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NOTES

Intracellular Transport and Leukemogenicity of Spleen Focus-Forming Virus Envelope Glycoproteins with Altered Transmembrane Domains

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Friend murine spleen focus-forming virus (SFFV) encodes a glycoprotein designated gp52, which is responsible for the leukemogenic properties of the virus. gp52 lacks a cytoplasmic domain and is defective in its transport to the cell surface. We constructed a chimeric envelope gene which codes for a molecule with an external domain derived from the SFFV envelope gene and membrane-spanning and cytoplasmic domains derived from the Friend murine leukemia virus envelope gene. Like gp52, the chimeric protein was defective in its transport to the cell surface, indicating that the absence of a cytoplasmic tail is not responsible for the defective intracellular transport of SFFV gp52. However, unlike wild-type SFFV, the chimeric SFFV genome failed to induce erythroleukemia in adult mice. The results indicate that the altered membrane-spanning domain, lack of a detectable cytoplasmic tail in gp52, or both factors are prerequisites for the erythroleukemia-inducing properties of SFFV but are not responsible for the block in intracellular transport of the glycoprotein.

The replication-defective spleen focus-forming viruses (SFFV) found in stocks of Friend murine leukemia virus (F-MuLV) cause acute and fatal erythroleukemia in adult mice (21). The SFFV envelope gene encodes a 52-kilodalton glycoprotein (gp52) which has been identified as the sole determinant of erythroleukemia-inducing properties of SFFV (21, 26, 29, 30, 32). The gp52 glycoprotein is the product of a recombinant envelope gene derived from the putative parent—the envelope gene of F-MuLV—by a series of changes involving substitution, deletion, and insertion (1, 5, 31). The envelope gene of F-MuLV codes for a precursor glycoprotein (gPrENV) which is proteolytically cleaved into two proteins: an amino-terminal, glycosylated protein designated gp70 and a carboxy-terminal, nonglycosylated protein designated p15E, which spans the membrane bilayer (7). During virus maturation, p15E is further processed by a virus-encoded protease into p12E and a small peptide designated as the R peptide (25). In contrast, gp52 is the primary translation product of the SFFV *env* gene. Unlike the MuLV envelope proteins, gp52 is defective in its transport to the cell surface; only 3 to 5% of the total cellular gp52 is expressed on the surfaces of SFFV-infected cells (8). Interestingly, while some gp52 is expressed on the surfaces of cells infected with polycythemia-inducing strains of SFFV $(SFFV)_a$, no cell surface expression of gp52-related molecules is observed with anemia-inducing strains of SFFV $(SFFV)_P$ (20), and hence, the altered subcellular localization of gp52 is thought to be associated with induction of disease. Recent studies (1, 5, 31) indicate that the amino terminus of gp52 is closely related to the envelope gene of mink cell focus-forming viruses, which are thought to be derived by a recombination event involving ecotropic F-MuLV and endogenous MuLV sequences in the mouse chromosome. gp52 exhibits a major deletion of about 585 base pairs which encompasses the carboxy-terminal portion of the gp70 sequences and the amino-terminal portion of the p15E sequences. In addition, the sequences coding for the membrane-spanning region of gp52, as compared with similar domains in MuLV p15E, reveal the insertion of a 6-base-pair tandem repeat which adds two hydrophobic leucine residues; an additional single-base insertion is also observed in this domain which causes a frameshift mutation and results in premature termination of the molecule 34 codons before the termination codon in MuLV p15E (1, 5, 31). These changes result in a hydrophobic carboxy terminus which appears to be buried in the membrane, with no portion of the molecule exposed on the cytoplasmic side of the membrane (24). It is not certain which of these changes confers acute erythroleukemogenic properties on SFFV gp52. To investigate this question, we constructed a chimeric gene in which the entire amino-terminal external domain of the molecule was derived from the SFFV *env* gene, while sequences coding for the transmembrane and cytoplasmic domain were derived from the MuLV *env* gene. We show here that reconstructed viruses expressing such chimeric molecules, unlike wild-type SFFV, failed to induce acute erythroleukemia in adult mice, indicating a requirement for the carboxy-terminal domains of SFFV gp52 in disease induction. Furthermore, the chimeric molecules, like gp52, were defective in their transport to cell surfaces, indicating that altered subcellular localization, per se, does not account for the leukemogenicity of SFFV gp52.

Molecular clones of unintegrated circular DNAs from the F-MuLV (13) and $SFFV_P$ (16) genomes were obtained from Allen Oliff and David Linemeyer, respectively, and used to construct the chimeric SFFV envelope gene. Analyses of the published nucleotide sequences of the SFFV (31) and F-MuLV (11) envelope genes revealed the presence of a unique *Asu*II restriction site 5′ to the sequences coding for the transmembrane domains of the glycoproteins and a unique

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*Kpn*I site 3′ to the envelope gene located in the long terminal repeat (LTR) sequences. Accordingly, the construction of the chimeric gene was accomplished by substitution of the *Asu*II-*Kpn*I fragment from the MuLV genome into the SFFV genome according to standard procedures. Briefly, an *Xba*I-*Kpn*I fragment containing the MuLV *env* gene was first subcloned from plasmid p231 into plasmid vector pGEM3, and the resulting plasmid was designated as pGEM.XK. Plasmids p4 (containing the SFFV *env* gene) and pGEM.XK (containing the MuLV *env* gene) were digested to completion with the restriction enzymes *Asu*II and *Kpn*I, and the resulting fragments were purified by electrophoresis on low-melting-point agarose gels. The large fragment derived from the SFFV clone, containing sequences that code for the ectodomain of gp52, was ligated to the small fragment from the MuLV clone which contains sequences that code for the transmembrane and cytoplasmic domains of p15E. The resulting construct was designated Ch.SFFV, and its identity was confirmed by restriction maps, hybridization under stringent conditions to oligonucleotides complementary to unique regions of the MuLV or SFFV *env* gene encoding the transmembrane domains (data not shown), and limited nucleotide sequencing. The restriction maps of the different plasmids and a limited nucleotide sequence (and deduced amino acid sequence) of the wild-type and chimeric SFFV molecules are summarized in Fig. 1 A and B. These results indicate that the chimeric envelope gene would code for a molecule with an ectodomain corresponding to that of SFFV gp52, while the transmembrane and cytoplasmic domains correspond to those of MuLV p15E.

The chimeric and wild-type SFFV clones contain a complete (although permuted) viral genome with a single LTR. Previously, it has been shown that such molecules are expressed in eucaryotic cells upon transfection and can be rescued by helper viruses to obtain infectious SFFV genomes (13). Accordingly, we excised the viral sequences from the flanking plasmid sequences by digestion with *Hin*dIII and generated concatemers by treatment with T4 DNA ligase to obtain molecules with sequences colinear with proviral DNA and to facilitate efficient integration into cells. These molecules were used to transfect NIH 3T3 cells by the calcium phosphate precipitation method (9). The pSV2.Neo plasmid, which confers resistance to the antibiotic geneticin (G418; GIBCO Laboratories) was used as a cotransfectant in these experiments, and transfectants were selected by growing the cells in the presence of medium containing G418 (23). Neomycin-resistant colonies were clonally isolated, expanded, and screened for the expression of chimeric molecules by a radioimmune precipitation assay by using a monoclonal antibody prepared against SFFV gp52 (28). One of the clones, designated Ch5.2, was found to express a gp52-related chimeric molecule and was chosen for further studies. On the basis of the predicted structure, the chimeric molecule was expected to migrate slightly slower than wild-type SFFV gp52, with an apparent molecular mass of about 57 kilodaltons, as well as to possess determinants which would be recognized by an antiserum to the carboxy-terminal R peptide of MuLV (25). Radioimmune precipitation of [^{3}H]leucine-labeled cell lysates with anti-gp70 serum revealed a chimeric SFFV gp52-related molecule that migrated slightly slower than wild-type gp52 (Fig. 2, lanes 1 and 2). Also, the chimeric molecule, but not wild-type gp52, was observed in immune precipitates with an anti-R peptide serum (Fig. 2, lanes 3 and 4). These results demonstrate that the chimeric molecule has a cytoplasmic domain corresponding to that of the MuLV envelope protein.

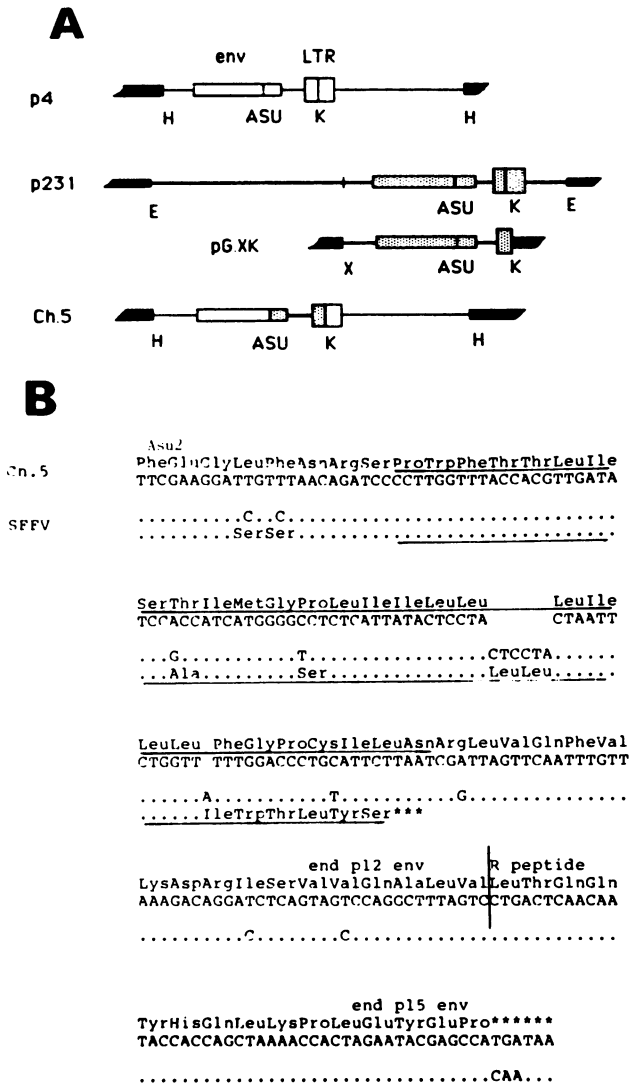

FIG. 1. (A) Restriction endonuclease maps of original and constructed DNAs. Locations of the coding regions for the envelope glycoproteins (env) and the LTRs in the SFFV (p4) and the MuLV (p231) genomes are shown. An *Xba*I-*Kpn*I fragment from MuLV plasmid p231, carrying the entire MuLV envelope gene, was first subcloned into plasmid vector pGEM3. The large fragment obtained from *Asu*II-*Kpn*I digestion of SFFV plasmid p4 was ligated with the small fragment derived by *Asu*II-*Kpn*I digestion of plasmid pG.XK, and the resulting plasmid was designated Ch.5. Heavy bars and shaded boxes represent MuLV-derived sequences, and light bars and open boxes represent SFFV sequences. Flanking plasmid sequences are indicated by interrupted dark boxes. Abbreviations: H, *Hin*dIII; K, *Kpn*I; X, *Xba*I; E, *Eco*RI; ASU, *Asu*II. DNA fragments used for ligation were prepared from cesium chloride gradient-purified plasmid DNA by restriction endonuclease digestion and separation on low-melting-point agarose gels. (B) Confirmation of the construction of chimeric SFFV by partial sequence analysis of the *Asu*II-*Kpn*I fragment from the chimeric (Ch.5) and wild-type SFFV (p4) genomes. The nucleotide sequence and the deduced amino acid sequences are shown. The hydrophobic, putative membrane-spanning regions are underlined. The asterisks indicate the termination codons. The vertical line indicates the site of cleavage of the R peptide.

A small proportion of SFFV gp52 undergoes oligosaccharide processing to yield a higher-molecular-weight form, designated gp65, which may be detected on the cell surfaces (20, 21). We therefore examined lysates from cells labeled with radioactive sugar precursors for the presence of processed forms of viral glycoproteins. Cells were labeled with [^{3}H]glucosamine (250 μCi/ml) for 3 h, and the cell lysates were immune precipitated with anti-gp70 serum. Under these labeling conditions, gp65, the processed form of gp52, was readily detected in cells infected with wild-type SFFV (Fig. 2, lane 5). Similarly, a higher-molecular-weight protein corresponding to the processed form was detected in cells infected with chimeric SFFV (Fig. 2, lane 6). The ratio of the radiolabel incorporated into the processed and unprocessed forms of the viral glycoproteins was determined by scanning densitometry of the autoradiograms, and the processed forms represented about 10% of the glycoprotein in both wild-type SFFV- and chimeric SFFV-infected cells. The low efficiency at which the chimeric molecule was processed into a higher-molecular-weight form indicates that this molecule, like wild-type gp52, may be defective in its intracellular transport.

The processed forms of gp52 from $SFFV_P$-infected, but not $SFFV_a$-infected, cells were found to be expressed on the cell membranes (20). We therefore compared the surface expression of the wild-type and chimeric molecules on NIH 3T3 cells by indirect immunofluorescence. The intracellular staining pattern of cells that expressed the chimeric molecule was similar to the pattern observed with wild-type SFFV- or MuLV-infected cells (Fig. 3a to c). Both wild-type SFFV-infected cells (Fig. 3f) and chimeric-SFFV- infected cells (Fig. 3g) displayed definite surface immunofluorescence, suggesting that the processed form of chimeric SFFV, like SFFV gp65, is expressed on the surfaces of infected cells. However, the intensity of surface immunofluorescence was significantly lower than that of gp70 on MuLV-infected cells (Fig, 3e), indicating inefficient transport of chimeric and wild-type gp52 molecules to the cell surface. These results, together with the results from sodium dodecyl sulfate-polyacrylamide gel electrophoretic analyses, indicate that alterations in the transmembrane domain and lack of a cytoplasmic tail, per se, do not account for the transport defects and altered subcellular localization of SFFV gp52 and implicate the changes in the external domain in the transport defect.

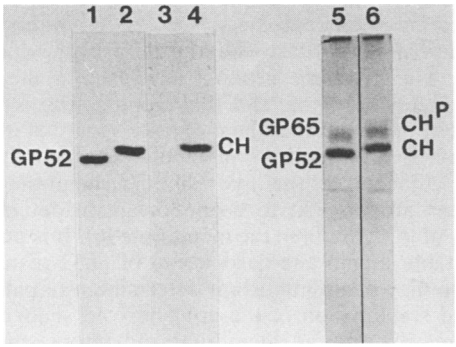

FIG. 2. Expression of gp52-related chimeric protein in transfected NIH 3T3 cells. Wild-type SFFV-transfected cells (lanes 1 and 3) or chimeric SFFV-transfected cells (lanes 2 and 4) were labeled with [^{3}H]leucine (500 μCi/ml) for 1 h, and the cell lysates were analyzed by immune precipitation with goat anti-gp70 serum (lanes 1 and 2) or rabbit anti-R peptide serum (lanes 3 and 4). Lanes 5 and 6 show the immune precipitation of [^{3}H]glucosamine-labeled cells that expressed the wild-type (lanes 5) or chimeric (lanes 6) SFFV genome with anti-gp70 serum.

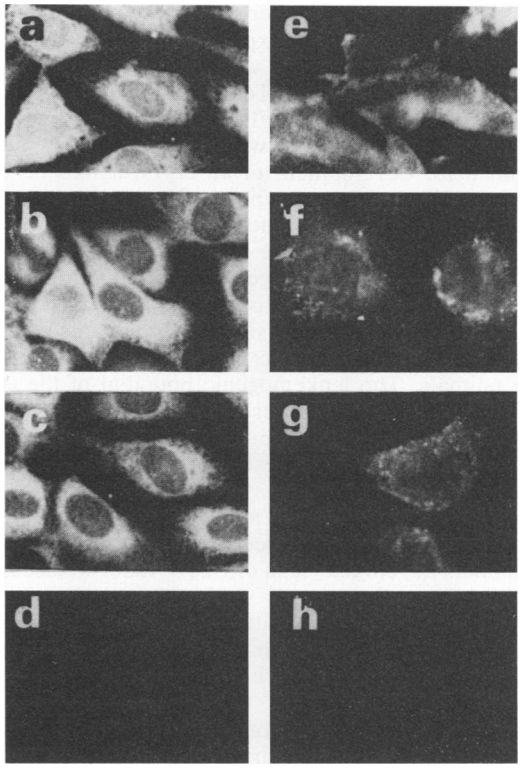

FIG. 3. Surface and intracellular localization of wild-type and chimeric SFFV gp52 molecules. Indirect immunofluorescence was carried out on ethanol-acetic acid (95:5)-fixed (panels a to d) or unfixed (panels e to h) monolayers of NIH 3T3 cells with a goat antiserum prepared against MuLV gp70 and fluorescein-conjugated rabbit anti-goat immunoglobulins. Panels: a and e, F-MuLV infected; b and f, wild-type SFFV infected; c and g, chimeric SFFV infected; d and h, uninfected NIH 3T3 cells.

SFFV gp52 is responsible for the acute erythroleukemia-inducing properties of the virus. We therefore wished to determine the effects of the domain-specific modifications on the erythroleukemia-inducing properties of gp52. To prepare infectious virus stocks suitable for inoculation into mice, we cotransfected NIH 3T3 cells with concatemerized wild-type or chimeric clones and an infectious clone of Moloney MuLV (pMov-3) (10) as a helper virus. The transfected cells were passaged several times to facilitate the spread of virus among the cells until nearly all of the cells in the monolayer were infected with the virus, as determined by an infectious-center assay based on the fusogenic property of ecotropic viruses (17). Culture supernatants from such cells were

TABLE 1. Pathogenicity of wild-type and chimeric SFFV genes[a]

Virus	Deaths/total no. of mice	Avg spleen wt (g)	Mean hematocrit
SFFV	5/9	2.85	63
Ch.SFFV	0/10	0.12	48
None	0/5	0.12	48

[a] Male NIH Swiss mice (5 to 6 weeks old) were inoculated intravenously with 0.5 ml of virus preparation, sacrificed at 5 weeks postinfection, and examined for signs of SFFV disease.

found to express both helper and SFFV viral genomes, as shown by their ability to express both SFFV gp52-related molecules and MuLV gp70 when used to infect fresh NIH 3T3 cells (data not shown). To assess the in vivo pathogenic potential of the chimeric SFFV genomes, NIH Swiss mice were injected intravenously with undiluted culture fluids and examined for development of an erythroleukemic disease. Mice inoculated with wild-type SFFV were found to develop acute erythroleukemia characterized by marked splenomegaly and polycythemia (Table 1). In contrast, no evidence of disease was observed in mice infected with chimeric SFFV (Fig. 4). The absence of disease was not due to lack of establishment of infection, since we could recover viruses that expressed the chimeric molecule from the spleens of these animals. The leukemogenic potential of the chimeric SFFV molecules was further investigated by inoculation of mouse-passaged virus into newborn animals. Two litters of NIH Swiss mice, each consisting of six newborn mice, were inoculated intraperitoneally with 0.2 ml of a 10% spleen suspension prepared from mice previously infected with chimeric SFFV. Over a 4-week observation period, none of the animals developed signs of SFFV-induced disease characterized by splenomegaly and polycythemia (data not shown). These results indicate that substitution of the SFFV gp52 transmembrane domain with the transmembrane and cytoplasmic domains of MuLV p15E markedly reduced the leukemogenicity of SFFV.

During the construction of the chimeric SFFV genome, part of the 3′ LTR sequences from F-MuLV were also transferred to the SFFV genome (Fig. 1). Accordingly, the chimeric SFFV LTR contains sequences derived from the MuLV genome. Nevertheless, these differences are not likely to influence the pathogenicity of these viruses. The LTRs of F-MuLV and SFFV are very closely related (3, 4, 27). However, the F-MuLV LTR contains two copies of the enhancer sequences, while only one copy of the enhancer sequences is found in the SFFV LTR. Previous studies, however, have shown that the acute erythroleukemia induced by SFFV is dependent entirely on the expression of gp52 glycoprotein and is not affected when the SFFV LTR is replaced by analogous LTRs from Friend or Moloney ecotropic or mink cell focus-forming virus (29, 30, 32). Our results, therefore, indicate that the insertions found in the 3′ region of the SFFV *env* gene, which are responsible for changes in the structure of the membrane-spanning and cytoplasmic domains of gp52, are essential for erythroleukemia induction by SFFV.

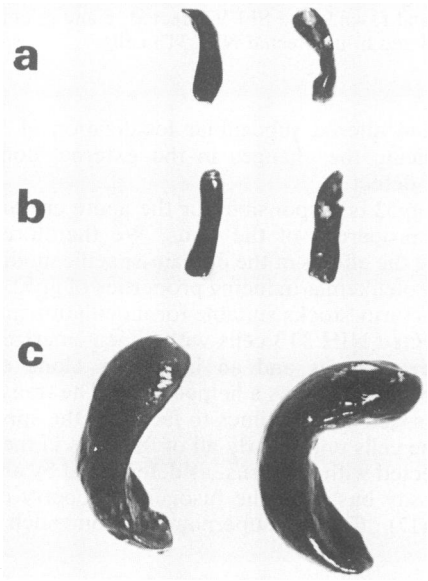

FIG. 4. Induction of splenomegaly in mice injected with M-MuLV or M-MuLV pseudotypes of the wild-type and chimeric SFFV genomes. Adult male NIH Swiss mice were injected intravenously with 0.5 ml of helper M-MuLV or M-MuLV pseudotypes of the wild-type SFFV or chimeric SFFV genome. At 5 weeks later, animals were sacrificed and the spleens were examined. Panels: a, the size of spleens from helper virus (M-MuLV)-infected mice, b, spleens from mice infected with M-MuLV pseudotypes of the chimeric SFFV genome; c, spleens from mice infected with M-MuLV pseudotypes of the wild-type SFFV genome.

The reasons for the lack of leukemogenicity of chimeric SFFV are not clear. Nucleotide sequence analyses of spontaneous nonleukemogenic mutants of SFFV and their revertants show deletions or changes in several distinct regions of the envelope gene (12, 14, 15, 22), and all of these changes may be mapped to the ectodomain of gp52. The lack of leukemogenicity of these mutants is thought to be due either to changes in a functionally important domain(s) of the molecule or alterations in protein folding and tertiary structure of the molecule caused by these mutations. Previous studies have indicated that cytoplasmic domains of cellular and viral integral membrane proteins can substitute for the cytoplasmic domain of the vesicular stomatitis virus glycoprotein (19). Thus, the domain-specific changes conferred on gp52 in this study are less likely to affect the tertiary structure of the external domain, although we cannot rule out that possibility. If, indeed, the lack of leukemogenicity is due to alterations in protein folding and tertiary structure, it may be inferred that the conserved changes undergone by different SFFV genomes to generate the altered transmembrane domain and delete the cytoplasmic domain are essential for proper folding and function of the gp52 ectodomain. On the other hand, if the tertiary structure of the gp52 ectodomain is not significantly affected by the changes found in chimeric SFFV, the present results indicate that the pathogenic potential of the molecule is mediated by the altered transmembrane domain. Recent studies indicate that a point mutation altering the transmembrane region of the *neu* gene product, p185, is involved in the activation of this proto-oncogene (2). In this case, the observed mutation causes a valine residue in the transmembrane domain of p185 to be replaced by a glutamic acid residue. The *neu* gene product is related to the epidermal growth factor receptor, and this modification is thought to alter the conformation of the molecule such that it resembles a ligand-activated receptor molecule. The charged residues located adjacent to the hydrophobic membrane-spanning sequences are thought to be important in determining the stability of the protein in the membrane (6). It is possible that the unstable membrane association of gp52 resulting in its secretion may be an important determinant of pathogenicity (18), and stabilization of the gp52-derived sequences in the membranes by the transmembrane and cytoplasmic domains derived from MuLV may prevent release of the chimeric molecule from the cell and thus account for the lack of leukemogenicity. We are currently investigating several additional mutants to test these possibilities.

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Lerner, and the monoclonal gp52-specific antibody was a generous gift from S. Ruscetti.

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ADDENDUM

After this article was submitted, Li et al. (J.-P. Li, R. K. Bestwick, C. Spiro, and D. Kabat, J. Virol. **61:** 2782–2792, 1987) generated mutants of SFFV by in-phase linker insertion and reported that all leukemogenic mutants expressed gp52-related proteins on cell surfaces, whereas nonleukemogenic mutants failed to express such molecules on cell surfaces, suggesting that surface expression is required for leukemogenesis. Our results indicate that surface expression of gp52-related molecules per se is not sufficient for induction of the disease process.

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