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Mutations within a Putative Cysteine Loop of the Transmembrane Protein of an Attenuated Immunodeficiency-Inducing Feline Leukemia Virus Variant Inhibit Envelope Protein Processing

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A replication-defective feline leukemia virus molecular clone, 61B, has been shown to cause immunodeficiency in cats and cytopathicity in T cells after a long latency period when coinfected with a minimally pathogenic helper virus (J. Overbaugh, E. A. Hoover, J. I. Mullins, D. P. W. Burns, L. Rudensey, S. L. Quackenbush, V. Stallard, and P. R. Donahue, Virology 188:558–569, 1992). The long-latency phenotype of 61B has been mapped to four mutations in the extracellular domain of the envelope transmembrane protein, and we report here that these mutations cause a defect in envelope protein processing. Immunoprecipitation analyses demonstrated that the 61B gp85 envelope precursor was produced but that further processing to generate the surface protein (SU/gp70) and the transmembrane protein (TM/p15E) did not occur. The 61B precursor was not expressed on the cell surface and appeared to be retained in the endoplasmic reticulum or Golgi apparatus. Two of the four 61B-specific amino acid changes are located within a putative cysteine loop in a region of TM that is conserved among retroviruses. Introduction of these two amino acid changes into a replication-competent highly cytopathic virus resulted in the production of noninfectious virus that exhibited an envelope-protein-processing defect. This analysis suggests that mutations in a conserved region within a putative cysteine loop affect retroviral envelope protein maturation and viral infectivity.

Retroviral extracellular surface glycoprotein (SU) and transmembrane protein (TM) envelope polypeptides are synthesized as a single polyprotein precursor that is proteolytically cleaved to produce the mature functional envelope proteins. The envelope precursor enters the secretory pathway during translation, the signal sequence is cleaved, and the precursor is glycosylated in the endoplasmic reticulum. After glycosylation, the envelope precursor protein oligomerizes and the complexes are transported to the Golgi apparatus. In the Golgi apparatus, high-mannose oligosaccharides are modified to complex and hybrid carbohydrate side chains, and the glycoprotein precursor is cleaved by a host cell protease to generate SU and TM. The mature envelope proteins are then transported to the cell surface as an oligomeric complex (for a review, see reference 27).

The envelope protein has been implicated as the determinant of disease specificity for variants of feline leukemia virus (FeLV) that cause immunodeficiency (12, 45). Viral chimeras that encode the SU of immunodeficiency-inducing replication-defective variants 61C or 61B in the context of a mildly pathogenic, replication-competent provirus, 61E, cause cytopathic effects in feline T cells and immunodeficiency disease in cats (12, 39, 45). However, 61B is delayed compared with 61C in its ability to cause cytopathic effects in T cells and immunodeficiency in cats as a result of four amino acid differences in the envelope TM protein (40, 53). Two of the changes should introduce positively charged residues in place of negatively charged residues in a region of TM that is highly conserved in FeLV and among other retroviruses (10, 41, 50).

In this study, we have investigated the effect of the predicted amino acid differences in 61B and 61C TM on envelope protein synthesis and processing using stable cell lines expressing 61C, 61B, or chimeric envelope proteins. This analysis shows that the 61B envelope precursor protein is not processed to SU and TM and that 61B-specific TM sequences between two highly conserved cysteine residues confer the envelope-processing defect. The 61B envelope precursor protein cannot be detected on the cell surface and appears to be sequestered in the endoplasmic reticulum or Golgi apparatus. In addition, the two 61B-specific amino acid changes in TM abolish the infectivity of an infectious molecular clone.

MATERIALS AND METHODS

Plasmid constructions and mutagenesis. The plasmids p61C, p61B, pEECC, and pCCC([CB]C) have been described previously (39, 53) and are diagrammed in Fig. 1. Chimera CCC([CB]C]) consists of 61C proviral DNA that contains 154 amino acids of the 61B TM.

The plasmid pEECC-env$K_{536}K_{537}$ was constructed by the following procedure: two nucleotide changes were introduced into p61C by a ligase-mediated PCR technique (35) using a phosphorylated mutagenic primer, *61B-env24* (5′GAAG CAACATT**T**TTT**T**TTTTAATGCGGCACAG3′). This primer contained 61B-specific sequences at *env* nucleotides 1606 and 1609 (i.e., amino acids 536 and 537 of the envelope precursor), which introduced T changes (underlined and in boldface for the primer) relative to 61C. Primers that hybridize to the 3′ end of SU (61C [GenBank M18246] *env* nucleotides 1040 to 1060) and to the 3′ end of TM (61C *env* nucleotides 1904 to 1929) were used to generate a 890-bp fragment containing the mutagenic primer.

PCR was performed as described by Michael (35). The desired 890-bp product was purified after gel electrophoresis and then cloned into a 3′ 61C subclone (p3′CC [39]) using conserved *Nco*I and *Rsr*II sites. To reconstruct a full-length provirus, the *Eco*RI-*Xho*I long terminal repeat (LTR)-*gag-pol* from 61E (6.5 kb) was ligated into p3′CC-env$K_{536}K_{537}$. The structure of the plasmid (pEECC-env$K_{536}K_{537}$) was verified by restriction fragment analysis. The PCR-amplified portion of the genome was sequenced by using the Sanger dideoxy chain termi-

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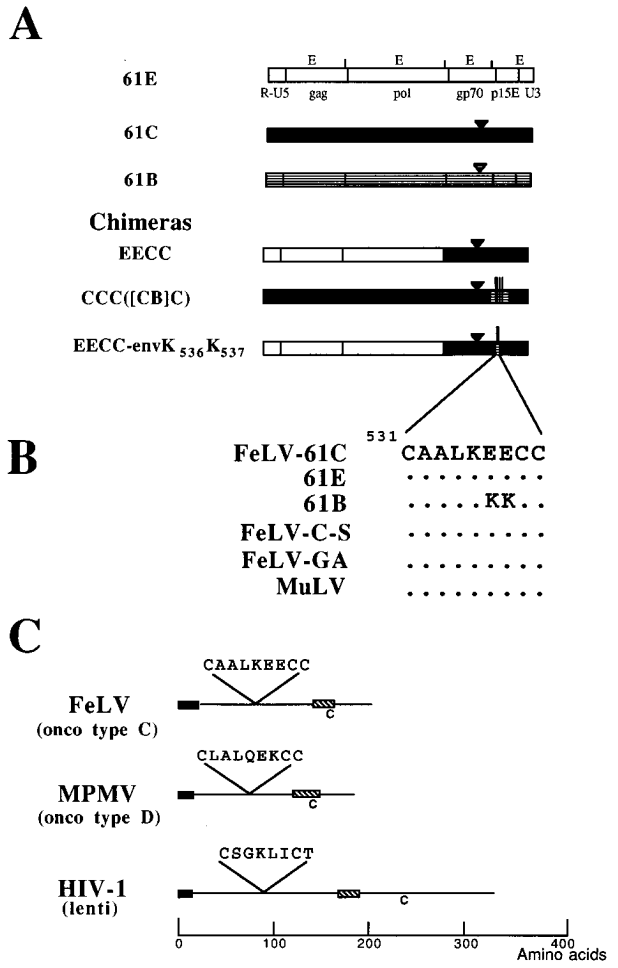

FIG. 1. (A) Schematic diagram of 61E, 61C, and 61B viral genomes and chimeras. The chimeras are named according to the system used previously (12, 40, 46). For the chimeras with four-letter names, the letters designate, respectively, the 5′ LTR-*gag* segment; the *pol* segment; the *Xho*I-*Nco*I fragment encoding a small segment of 3′ *pol* and most of *env*; and the *Nco*I-*Eco*RI fragment encoding the C-terminal end of gp70, all of p15E, and the 3′ LTR. Parentheses in the names of subsequent chimeras indicate a subdivision of the fourth segment, and brackets denote a further subdivision of part of that segment. The triangle denotes the location of the six-amino-acid insertion that is a primary determinant of pathogenicity (12, 45), and the vertical lines indicate the locations of amino acid differences in 61B relative to 61C or 61E in TM (53). (B) Amino acids identical to those in the 61C reference sequence are denoted by dots. The 61C sequence starts at amino acid 531 of the gp85 coding region. Shown are sequences for FeLV 61E, 61C, and 61B (39, 40), FeLV-C-Sarma (FeLV-C-S) (47), FeLV-GA (subgroup B FeLV, Gardner-Arnstein isolate) (18), and murine leukemia virus (MuLV; the sequence is identical for Moloney murine leukemia virus [49], Akv murine leukemia virus [31], and Friend murine leukemia virus [28]). (C) Diagram of representative TM proteins. The black boxes denote the fusion peptides, and the cross-hatched boxes represent the membrane-spanning regions. onco, MPMV oncovirus (50); lenti, HIV-1 lentivirus (Bru isolate) (55).

nation method (48) to verify that the desired changes were present and that there were no additional mutations introduced during amplification.

Transfection and isolation of stable cell lines. AH927 feline embryonic fibroblasts and 3201 T cells were cultured as described previously (40). Stable AH927 cell lines that contain 61C and 61B proviral DNA have been described previously (39). We used a similar strategy to establish cell lines that contained various FeLV chimeras. Briefly, FeLV proviral DNA plus pMOneo (20) was transfected into AH927 cells at a 3:1 molar ratio using calcium phosphate (Stratagene, La Jolla, Calif.) (6). Single-cell clones were isolated after selection in 0.6 mg of G418 (Gibco/BRL, Grand Island, N.Y.) per ml for 2 to 3 weeks. Individual colonies were analyzed for the presence of proviral DNA by Southern blotting (40) and for the expression of $p27^{gag}$ by enzyme-linked immunosorbent assaying (ELISA) (Virachek/FeLV; Synbiotics, San Diego, Calif.). Total cellular RNA, prepared by the guanidinium isothiocyanate procedure (8), was analyzed by Northern (RNA) blotting using the LTR-specific exU3 probe (37) to ensure that the expected full-length and subgenomic mRNAs were expressed.

3201 cells were transfected using Lipofectamine (Gibco/BRL) and 2 μg of pEECC or pEECC-env$K_{536}K_{537}$ plasmid DNA according to the supplier's instructions. Cultures were monitored by FeLV $p27^{gag}$ ELISA for virus replication and spread at 3- to 4-day intervals for 5 weeks after transfection.

Metabolic labeling and radioimmunoprecipitation of cellular lysates. Radioimmunoprecipitation analysis (RIPA) was performed with minor modification of a method described previously (43). Cells were labeled for lengths of time specified in the figure legends in methionine- and cysteine-deficient Dulbecco's modified Eagle's medium supplemented with 100 μCi of [^{35}S]methionine plus [^{35}S]cysteine per ml (Trans-Label; ICN, Irvine, Calif.). After labeling, cells were washed twice in cold phosphate-buffered saline and then lysed in cold lysis buffer A (25 mM Tris hydrochloride [pH 8.0], 0.15 M NaCl, 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride [56]). Cellular lysates were clarified by centrifugation at 15,000 × *g* at 4°C for 5 min, which was followed by preclearing with protein A-Sepharose beads overnight with rocking at 4°C. Incorporated radioactivity was determined by precipitation using trichloroacetic acid. An equal number of trichloroacetic acid-precipitable counts was added to each RIPA reaction mixture.

Antibodies were obtained from Custom Monoclonals, Sacramento, Calif. (anti-SU monoclonal antibody C11D8 and anti-TM monoclonal antibody PF6J-2A) or Quality Biotech Inc., Resource Lab, Camden, N.J. (serum no. 77S000301). Serum no. 77S000301 is a goat polyclonal antiserum that was made using denatured FeLV virion as immunogen.

Antibody was mixed with protein A-Sepharose beads for 30 min at 4°C, which was followed by washing once in 50 mM Tris (pH 8.0)–150 mM NaCl. Cell lysates were added to antibody-protein A beads and incubated with rocking for 3 h at 4°C. RIPA reaction mixtures were washed five times in 1 ml of cold wash buffer (0.05 M Tris [pH 7.2], 0.15 M NaCl, 0.1% sodium dodecyl sulfate [SDS], 0.1% Triton X-100) on ice, and then the beads were resuspended in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and analyzed by SDS-PAGE (30) and fluorography.

For endo-β-*N*-acetylglucosaminosidase H (endo H) digestions, beads containing immunoprecipitated proteins were boiled for 3 min and then subjected to digestion using conditions described by the supplier (Genzyme, Cambridge, Mass.). Endo H digestion was performed at 37°C for 1 h, additional enzyme was added, and digestion was continued overnight.

Metabolic labeling and radioimmunoprecipitation of culture supernatant. For analysis of proteins present in the culture supernatant, subconfluent cells were labeled in methionine- and cysteine-deficient medium that contained 300 μCi of [^{35}S]methionine plus [^{35}S]cysteine per ml for 1 h, which was followed by the addition of an equal volume of complete medium and incubation for 22 h. Culture supernatant was clarified by centrifugation, and 5× detergent mix (56) was added before RIPA was performed.

FACS. Immunostaining and fluorescence-activated cell sorting (FACS) were performed by standard methods. Cells were incubated with primary antibody (C11D8) at a concentration of 34 μg/ml at 37°C. Cells were incubated at 4°C with 50 μg per ml of secondary antibody, an anti-mouse immunoglobulin G2B-fluorescein isothiocyanate (Fisher, Pittsburgh, Pa.).

RESULTS

Comparison of envelope protein processing in cell lines expressing 61C and 61B. To define the biochemical defect associated with a replication defect encoded by the 61B envelope gene, we examined the envelope protein produced by the 61B provirus. For this purpose, we used stable cell lines that contain 61B or 61C replication-defective proviral genomes. In these cell lines, full-length viral RNA and subgenomic envelope RNA are expressed but no infectious virus is produced, because of a mutation(s) in the 5′ LTR, *gag*, or *pol* (39). Envelope proteins were analyzed by RIPA of lysates of cells labeled with [^{35}S]methionine and [^{35}S]cysteine for 7 h (Fig. 2A). Immunoprecipitation was performed using C11D8, an antibody that recognizes an epitope (MGPNL) found in the FeLV SU protein (25). This antibody did not recognize any AH927 cellular proteins in an immunoprecipitation reaction (Fig. 2A, lane 1). The gp85 envelope precursor and the mature SU protein, gp70, were precipitated from the lysates of cells expressing 61C (lane 3). The 61C lysate contained approximately equal amounts of gp70 and gp85. Similar results were obtained for 61E-infected cells (Fig. 2A, lane 2). However, in cell lines expressing the 61B envelope protein (lanes 4 and 5), only the gp85 envelope precursor was detected. Mature 61B

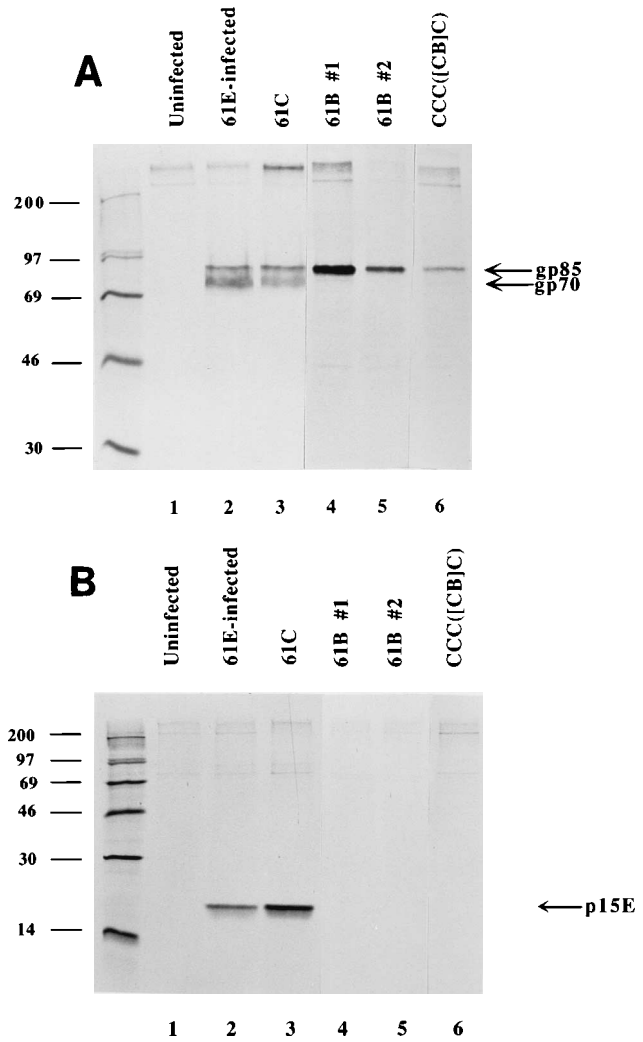

FIG. 2. Immunoprecipitation of envelope proteins from AH927 cell clones expressing defective 61C, 61B, or chimeric viral genomes. AH927 cells were labeled with [^{35}S]methionine plus [^{35}S]cysteine for 7 h. The viral genome expressed in each cell line is indicated above each lane. 61B nos. 1 and 2 are independent cell lines expressing 61B. Molecular weight markers (in kilodaltons) are labeled adjacent to the bands, as are gp85, gp70, and p15E. (A) Cellular lysates immunoprecipitated with the anti-gp70 and -gp85 antibody C11D8 and analyzed by SDS-9% PAGE; (B) Cellular lysates immunoprecipitated with the anti-p15E antibody PF6J-2A and analyzed by SDS-12.5% PAGE.

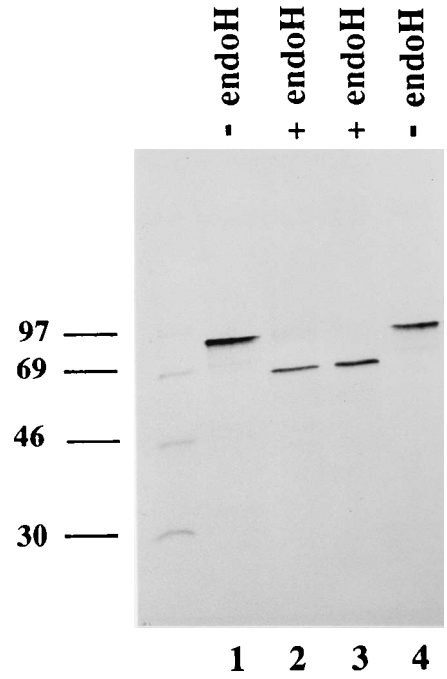

FIG. 3. Endo H analysis of 61B envelope protein. AH927 cells expressing 61B envelope protein were labeled with [^{35}S]methionine plus [^{35}S]cysteine for 7 h. Cellular lysates were immunoprecipitated using C11D8 antibody. Precipitated proteins were incubated in duplicate overnight in the presence (lanes 2 and 3) or absence (lanes 1 and 4) of endo H and analyzed by SDS-9% PAGE.

SU protein was not detected by Western blot (immunoblot) analysis or in pulse-chase experiments, even at times when most of the 61C envelope protein had been processed to gp70 (data not shown).

Figure 2B shows the results of RIPA of cellular lysates using an anti-TM antibody, PF6J-2A, which recognizes an epitope in the amino terminus of FeLV TM, upstream of the region where 61B and 61C sequences differ (Custom Monoclonals); however, this antibody reacts poorly with the gp85 envelope precursor. PF6J-2A antibody did not precipitate any labeled proteins from uninfected AH927 cells (lane 1). The expected TM protein (p15E) was detected in lysates of cells expressing 61C envelope (lane 3) and in 61E-infected cells (lane 2). In contrast, no p15E was immunoprecipitated in the cells expressing 61B envelope (lanes 4 and 5), and a longer exposure of the gel did not show any detectable p15E in these lysates (data not shown). Taken together, the absence of mature 61B SU and TM protein in cellular lysates suggests that 61B envelope processing is defective.

Localization of 61B envelope protein. Because mature 61B envelope proteins could not be detected, we wanted to determine which step in the processing pathway was defective. Endo H analysis was used to determine if the envelope precursor contained the types of oligosaccharides found on proteins at early steps in the oligosaccharide-processing pathway or types of oligosaccharides characteristic of completely processed proteins. Figure 3 shows the products of immunoprecipitation of 61B envelope using C11D8 anti-SU antibody and endo H digestion. Incubation of the immunoprecipitate in the absence of endo H did not change the mobility of the gp85 precursor protein (lanes 1 and 4). The 61B gp85 was sensitive to endo H, as indicated by a change in the mobility of the 61B envelope precursor protein (lanes 2 and 3). The size of the digested protein is consistent with the predicted size of the fully deglycosylated protein, which suggests that the envelope precursor has not been exposed to the processing enzymes in the Golgi apparatus that convert high-mannose residues to complex and hybrid residues. Therefore, the 61B envelope precursor is likely located in the endoplasmic reticulum or proximal Golgi apparatus.

To test for envelope protein on the cell surface, we performed indirect immunofluorescence using anti-SU primary antibody, incubation with a fluorescein isothiocyanate–anti-mouse secondary antibody, and FACS analysis (Fig. 4). As shown in Fig. 4A, 61C envelope could be detected on the surface of cells transfected with 61C proviral DNA. Envelope protein was detected on the surface of FeLV-infected AH927 cells but not on uninfected AH927 cells (Fig. 4B). In contrast, cells expressing 61B envelope did not exhibit fluorescence under similar conditions (Fig. 4C). The absence of 61B envelope on the cell surface is consistent with the endo H analysis, which

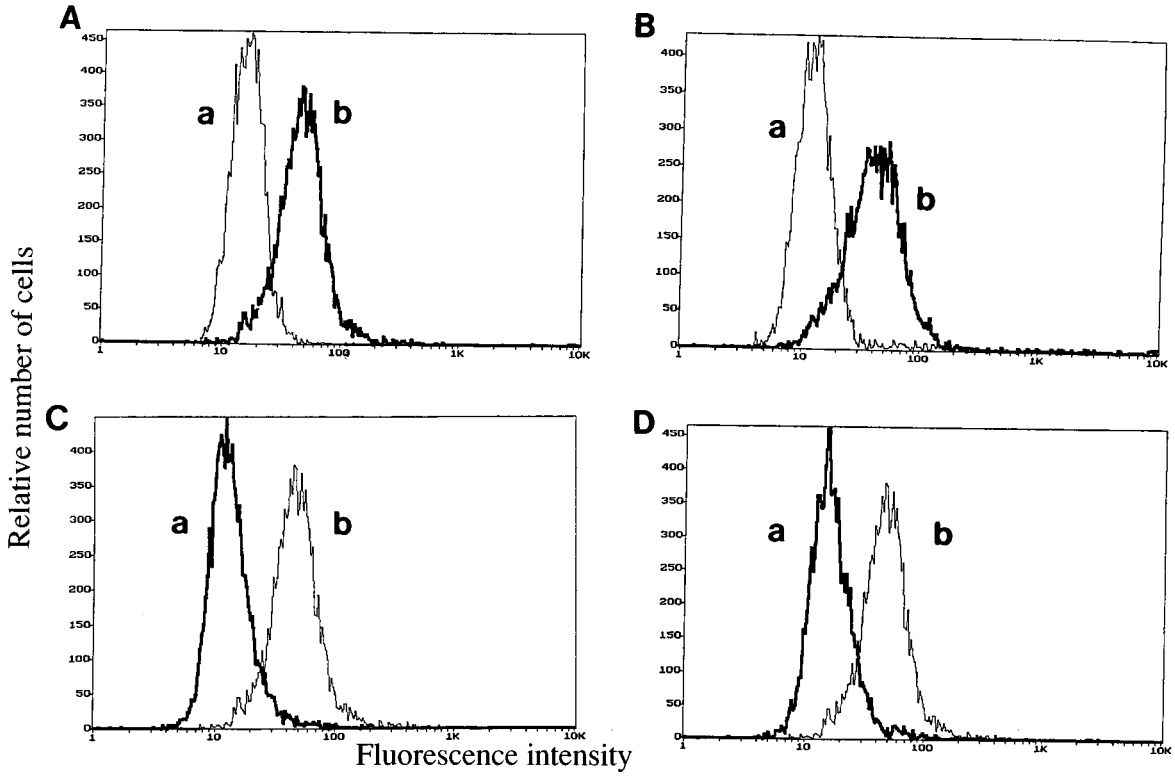

FIG. 4. FACS analysis of cells expressing 61C, 61B, and chimeric 61C/61B envelope proteins. AH927 cells were incubated sequentially with C11D8 antibody (primary antibody) and mouse anti-immunoglobulin G2B–fluorescein isothiocyanate (secondary antibody) and then FACS analyzed. In each panel, graph b represents cells expressing envelope (61C or 61E) incubated with primary and secondary antibody. (A) AH927 cells expressing 61C envelope incubated with secondary antibody only (graph a) or with primary and secondary antibody (graph b); (B) uninfected AH927 cells incubated with primary and secondary antibody (graph a) and AH927 cells infected with 61E incubated with primary and secondary antibody (graph b); (C) AH927 cells expressing 61B envelope incubated with primary and secondary antibody (graph a) and AH927 cells expressing 61C envelope incubated with primary and secondary antibody (graph b, included for comparison); (D) AH927 cells expressing CCC([CB]C) chimeric envelope incubated with primary and secondary antibody (graph a) and AH927 cells expressing 61C envelope incubated with primary and secondary antibody (graph b, included for comparison).

suggests that the envelope precursor protein was trapped in the endoplasmic reticulum or Golgi apparatus.

Mapping the envelope-processing defect of 61B. We were interested in the role of specific 61B envelope determinants in defective envelope processing because the long-latency phenotype and a replication defect colocalized to 61B TM. To investigate this, we analyzed envelope expression of several replication-defective 61C/61B chimeras encoding 61B TM (see Fig. 1 for a diagram of the clones.) Chimera CCC([CB]C) contains 61C proviral DNA with an 154-amino-acid segment of 61B TM and has been shown to exhibit the long-latency phenotype and a replication defect (53). Figure 2A, lane 6, shows that anti-SU antibody precipitated gp85, but not gp70, in lysates of AH927 cell lines expressing this chimera. Similarly, no p15E was immunoprecipitated by anti-TM antibody (Fig. 2B, lane 6). In addition, no envelope protein was detected on the surface of cells that contain chimeric FeLV genomes, as depicted in Fig. 4D (graph a). These experiments indicate that the envelope-processing defect was, like the replication defect and the long-latency phenotype, conferred by 61B TM sequences.

Site-specific mutagenesis of 61C TM to test the infectivity of EECC-env$K_{536}K_{537}$. The region of 61B TM that confers the long-latency phenotype and a defect in envelope protein processing contains four predicted amino acid differences relative to 61C. Two of the mutations encode nonconservative changes in a conserved region of the TM protein. In this region, the 61B sequence contains a positively charged lysine in place of a negatively charged glutamate at two adjacent positions between two cysteines that are present in the extracellular domain of all retroviral TMs (Fig. 1) (23, 24, 38, 41). Thus, we predicted that these two changes confer the defective-envelope-processing phenotype, and we introduced them into the TM protein of the EECC chimera, a clone that encodes the 5′ LTR, *gag*, and *pol* genes of 61E and the *env* gene and 3′ LTR of 61C (39). The mutations were introduced into EECC because EECC virus is replication competent and highly infectious for feline T cells; thus, the effect of the mutations on infectivity could be examined. The resulting chimera, EECC-env$K_{536}K_{537}$, contained 61B-specific sequences at two positions, which introduced lysine residues at amino acids 536 and 537 in the envelope precursor.

In order to determine if the mutations affected replication and/or infectivity, we established stable AH927 cell lines that contained EECC-env$K_{536}K_{537}$ provirus and tested for infectious virus by transferring cell-free culture supernatants to 3201 cells. No $p27^{gag}$ was detected in the 3201 recipient cells during a 7-week period, whereas the EECC positive control produced high levels of $p27^{gag}$ and exhibited cytopathic effects in 3201 cells at 1 week postinfection. This suggests that the glutamate-to-lysine mutations in TM made the virus replication defective. In addition, transfection of EECC-env$K_{536}K_{537}$ plasmid into 3201 T cells did not result in the production of infectious virus during a 5-week period, although replication of EECC was detected by $p27^{gag}$ ELISA at 10 days posttransfection.

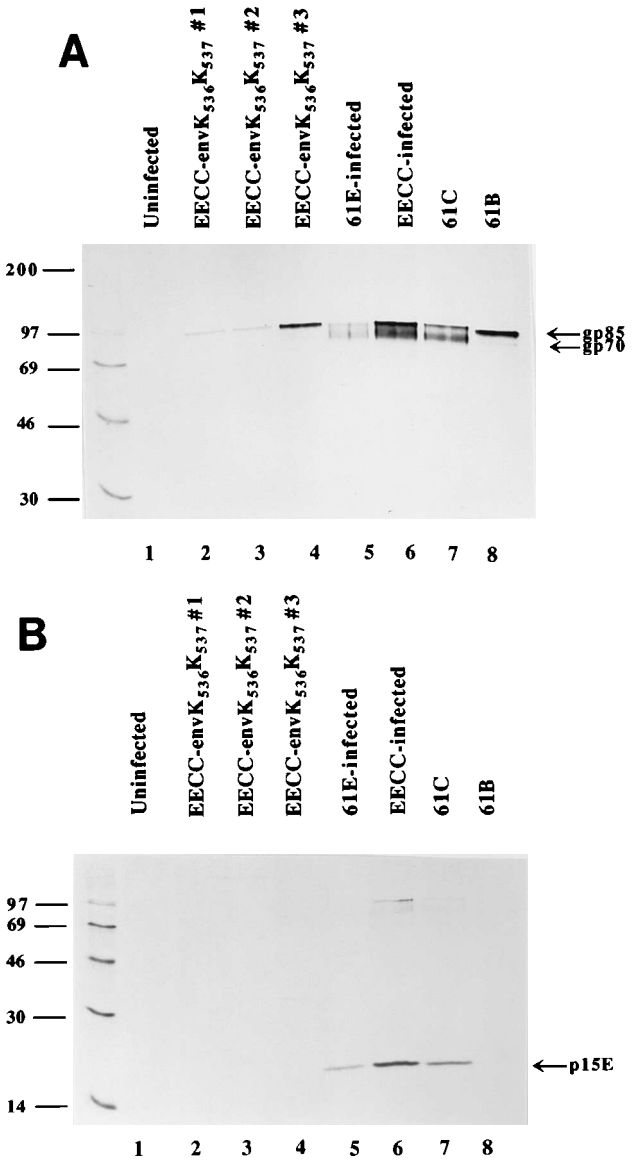

FIG. 5. Immunoprecipitation of envelope proteins from AH927 cell clones expressing EECC-env$K_{536}K_{537}$. Experimental procedures and the layout of the figure are as described in the legend to Fig. 2. (A) Cellular lysates immunoprecipitated with the anti-gp70 and -gp85 antibody C11D8; (B) cellular lysates immunoprecipitated with the anti-p15E antibody PF6J-2A.

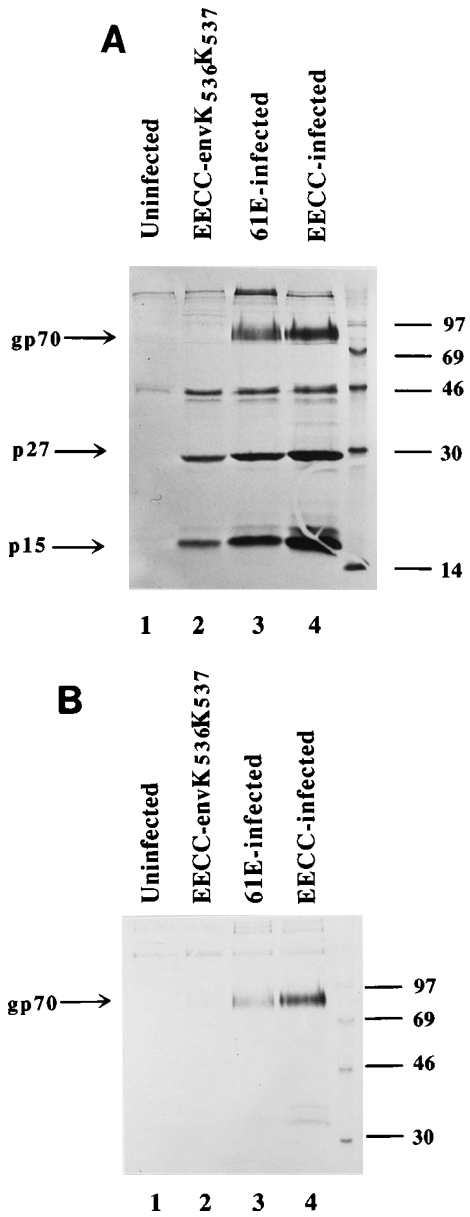

FIG. 6. Immunoprecipitation of viral proteins in culture supernatant from AH927 cells expressing EECC-env$K_{536}K_{537}$. AH927 cells were labeled with [^{35}S]methionine plus [^{35}S]cysteine for 22 h. (A) Clarified culture supernatants immunoprecipitated with anti-FeLV antibody and analyzed by SDS-12.5% PAGE; (B) clarified culture supernatants immunoprecipitated with anti-SU antibody and analyzed by SDS-9% PAGE.

Envelope protein processing of EECC-env$K_{536}K_{537}$. In order to evaluate the effect of the two glutamate-to-lysine mutations on envelope protein processing, we performed RIPA of the AH927 cell lines expressing EECC-env$K_{536}K_{537}$. In each of the control extracts, including cells expressing 61E, 61C, and EECC, both gp70 and gp85 were precipitated as expected (Fig. 5A, lanes 5 to 7). Similarly, p15E was detected in these lysates (Fig. 5B, lanes 5 to 7). Immunoprecipitates of cells expressing EECC-env$K_{536}K_{537}$ contained gp85 but did not contain any detectable gp70 or p15E protein (Fig. 5A and B, lanes 2 to 4).

Cell lines expressing EECC-env$K_{536}K_{537}$ were examined in order to determine whether they produced virus particles that contain envelope proteins. RIPA of the culture supernatant was performed using an anti-virion antiserum that precipitated Gag proteins ($p27^{gag}$ and $p15^{gag}$) and Env protein (gp70) in culture supernatant of cells infected with 61E (Fig. 6A, lane 3) or EECC (lane 4). In culture supernatant from stable cell lines expressing EECC-env$K_{536}K_{537}$, Gag proteins were detected, but no gp70 was present. The level of Gag proteins in EECC-env$K_{536}K_{537}$ culture supernatant was approximately threefold lower than the level of Gag proteins in the culture supernatant of 61E-infected cells. However, a longer exposure of the gel did not reveal any gp70 in immunoprecipitates of env$K_{536}K_{537}$ culture supernatant (data not shown), demonstrating that the absence of gp70 in cell lysates is not due to shedding from the cell surface or from virus particles. RIPA using anti-SU antibody yielded similar results, except that a small amount of protein, approximately the size of gp70, was observed in immunoprecipitates of the culture supernatant of env$K_{536}K_{537}$

cell lines (Fig. 6B). It is possible that processing occurs very slowly and that some gp70 accumulates in the culture supernatant during the long labeling period.

DISCUSSION

Retroviral TM proteins consist of a large extracellular domain, a membrane-spanning domain, and a cytoplasmic tail of variable length. A fusion domain is located in the extracellular domain of TM (1, 29), as are sequences involved in oligomerization (15–17) and interaction with SU (3–5, 29). Within the extracellular domain of TM there is a region that is conserved among retroviral TM proteins (10, 41, 47, 50) that includes both the conserved cysteines depicted in Fig. 1 and an upstream region that has been referred to as the immunosuppressive peptide (9) or as a leucine zipper (14). The function of the conserved region of TM is not completely understood. The position of these cysteines, relative to the fusion peptide and the membrane-spanning domain, is conserved in TM proteins from widely divergent retroviruses (human T-cell leukemia virus, human immunodeficiency virus [HIV], Rous sarcoma virus, murine leukemia virus, and Mason-Pfizer monkey virus [MPMV]) (10, 11, 41, 51). More extensive alignments of retroviral TM proteins, which involved comparison of sequences for several retroviral TMs in the region spanning the cysteine residues, have been presented previously (11, 23, 41). Analysis of the immunogenicity and structure of this region of HIV TM has suggested that disulfide bonds form between these cysteines (24, 38), and investigators have inferred that a similar structure exists for FeLV (23). The present study defines amino acids between the cysteines that play an important role in envelope protein processing.

In the oncovirus MPMV, deletion analyses implicated a 35-amino-acid region including the cysteine loop as important in envelope protein processing (3). Recent studies have shown that mutation of the conserved cysteine residues to glycine or serine in the HIV type 1 (HIV-1) TM protein disrupts envelope protein processing and abolishes infectivity (11, 51). Our data suggest that the putative cysteine loop is important for proper envelope protein processing for FeLV as well as HIV-1, even though there are few amino acid similarities within the loop sequences of these viruses (Fig. 1C). More importantly, our experiments demonstrate that not just the cysteines that form the disulfide bridge but also specific sequences within the loop are required for proper envelope protein maturation.

The absence of 61B envelope on the cell surface and the endo H analysis of the 61B envelope precursor suggest that the processing defect is probably at the level of oligomerization or subsequent transport from the endoplasmic reticulum to the Golgi apparatus. A similar phenotype was observed for the MPMV envelope containing a 35-amino-acid deletion in the extracellular domain of TM (3). Similar results have also been observed in other retroviral systems due to changes in SU, not TM (19, 33, 36, 52). Analysis of these mutant precursors suggests that when the block in the envelope processing pathway occurs in the endoplasmic reticulum, the precursor is not transported to the cell surface. On the other hand, amino acid changes that affect later steps in the processing pathway, such as cleavage site mutations, usually result in uncleaved envelope precursors that are transported to the cell surface and, in some cases, incorporated into virions (2, 13, 21, 26, 34, 42). In all cases, cleavage of the envelope precursor is required for virus infectivity (2, 7, 11, 13, 22, 26, 32, 34, 54). The studies presented here demonstrate that point mutations in TM can result in a block in envelope processing in the endoplasmic reticulum or proximal Golgi apparatus and prevent subsequent transport of the precursor to the cell surface.

Previous studies have shown that envelope processing is delayed in the immunodeficiency-inducing, highly cytopathic FeLV variant 61C (43, 44). The defect in 61B envelope protein processing, in contrast, is more pronounced than that for 61C, with no detectable gp70 produced. The envelope-processing phenotypes of 61C and 61B are further distinguished by the fact that the delayed-processing phenotype of the 61C envelope is conferred by SU sequences, whereas the defective-processing phenotype of the 61B envelope is conferred by TM sequences. The present study suggests that a recombinant virus encoding the 61B SU and 61E TM, such as was observed late in 61B (61E) mixed infections (53), would express functional SU and TM proteins. This recombinant virus would have a selective advantage in T cells because the 61B SU, like the 61C SU, confers on the virus the ability to replicate to high levels in T cells. The high level of replication, then, would lead to cytopathic effects in T cells and to consequent immunodeficiency disease. Thus, the presence of many defective variants during FeLV infection may allow recombination to generate viruses with unique growth properties, which eventually determine the timing and outcome of disease.

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