine zipper region of MLV TM protein p15E. Nine mutations have been introduced into this region and the effects on protein expression, viral envelope incorporation, envelope-mediated fusion, and infectivity have been analyzed. Our data show the importance of this region for infectivity as even conservative changes to a number of key hydrophobic residues adversely affect the ability of the envelope proteins to mediate virus infectivity. Furthermore, our analysis has functionally separated the phenomena of infection and XC cell fusion.

MATERIALS AND METHODS

Cell lines and culture

XC-1, NIH3T3, and 293T (293/tsa 1609 neo) (Dubridge *et al.,* 1987) cells were maintained in Dulbecco's modified Eagle's medium (GIBCO BRL) containing 10% FCS and supplemented with antibiotics. The 293T cells were obtained from D. Baltimore, Rockefeller University.

Mutagenesis and production of p15E mutant envelope expression plasmids

Nucleotide and amino acid positions for the ecotropic envelope correspond to the numbering of Shinnock *et al.* (1981). The MLV p15E coding region was PCR amplified from penv (Markowitz *et al.,* 1988) using the following primers: N-terminal primer 5′ AGGCCTGAATTCGGATCCATGGAACCGGTGTCGTTAACCCGGCC 3′ and C′ terminal primer 5′ AGGCCTGAATTCGGATCCCTATGGCTCGTACTCTATAG GCTTCA G 3′. The PCR product was digested with *Eco*RI and subcloned into pBluescript (KS^+) (Stratagene). The p15E insert was then fully sequenced.

The p15E insert was then excised from pBluescript with *Bam*HI and subcloned into pALTER-1 (Promega) and site-directed mutagenesis was performed using the Altered Sites mutagenesis system according to manufacturer's recommendations (Promega). All mutants were confirmed by dideoxy sequencing using the Sequenase system (U.S.B.). The whole p15E insert was then sequenced to ensure that the insert contained only the desired mutation.

Plasmid pRV187 contained the entire 2.4-kb ecotropic envelope gene from penv (Markowitz *et al.,* 1988). pRV187 was partially digested with *Nco*I (7229 bp) and digested to completion with *Bsm*I (7665 bp) in the envelope, respectively. The corresponding *Nco*I/*Bsm*I fragment from the pALTER-1 mutants was then subcloned into the pRV187 vector and the construct verified by dideoxy sequencing. The reconstructed mutant envelopes were then subcloned into the *Eco*RI site of plasmid pGW1HG, a CMV promoter expression plasmid containing intron A and the SV40 ori (Soneoka *et al.,* 1995), the mutation again being verified by dideoxy sequencing.

Production of virus containing p15E mutations

Virus was produced by transient three-plasmid transfection of pHIT60 (a CMV-driven *gag–pol* expression plasmid), mutant envelope expression plasmid as described above and vector DNA pRV172. PRV172 was constructed by subcloning the luciferase gene from pGEM-luciferase (Promega) into the polycloning site of pLNCX (Miller and Rosman, 1989) to generate pRV156. pRV156 was then digested with *Kpn*I and the proviral fragment ligated to the backbone of pRV109 (Soneoka *et al.,* 1995), resulting in plasmid pRV172. Briefly, 9 hr prior to transfection 293T cells were split onto 10-cm dishes so that the cells were 50% confluent before transfection. The cells were then transfected by overnight $CaPO_4$ transfection with a total of 30 mg DNA: 10 mg of the envelope expression plasmid, 10 mg pHIT 60, and 10 mg pRV172. The next day, the $CaPO_4$ precipitate was removed and 10 ml medium containing sodium butyrate to a final concentration of 10 m*M* was added. The cells were treated with sodium butyrate for 10 hr after which the cells were washed with PBS to remove any contaminating sodium butyrate and refed with 5 ml medium. Viral supernatants were harvested 12–16 hr later and polybrene (Sigma) was added to a final concentration of 8 mg/ml. The supernatant was filtered through a 0.2-μm filter and the infectivity assayed by infection of NIH3T3 cells. For infectivity assays to be standardized and to allow analysis of the expression of the mutant envelopes, the 293T producer cells were harvested by trypsinization and divided equally between two tubes. The cells in each tube were pelleted by centrifugation and washed with cold PBS followed by a further centrifugation. One pellet was resuspended in protein lysis buffer (9 *M* urea, 50 m*M* Tris, pH 7.4, 1% β-mercaptoethanol) and the other pellet was resuspended in luciferase lysis buffer (1% v/v Triton X-100, 25 m*M* Tris–phosphate, pH 7.75, 15% glycerol, 8 m*M* $MgCl_2$, 0.1 m*M* EDTA, 1 m*M* DTT, and 1% w/v BSA).

Infection of cells with virus

NIH3T3 and XC cells were split onto 5-cm dishes 24 hr prior to infection so that on the day of infection they were 70% confluent. Medium was removed, 1 ml of viral supernatant added, and the cells were placed in the incubator for 2–4 hr with gentle rocking every 30 min, after which 5 ml growth medium was added. The infection results were standardized by the measurement of luciferase activity in the transfected 293T cells for each mutant. Analysis of the luciferase activity in the 293T transfected cells allowed the results to take into account any difference in transfection efficiency and also any difference in the number of 293T virus producer cells. The

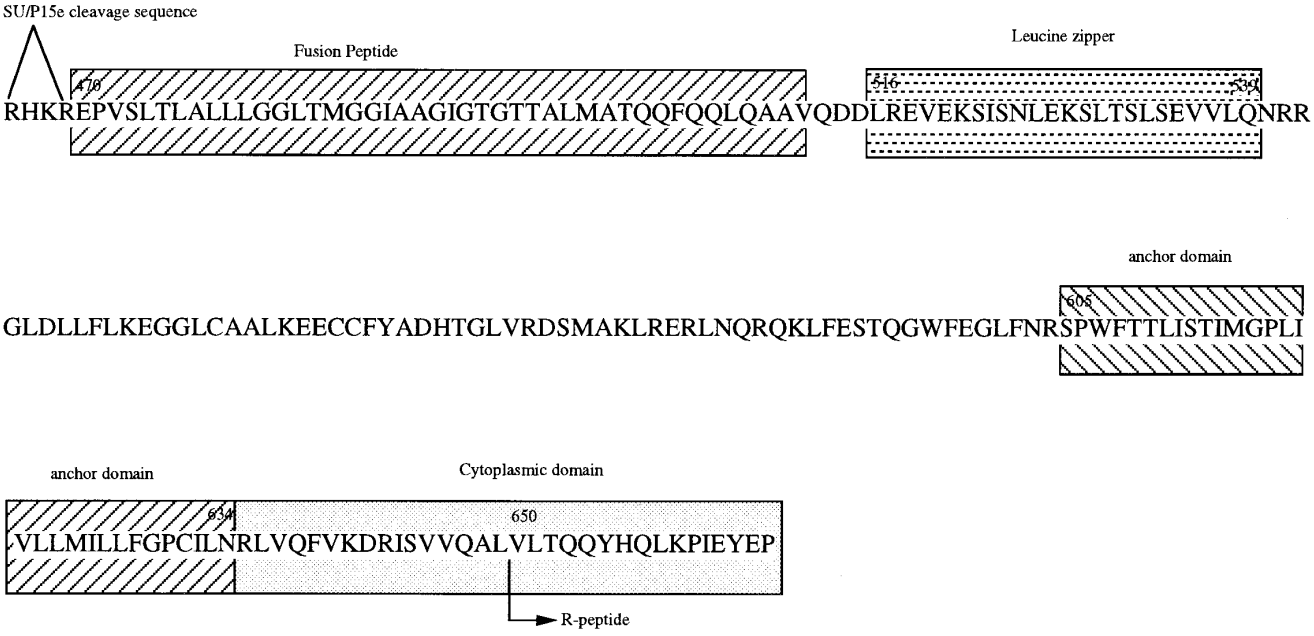

FIG. 1. MLV transmembrane protein p15E sequence (Rein *et al.,* 1994) showing the putative domains of the protein as boxed areas. The fusion peptide has been located to the N-terminal 40 amino acids (Green *et al.,* 1981) which is followed by a putative leucine zipper motif (Chen *et al.,* 1993). The protein contains a membrane anchoring region and a cytoplasmic domain which has the C-terminal 16 amino acids cleaved by a viral protease to transform the protein from a fusion-incompetent to a fusion-competent form (Morgan *et al.,* 1993; Pinter and Flessner, 1979).

same number of 293T cells were transfected but after several washes preceding transfection and sodium butyrate treatment a variable loss of 293T cells may have occurred. Therefore by measuring the luciferase activity of the 293T cells, an accurate measure of the transfection efficiency of the actual cells from which virus was obtained was accounted for. Within each experiment the luciferase activity of the transfected 293T cells never varied more than twofold.

Analysis of infection

Forty-eight and twenty-four hours postinfection the infected NIH3T3 or XC cells, respectively, were washed with PBS and harvested in 500 ml of luciferase lysis buffer. One milliliter of the 500 ml of 293T lysate and 50 ml of the 500 ml of the NIH3T3 lysate were analyzed for luciferase activity by diluting to 250 ml in 25 m*M* Tris–phosphate, pH 7.75, in a luminometer cuvette with 4 ml 40 m*M* ATP and assayed by injection of 100 ml of 2 m*M* luciferin (Promega) in a Lumac M2010 Biocounter. The light units/second were recorded. The luciferase activity of the 293T cells was then used to adjust the luciferase activity observed in the infected cells and all results were expressed relative to wild type which denoted a 100% infectivity.

Cocultivation fusion assay

A 1 × 5-cm dish of 70% confluent 293T cells was transfected with 10 mg of the mutant envelope expression plasmid by $CaPO_4$ transfection overnight and sodium butyrate treatment the following day as described for virus production. The next day the 293T cells were harvested and along with 10^6 XC cells were plated onto a 1 × 5-cm dish. Twenty-four hours postcoculture the cells were stained with 10% methylene blue in methanol. Squares (10 × 1 mm^2) were scored microscopically for syncytia (cells containing five or more nuclei).

Analysis of viral proteins

One milliliter of viral supernatant was centrifuged in a microcentrifuge tube for 30 min at 4°, and the pellet was then resuspended in cold PBS and recentrifuged. The washed pellet was resuspended in 50 ml protein lysis buffer (9 *M* urea, 50 m*M* Tris, pH 7.4, 1% β-mercaptoethanol) and stored at −20°. Aliquots were diluted in SDS loading buffer, heated at 90° for 5 min, and analyzed by SDS–PAGE. Specific MLV proteins were detected by Western blotting. gp70 was detected using a 1:3000 dilution of an antibody raised against RLV gp69/71 (Quality Biotech Inc., NJ). The gag protein was detected using an anti-p30 rat monoclonal obtained from the ATCC (CRL 1912) and used at a dilution of 1:4. The p15E and p12E proteins were detected using a 1:500 dilution of a rabbit polyclonal serum containing antibodies raised against the full-length gp80 protein molecule. The secondary antibodies used were horseradish peroxidase-conjugated anti-goat (Vector), anti-rat, or anti-rabbit (Sigma) immunoglobulins used at dilutions of 1:1000. Specific proteins were visualized using the enhanced chemiluminescence detection system (Amersham).

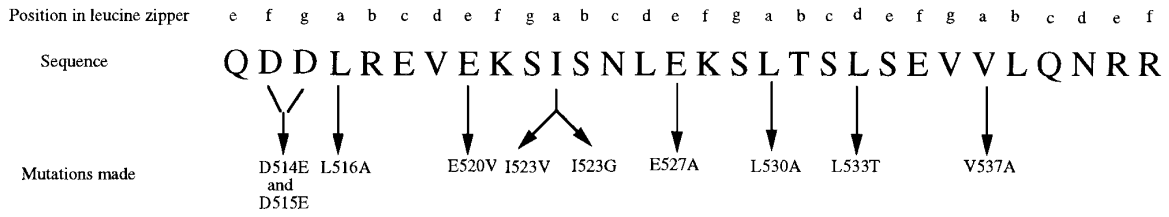

FIG. 2. The putative leucine zipper motif of MLV transmembrane protein p15E showing the mutations introduced for this study. The mutations are identified by the amino acid code and the position in the full-length envelope protein (Rein *et al.,* 1994) followed by the amino acid code in the mutated construct. The positions in the heptad repeat are shown (positions a–g).

RESULTS

Generation of virus containing p15E mutations

The putative domains of p15E are shown in Fig. 1. Nine mutations were made by site-directed mutagenesis in order to examine the role of the proposed leucine zipper region in virus infectivity (Fig. 2). Eight of the mutations were single point mutations at either the hydrophobic residues (positions a or d) or the charged hydrophilic residues (position e), and one mutation was a double mutation, D514E/D515E. This mutation was made because analysis of the p15E protein sequence using the PC/Gene beta turn prediction program suggested that the two aspartic acid residues at these positions could form a beta bend. By analogy with the influenza hemagglutinin structure it was considered possible that this beta bend could be present in a fusion-inactive form which upon activation forms an alpha-helical structure expelling the fusion peptide (Carr and Kim, 1993). Mutations to glutamic acids were made because it was hoped that this would have no effect on the charge of the protein but would prevent the formation of a beta bend and allow the adoption of a fusion-active p15E conformation. Mutations are identified by the amino acid code and the position in the full-length envelope (Shinnock *et al.,* 1981) followed by the amino acid code in the mutated construct (Fig. 2). The mutant envelopes were inserted into the mammalian expression vector pGW1HG and virus was generated by transiently transfecting 293T cells with the mutant envelope expression plasmid, a *gag–pol* expression cassette (pHIT60), and a luciferase vector (pRV 172) as described under Materials and Methods.

In order to determine whether each mutant envelope was expressed at the same level as the wild-type envelope and to assess whether the mutation adversely affected protein stability, transfected 293T cells were harvested and 10 mg of total cell protein was subjected to SDS–PAGE followed by Western blotting. Probing for gp80 demonstrated that envelope expression for all mutants was equivalent to wild-type expression (Fig. 3). This demonstrated that the point mutations had no effect on either the expression of the envelope protein or its stability. The data in Fig. 3 suggest that there may be some differences in processing to gp70, but this was not reproducible and therefore not significant.

Analysis of infectivity in NIH3T3 cells of viruses containing p15E mutations

Mutant viruses produced by the transfected 293T cells were tested for their ability to infect NIH3T3 cells as described under Materials and Methods. Briefly, 48 hr postinfection the NIH3T3 cells were harvested in luciferase lysis buffer and assayed for luciferase activity. The results are shown in Fig. 4. The results demonstrate with one exception (V537A) that changes, including conservative changes, to the hydrophobic residues of the putative leucine zipper region had gross effects on the viral infectivity. Mutations L516A, I523G, L530A, and L533T all showed complete inhibition of infectivity and mutation I523V showed a reproducible infectivity of 0.16% of wild-type infectivity. In contrast, V537A showed a slight increase over wild-type levels of infection. Mutations to charged hydrophilic residues reduced infectivity to varying degrees. The D514E/D515E completely abolished infectivity, supporting the notion that these residues are within a turn region. Mutant viruses E520V showed 53% infectivity compared to wild type and mutant E527A approximately 10% infectivity. Unlike the hydrophobic residues, therefore, in some cases the charged hydrophilic residues could be mutated even nonconservatively without completely abolishing infection.

Analysis of mutant envelope incorporation into viral particles

In order to ensure that the differences in infectivity were not a result of differences in the mutant envelope incorporation into viral particles, the surface and gag-p30 levels were analyzed in the virus (Fig. 5a). For all mutant envelopes the ratio of gp70 to p30 was approximately equal. This demonstrated that all the mutant envelope molecules were processed normally, transported to the cell membrane, and incorporated into the virus envelope as efficiently as the wild-type envelope. Figure 5 shows slight differences in the processing of the gp80 precursor to the mature surface gp70 molecule between different envelope mutants as was the case for the cell extracts (Fig. 3). Again, these differences were not reproducible, and the same mutant often showed small differences in the extent of apparent processing with each virus preparation.

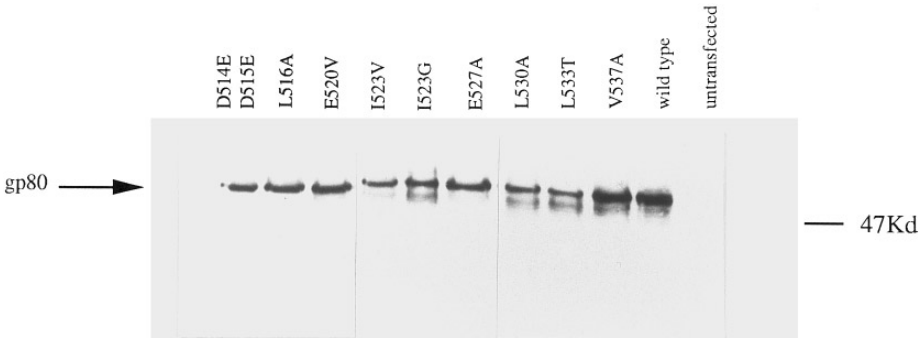

FIG. 3. gp80 expression in transfected 293T cells. 293T cells transfected with mutant envelope were harvested in protein lysis buffer as described under Materials and Methods and the protein was then subjected to SDS–PAGE followed by Western blotting probing for gp80. Envelope gp80 protein was detected using a 1:3000 dilution of an antibody raised against RLV gp69/71 in goat (Quality Biotech Inc., NJ). The secondary antibody used was a horseradish peroxidase-conjugated anti-goat (Vector) and specific proteins were visualized using the enhanced chemiluminescence detection system (Amersham).

Recently, it has been shown that removal of the R-peptide from the C-terminus of p15E to form p12E is important for syncytium formation in a cell fusion assay and it has been suggested that this cleavage event activates the molecule for fusogenesis (Ragheb and Andersen, 1994; Rein *et al.,* 1994). To demonstrate that the mutant envelope molecules were capable of undergoing this p15E:p12E cleavage, virus was analyzed by Western blotting using a probe that would detect p15E and p12E (Fig. 5b). This demonstrated that all mutant envelope molecules cleaved to form approximately equal ratios of p15E and p12E to that observed for wild-type virus. It was therefore concluded that any difference in the infectivity of the different mutants was not a result of an inability to cleave at the p15E:p12E cleavage site.

Analysis of fusion ability of p15E mutants

Taken together these data show that the defects in the mutants were not due to a disruption of the integrity or stability of p15E, its cleavage to p12E, or its ability to be incorporated into virus. We asked therefore if p15E mutants were compromised in infectivity as a result of an inability to mediate fusion. Mutant envelopes were analyzed for their ability to fuse in an XC fusion assay. XC cells have been widely used to study the ability of different ecotropic envelopes to induce fusion (Jones and Risser, 1993; Morgan *et al.,* 1993; Ragheb and Andersen, 1994). XC cells can be infected by MLV in a pH-independent manner and have the ability to fuse and form syncytia with cells which express the ecotropic envelope on

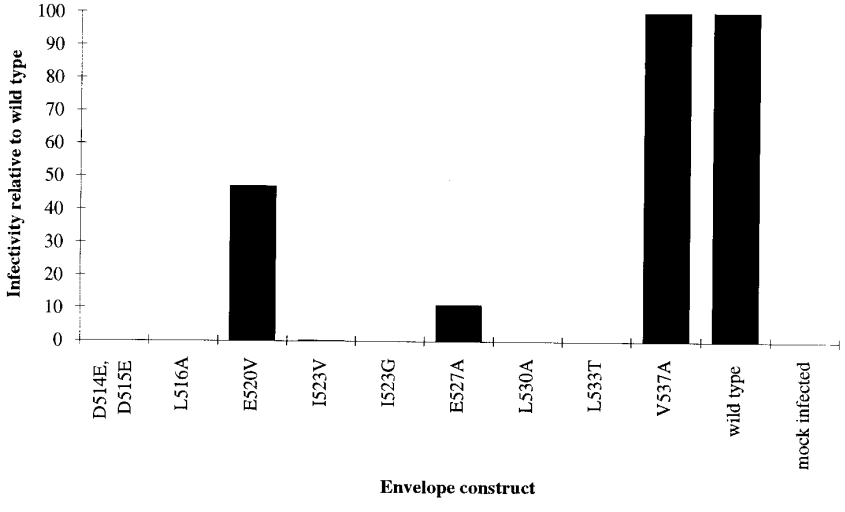

FIG. 4. Relative infectivities in NIH3T3 cells of the different p15E mutants. Expression constructs were generated by inserting the mutant *Nco*I (7229 bp)–*Bsm*I (7665 bp) p15E fragment into the full-length envelope sequence which was then subcloned into pGW1HG. Virus was then rescued using the three-plasmid cotransfection method as described under Materials and Methods and was used to infect NIH3T3 cells. Luciferase activity in the infected cells 48 hr postinfection was measured. The 293T producer cells were also assayed for luciferase activity so that the infectivity results could then be normalized to wild type accounting for slight variability in the efficiency of 293T transfection and any variation in the number of virus producer cells. This figure shows the averaged results of three independent experiments. The errors observed over three experiments were within ±30% for all mutants.

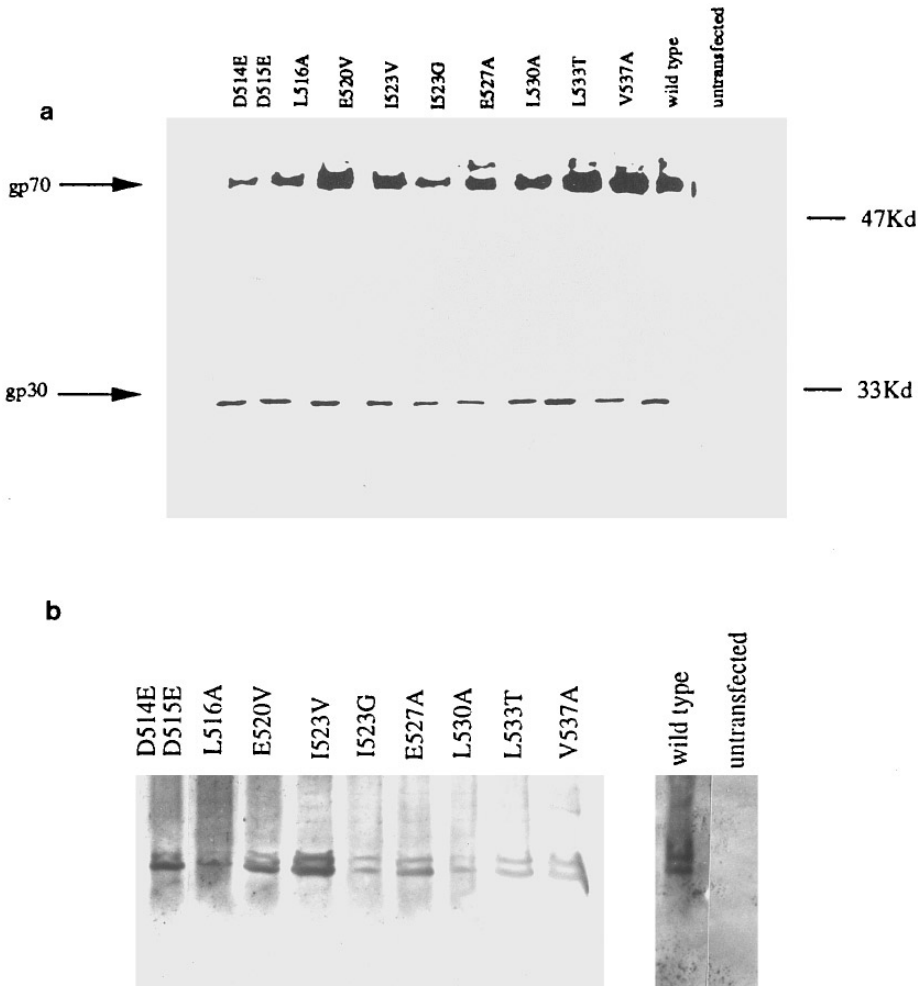

FIG. 5. Virus generated by a three-plasmid cotransfection was pelleted as described under Materials and Methods. The virus was resuspended in protein lysis buffer. For the detection of gp80 and p30 proteins were subjected to SDS–PAGE followed by Western blotting. For the detection of p15E and p12E protein was subjected to Tricine–SDS–PAGE (Rein *et al.,* 1994) followed by Western blotting. (a) gp80 and p30 expression in virus particles. Envelope gp70 protein was detected using a 1:3000 dilution of an antibody raised against RLV gp69/71 in goat (Quality Biotech Inc., NJ) and gag protein was detected using an anti-p30 rat monoclonal obtained from the ATCC (CRL 1912) used at a dilution of 1:4. The secondary antibodies used were horseradish peroxidase-conjugated anti-goat (Vector) and anti-rat (Sigma) immunoglobulins used at dilutions of 1:1000. Specific proteins were visualized using the enhanced chemiluminescence detection system (Amersham). (b) p15E and p12E expression in virus particles. p15E and p12E were detected using a 1:500 dilution of a rabbit polyclonal sera raised against gp80. The secondary antibodies used were horseradish peroxidase-conjugated anti-rabbit (Sigma) immunoglobulins used at dilutions of 1:1000. Specific proteins were visualized using the enhanced chemiluminescence detection system (Amersham).

their cell surface (McClure *et al.,* 1990). The results with our mutant envelopes are summarized in Fig. 6. The average number of syncytia formed for wild-type envelope over three experiments was 5873 syncytia/mg transfected DNA. Interestingly the hydrophobic residue mutations which showed no infection also showed no fusion. Similarly, mutant I523V which had a severely reduced infectivity of 0.16% in NIH3T3 cells also had a reduced ability to induce XC cell fusion at 5% of wild-type activity. The D514E/D515E mutant showed no fusion activity, which matched its infectivity in 3T3 cells. In contrast, mutants E520V and E527A showed reduced infectivites in NIH3T3 cells (53 and 9.3% infectivity relative to wild type, respectively) but demonstrated 100% XC cell fusion. In the case of these mutants therefore the ability to efficiently fuse in XC cells did not correlate with their low infectivity in NIH3T3 cells. This could reflect a difference between the fusion process in XC cells and the fusion event occurring upon infection or alternatively, and more likely, the results support the notion that the fusion event is not the only event required for infection.

Analysis of infectivity in XC cells of viruses containing p15E mutations

It was interesting to observe a discrepancy between the XC cell fusion and NIH3T3 infectivities of mutants E520V and E527A. This discrepancy was only slight for mutant E520V but was reproducible and substantial for

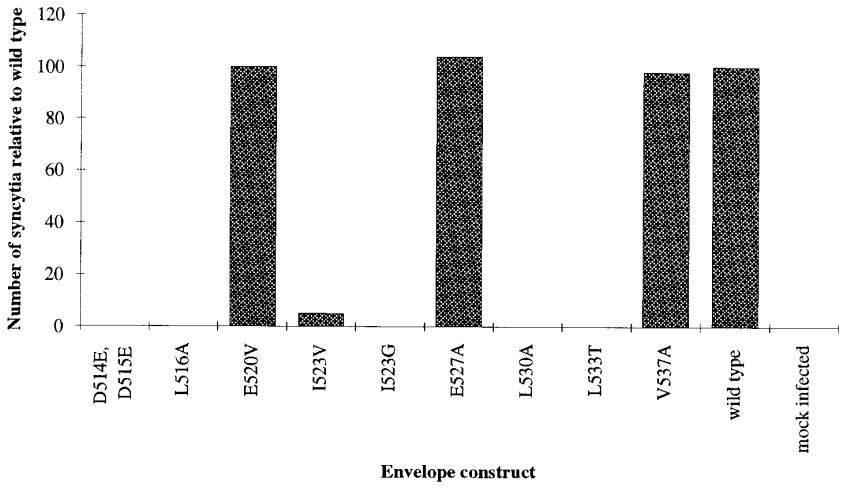

FIG. 6. XC cell fusion assay. The mutant envelope expression constructs were transfected into 293T cells and 48 hr posttransfection were cocultivated with XC cells. 24 hr later the cells were stained with 10% methylene blue in methanol. 10 $\times$ 1 mm^2 of the tissue culture dish was scored microscopically for syncytia (cells containing 5 or more nuclei) and the averaged results of three independent experiments are shown graphically. The fusion of each mutant envelope was compared to wild-type envelope which was designated a 100% fusion ability. The errors observed over the three experiments were within $\pm$0% for all mutants.

mutant E527A, which demonstrated an infectivity of 9% but an XC fusion ability of 104% relative to wild type. In order to investigate whether this XC fusion ability of the envelope mutants corresponded to the infectivity in XC cells, mutant viruses which were able to induce at least some fusion in the XC fusion assay were tested for their ability to infect XC. This was done using the same viral supernatants as were used for two of the NIH3T3 infections. Luciferase activity in the XC cells was quantitated and the results were expressed relative to wild type as shown in Fig. 7. These data show that in general there is a good correlation between the ability to fuse XC cells and XC cell infectivity, but this does not extend to an ability to infect NIH3T3 cells. Mutants E527A and I523V in particular showed XC fusion abilities which matched XC infectivity but not NIH3T3 infectivity and these mutants therefore highlight a difference between the infection process occurring in XC cells and that in NIH3T3 cells. This may be a function of the fact that infection in XC cells occurs independent of a reduction in pH while in NIH3T3 cells a pH drop is required for infection. Mutants E527A and I523V may not be able to undergo the low-pH step essential for NIH3T3 infection but in XC cells, where this step is not required, they show no aberrant phenotype. Alternatively, these data may simply reflect the fact that XC cells are more fusogenic and therefore more infectable.

DISCUSSION

It has been suggested that the extracytoplasmic domain of MLV transmembrane protein p15E has a struc-

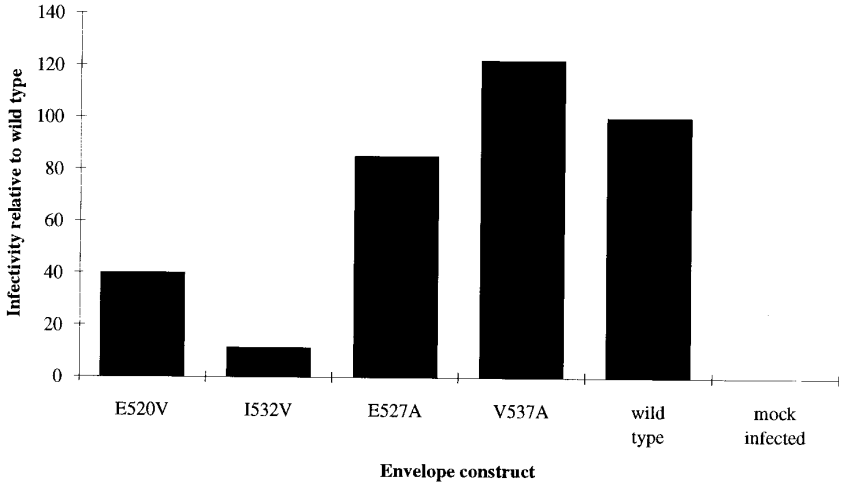

FIG. 7. Infectivities of mutant viruses in XC cells. Mutant envelopes which induced XC cell fusion were tested for their ability to infect XC cells. Averaged results for two experiments are expressed relative to wild type (100%). Errors over the two experiments were within $\pm$40%.

ture similar to that of the fusion proteins of other viruses (Chambers *et al.,* 1990). A number of p15E mutations in the hydrophobic residues at positions a and d (Fig. 2) were generated and the effect on fusion and infectivity in NIH3T3 and XC cells was studied. Most of the mutations of the hydrophobic residues resulted in complete or substantial inhibition of infectivity, the exception being V537A, which had wild-type infectivity. It is interesting that even conservative changes such as L530A and I523V had gross effects on infection. These phenotypes were not a result of differences in expression, disruption of incorporation of mutant proteins into the virus, or cleavage of p15E to form p12E and R-peptide. Interestingly, V537 tolerated a mutation to an alanine, which may suggest that this residue lies outside the functional leucine zipper region. However, V537A is the most conservative change of the hydrophobic residue mutations made in this study, the relative occurrence of valine at position a of the leucine zipper being only slightly higher than the relative occurrence of alanine at that position (Lupas *et al.,* 1991). This mutation may therefore have no effect on the leucine zipper structure.

The fusion results (Fig. 6) show that mutation of the hydrophobic residues results in an inability of these envelopes to fuse with XC cells. This suggests that the hydrophobic residues form a structure which is involved in the fusion event associated with infection. The working hypothesis is that these residues form a hydrophobic face of an alpha-helix which, as a trimer, forms all or part of a hydrophobic "pore" (White, 1992). Data presented here are consistent with the notion that the hydrophobic residues maintain the structure of this fusion "pore." Furthermore, taken together our data indicate that the putative leucine zipper of MLV has a more restricted structure than that of the HIV gp41 leucine zipper, for example, when conservative mutations to valine or leucine were made at the middle isoleucine of the leucine zipper of gp41 cell–cell fusion, infections were not blocked, and a mutation to alanine had an intermediate effect (Dubay *et al.,* 1992).

A number of events seem to be involved in MLV entry. Recently, it has been shown that removal of the R-peptide at the C-terminus of p15E results in increased levels of syncytium formation in a cell fusion assay (Ragheb and Andersen, 1994; Rein *et al.,* 1994). This cleavage event, by the viral protease, occurs during and after budding and it has been suggested that this cleavage activates the envelope molecule for fusogenesis (Ragheb and Andersen, 1994; Rein *et al.,* 1994). Interestingly, this cleavage is only 50% efficient in the viral particles (Andersen and Nexo, 1983; Pinter and Flessner, 1979) but, consistent with the idea that this R-peptide cleavage event is essential for infection, we have shown that where the R-peptide is completely deleted, particles which contain 100% p12E are twofold more infectious in NIH3T3 cells than wild type (data not shown). MLV infection also requires a low-pH step (Andersen and Nexo, 1983; McClure *et al.,* 1990) possibly occurring in the cellular endosomes after gp70 receptor binding. This is supported by McClure *et al.* (1990) who have demonstrated that acid treatment before adsorption did not activate MLV, suggesting that a reduction in pH does not actually induce a conformational change necessary for membrane fusion but instead it is required for a subsequent event. Work with lysomotropic agents supports the role of the endosome (Andersen and Nexo, 1983; Marsh and Helenius, 1989; Wilson *et al.,* 1992) as do recent data with fluorescently labeled ecotropic virions (Rein *et al.,* 1994). Low pH may result in a conformational change to directly activate fusogenesis and infection in a manner similar to the mechanism of Influenza infection or, alternatively, it has been suggested that low pH may alter the conformation of the envelope proteins to allow the access of a proteolytic enzyme which acts upon gp70, this cleavage being required for a subsequent step in infection (Andersen and Nexo, 1983).

XC cells are particularly sensitive to MLV-induced fusion (Klement *et al.,* 1969) but unlike other cells which MLV infects, infection in XC cells occurs by a pH-independent route. One possible explanation for this is that in XC cells there is a cell surface protease which acts upon the ecotropic envelope at neutral pH to mediate the gp70 cleavage event (McClure *et al.,* 1990).

We have shown that an ability to induce XC cell fusion does not necessarily correlate with infectivity, suggesting that these events are functionally distinct. It could be argued that the fusion event in an XC cell fusion assay may be unrelated to the fusion event occurring upon infection. This seems unlikely and indeed we have shown that an inability to fuse in this system generally correlates well with an inability of the virus to infect. This is demonstrated by mutants D514E/D515E, L516A, I523G, L530A, and L533T. However, where fusion was observed, the level of fusion did not always correlate with the level of infection in NIH3T3 cells as highlighted by mutant E527A which showed 100% fusion ability but only 9% NIH3T3 infectivity. This leads us to suggest that the adoption of a fusogenically competent envelope molecule is not the only step required for viral entry.

In summary, we have demonstrated the importance of the hydrophobic residues of the putative leucine zipper motif of p15E.

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