The Hydrophobic Membrane-Spanning Sequences of the gp52 Glycoprotein Are Required for the Pathogenicity of Friend Spleen Focus-Forming Virus

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Friend spleen focus-forming virus (SFFV) codes for a transport-defective envelope glycoprotein designated gp52, which is responsible for the leukemogenic properties of the virus. gp52 is a monotopic integral membrane protein anchored in the membrane by a stretch of hydrophobic amino acid residues located near the carboxy terminus of the molecule. We have constructed a mutant SFFV envelope gene in which the sequences that code for the hydrophobic membrane-spanning domain have been deleted, and we expressed this gene by using recombinant vaccinia virus vectors or retroviral vectors. The mutant SFFV envelope gene was found to encode a truncated glycoprotein (gp52t) which was also transport defective; a majority of gp52t remained cell associated, while a small proportion of the molecules underwent oligosaccharide processing. The processed form of gp52t was secreted from the cells. Retroviral vectors carrying the mutant SFFV envelope gene were found to be nonpathogenic in adult mice. These results indicate that the hydrophobic membrane-spanning region of gp52 is required for pathogenicity of SFFV and suggest that these sequences may play a role in signal transduction. The results also indicate that the transport defect of SFFV gp52 is due to structural features of the ectodomain of the molecule.

The Friend and Rauscher complexes of murine leukemia viruses (MuLVs) induce an acute and fatal erythroleukemia in adult mice, and a replication-defective spleen focus-forming virus (SFFV) contained in these stocks has been implicated in the disease process (27). Both Friend and Rauscher SFFV (F-SFFV and R-SFFV) code for an envelope glycoprotein with a molecular mass of about 52 kDa, designated gp52. Available evidence indicates that the expression of SFFV gp52 is essential and probably sufficient for disease production by these viruses (17, 19, 21, 35, 37). Nucleotide sequence analyses of envelope genes from F-SFFV indicate that gp52 is the product of a recombinant envelope gene, derived from the putative parent, the envelope gene of Friend MuLV (F-MuLV), by a series of changes involving substitutions, deletions, and insertions (1, 7, 34, 36). The amino terminus of gp52 is closely related to that of envelope glycoproteins from mink cell focus-forming viruses (MCFVs), which are thought to arise by a recombination between ecotropic MuLV and endogenous xenotropic MuLV sequences in the mouse chromosome. In comparison with MuLV and MCFV envelope genes, the SFFV envelope gene exhibits a 585-bp deletion, which encompasses the cleavage site between gp70 and p15E. Consequently, gp52 is expressed as an uncleaved protein. A single base insertion in the region coding for the membrane-spanning domain of gp52 causes a frameshift mutation leading to premature termination of the molecule, 34 codons prior to the termination codon in MuLV p15E. As a result, SFFV gp52 is expressed as a monotopic integral membrane protein, with no detectable cytoplasmic tail. Essentially similar modifications have been observed in the R-SFFV envelope gene,

suggesting that these modifications may be responsible for the erythroleukemia-inducing properties of gp52 (3, 11). In addition to these changes, an in-frame 6-bp insertion in the transmembrane domain coding regions is observed in polycythemia-inducing (F-SFFV$_P$) but not anemia-inducing (F-SFFV$_A$ and R-SFFV) strains of SFFV (34). Previous studies have indicated that the ability to generate erythropoietin-independent transformed cells, a characteristic feature of SFFV$_P$ isolates, maps to the region that codes for the transmembrane domain of SFFV$_P$ gp52 (5, 6).

SFFV gp52 is a transport-defective glycoprotein (30), with a majority of the SFFV gp52 remaining intracellular. However, a small proportion of the molecules are transported to the Golgi complex, where they undergo oligosaccharide processing. In SFFV$_P$-infected cells, the processed forms have a molecular mass of ~65 kDa (gp65) and are readily detected on the cell surface. However, in SFFV$_A$-infected cells, the processed forms of gp52 have a molecular mass of 60 kDa (gp60) and are not readily detected on the cell surface (28). Nevertheless, both gp65 and gp60 are secreted from erythroleukemia cells and SFFV-infected fibroblast cultures (25, 26). At least some of the secreted form of gp65 is ~3 kDa smaller than the cell-associated proteins, suggesting a proteolytic cleavage either before or after secretion (10, 26). On the basis of studies with leukemogenic and nonleukemogenic mutants of SFFV, it has been suggested that the surface expression of gp65 (and/or its release from cells) may be a prerequisite for the leukemogenicity of SFFV (2, 15, 31). We have been using domain-specific modification of SFFV envelope glycoproteins to investigate the role of specific structural features in determining its leukemogenicity or altered intracellular transport and secretion. In this study we have investigated the transport, secretion, and leukemogenicity of mutant SFFV gp52 molecules which lack the hydrophobic membrane-anchoring sequences.

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MATERIALS AND METHODS

Cells and viruses. NIH 3T3 cells were obtained from Stuart Aaronson, National Institutes of Health, Bethesda, Md., and maintained in Dulbecco's modification of minimal essential medium (DMEM) supplemented with 10% fetal calf serum. CV-1 cells and human TK⁻ cells were maintained in DMEM supplemented with 8% newborn calf serum. Wild-type vaccinia virus strain IHD-J and a recombinant vaccinia virus which expresses F-MuLV envelope proteins were kindly supplied by Bernard Moss, National Institutes of Health. Construction and characterization of recombinant vaccinia viruses that express SFFV gp52 (14), Friend MCFV (F-MCFV) envelope proteins (32), and truncated, anchor-minus MCFV envelope proteins (32), as well as chimeric SFFV envelope proteins (14), have been described before.

Plasmids and reagents. Molecular clones of unintegrated, proviral DNA from F-MuLV (24) and SFFV (18) were obtained from David Linemeyer and Alan Oliff, National Institutes of Health. Plasmid pMOV-3, which contains integrated proviral Moloney MuLV (M-MuLV) DNA, was provided by S. Goff, Columbia University, New York, N.Y. Plasmid pSC11, used to generate recombinant vaccinia vectors, was provided by B. Moss. Restriction endonucleases, the Klenow fragment of *Escherichia coli* DNA polymerase, and phage T4 DNA ligase were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. *Bam*HI linkers were obtained from New England BioLabs, Beverly, Mass. The reagents were used as specified by the suppliers.

Construction of a mutant SFFV envelope gene that codes for truncated SFFV gp52 molecules that lack a hydrophobic membrane anchor. A chimeric SFFV (Ch.SFFV) envelope gene, in which an *Asu*II-*Kpn*I fragment from the SFFV envelope gene had been substituted by an analogous fragment from the F-MuLV envelope gene, was used to construct the mutant SFFV envelope gene. Construction of this Ch.SFFV has been described before (31). Analyses of the restriction map and partial nucleotide sequence of this construct revealed that an *Asu*II restriction site is located at the 5' end of the region encoding the hydrophobic membrane anchor sequences of SFFV gp52, while a *Cla*I site is located at the 3' end of this region (Fig. 1). These two enzymes generate compatible cohesive ends, which upon religation expose a new stop codon, thus resulting in a mutated gene encoding a truncated gp52 molecule (gp52t) that lacks a membrane anchor (Fig. 1). Accordingly, a *Hin*dIII-*Kpn*I fragment from the Ch.SFFV genome was first subcloned into pUC19, doubly digested with *Asu*II and *Cla*I, and religated. The resulting constructs were verified by restriction mapping and partial nucleotide sequencing. In addition to the loss of membrane anchor sequences, six adjacent residues, Glu-Gly-Leu-Phe-Asn-Arg-Ser, amino terminal to the membrane-anchoring sequences are replaced with an Asp residue in the mutant envelope gene (Fig. 1).
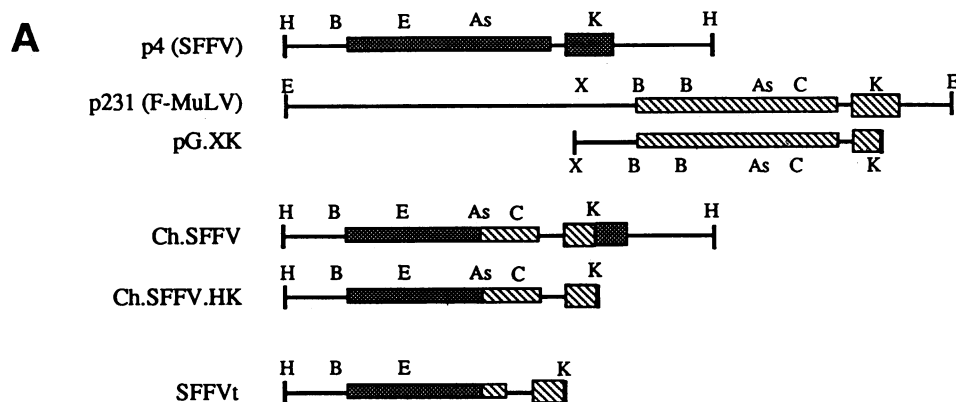
Construction of recombinant vaccinia virus vectors that express mutant SFFV envelope genes. The recombinant vaccinia virus vectors were generated as previously described (14). Briefly, a *Bam*HI-*Asp* 718 fragment containing the mutant SFFV envelope gene was excised from the plasmid, and the ends were blunted by a Klenow fill-in reaction and ligated with *Sma*I-digested pSC11. The resulting recombinants were screened by colony hybridization using an SFFV envelope gene-specific oligonucleotide probe, and the orientations of the inserts were determined by restriction enzyme analyses. Vaccinia virus (strain IHD-J)-infected TK⁻143 cells were transfected with a mixture of 10 μg of pSC11

containing the mutant SFFV envelope gene, 1 μg of vaccinia virus DNA, and 10 μg of sonicated salmon sperm DNA per ml, and recombinant viruses expressing the mutant SFFV envelope gene were selected according to previously described procedures (12).

Construction of recombinant retroviral vectors that express wild-type and mutant SFFV envelope genes and analysis of leukemogenicity. A unique *Cel*II restriction site is located downstream from the translation termination codon in the plasmid containing the mutant SFFV envelope gene. Plasmids p4.HK and SFFVt were linearized by digestion with *Cel*II, and *Bam*HI linkers were added at this site. From the resulting constructs, *Bam*HI fragments containing the entire mutant SFFV genome were excised and cloned into the retroviral expression vector pLXSN (23). The resulting recombinants were screened by colony hybridization using an SFFV envelope gene-specific oligonucleotide probe, and the orientations of the inserts were determined by restriction enzyme analyses. pLXSN plasmids containing the SFFV envelope genes in the proper orientation were prepared on a large scale and purified by cesium chloride density gradient centrifugation. NIH 3T3 cells were cotransfected with plasmid pMOV-3, which contains the infectious proviral clone of M-MuLV, along with the pLXSN vector containing wild-type or truncated SFFV envelope genes, by electroporation using a Cell-Porator (Bethesda Research Laboratories, Gaithersburg, Md.). The cells were propagated several times until the monolayers were uniformly infected with M-MuLV, as judged by an infectious center assay using XC indicator cells (31). The presence of viruses carrying both M-MuLV and the wild-type or mutant SFFV genome in culture supernatants was first confirmed by infecting NIH 3T3 cells prior to infection of animals. Clarified culture supernatants (0.2 ml) were inoculated intravenously in adult NIH Swiss mice (Charles River Breeding Co.), and the animals were sacrificed after 4 weeks and examined for hematocrit and splenomegaly.

Immunoprecipitation of radiolabelled proteins. NIH 3T3 cells infected with recombinant retroviral vectors, or CV-1 cells infected with recombinant viruses (at a multiplicity of 10 for 6 h), were radiolabelled with [³⁵S]methionine or [³H]glucosamine for the indicated time periods, and immunoprecipitations were carried out on culture media or cell lysates. For the preparation of cell lysates, monolayers were washed with phosphate-buffered saline (PBS, pH 7.2) and lysed with 0.25 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), and 20 mM EDTA. Culture supernatants and cell lysates were clarified by centrifugation in a microfuge prior to immunoprecipitation. To each sample was added 1 μl of goat anti-Rauscher MuLV (R-MuLV) gp70 serum, and the samples were incubated overnight at 4°C. The immune complexes were precipitated by the addition of protein A immobilized on agarose beads (Bethesda Research Laboratories). The precipitates were washed four times in lysis buffer, resuspended in sample buffer (with or without β-mercaptoethanol) and boiled for 5 min prior to analysis by SDS-polyacrylamide gel electrophoresis (PAGE) and fluorography.

Immunofluorescence studies. Monolayers of CV-1 cells grown on glass coverslips were infected with recombinant viruses (at a multiplicity of 10 for 6 h) and examined by immunofluorescence as previously described (31). For internal fluorescence, monolayers were washed with PBS and permeabilized with an ethanol-acetic acid mixture (95:5) for 20 min at −20°C. Cells were reacted with goat anti-R-MuLV

A

p4 (SFFV)

H   B   E   As   K   H

p231 (F-MuLV)

E   X   B   B   As C   K   E

pG.XK

X   B   B   As C   K

Ch.SFFV

H   B   E   As C   K   H

Ch.SFFV.HK

H   B   E   As C   K

SFFVt

H   B   E   K

B   *Chimeric SFFV*

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GTAAGAGATAGCATGGCCAAATTAAGAAAGAGACTCACTCAGAGACAAAAATTATTTGAGTCGAGCCGA
ValArgAspSerMetAlaLysLeuArgLysArgLeuThrGlnArgGlnLysLeuPheGluSerSerArg
              Asu2
GGATGGTTCGAAGGATTGTTTAACAGATCCCCCTGGTTTACCACGTTAATATCCACCATCATGGGGCCT
GlyTrpPheGluGlyLeuPheAsnArgSerProTrpPheThrThrLeuIleSerThrIleMetGlyPro
                            ClaI
CTCATTATACTCCTACTAATTCTGCTTTTTGGACCCTGCATTCTTAATCGATTAGTTCAATTTGTTAAA
LeuIleIleLeuLeuLeuIleLeuLeuPheGlyProCysIleLeuAsnArgLeuValGlnPheValLys

GACAGGATCTCAGTAGTCCAGGCTTTAGTCCTGACTCAACAATACCACCAGCTAAAACCACTAGAATAC
AspArgIleSerValValGlnAlaLeuValLeuThrGlnGlnTyrHisGlnLeuLysProLeuGluTyr

GAGCCATGATAAATAAAAGATTTTATTTAGTTTCCAGAAAAAGGGGGGAATGAAAGACCCCACCAAGTT
GluPro *  *

GCCGG
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Truncated SFFV

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GTAAGAGATAGCATGGCCAAATTAAGAAAGAGACTCACTCAGAGACAAAAATTATTTGAGTCGAGCCGA
ValArgAspSerMetAlaLysLeuArgLysArgLeuThrGlnArgGlnLysLeuPheGluSerSerArg

GGATGGTTCGATTAGTTCAATTTGTTAAAGACAGGATCTCAGTAGTCCAGGCTTTAGTCCTGACTCAAC
GlyTrpPheAsp *

AATACCACCAGCTAAAACCACTAGAATACGAGCCATGATAAATAAAAGATTTTATTTAGTTTCCAGAAA
AAGGGGGGAATGAAAGACCCCACCAAGTTGCCGG
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FIG. 1. (A) Restriction endonuclease maps of original and constructed DNAs. Locations of the coding regions for the envelope glycoproteins and the long terminal repeats in the SFFV (p4) and MuLV (p231) genomes are shown. An *XbaI-KpnI* fragment from the MuLV genome which contains the entire envelope gene and portions of the long terminal repeat was first subcloned into plasmid vector pGem2 (pG.XK). Plasmid Ch.SFFV was constructed by substituting the *AsuII-KpnI* fragment of p4 with a similar fragment derived from pG.XK. A *HindIII-KpnI* fragment from Ch.SFFV was first subcloned in to pUC19, and the resulting plasmid, ChSFFV.HK, was doubly digested with *AsuII* and *ClaI* and religated to obtain SFFVt. Abbreviations: H, *HindIII*; B, *BamHI*; E, *EcoRI*; As, *AsuII*; K, *KpnI*; X, *XbaI*; C, *ClaI*. DNA fragments used for ligation were prepared from cesium chloride gradient-purified plasmid DNA by restriction enzyme digestion and separation on low-melting-point agarose gels. (B) Partial nucleotide sequence and deduced amino acid sequence of chimeric and truncated SFFV. The hydrophobic, putative membrane-spanning regions are underlined. The asterisks indicate the termination codon. The amino acid residue in boldface indicates the residue not present in wild-type SFFV.

gp70 serum for 20 min and then with fluorescein-conjugated rabbit anti-goat immunoglobulin G. For surface immunofluorescence, unfixed, intact monolayers were used. After staining, the coverslips were mounted on a slide and examined under a Nikon Optiphot microscope equipped with a modified B2 cube.

RESULTS

Expression of wild-type and truncated SFFV gp52 molecules by recombinant vaccinia viruses. Recombinant vaccinia viruses that express wild-type SFFV gp52 molecules (rVV-SFFV) or truncated, anchor-minus SFFV gp52 molecules

(rVV-SFFVt) were used to infect CV-1 cells at a multiplicity of 10. After 4 h of infection, cells were labelled with [^{3}H]glucosamine (100 μCi/ml) for 16 h. At the end of the labelling period, the cells were lysed and the cell lysates were subjected to immunoprecipitation with goat anti-gp70 (R-MuLV) serum. In cells infected with rVV-SFFV, we observed gp52, the primary translation product of the SFFV envelope gene, as well as large amounts of gp65 which corresponds to the processed form of SFFV gp52 (Fig. 2, lane A). A major band migrating slightly faster than SFFV gp52 was observed in cells infected with rVV-SFFVt. The gp52t molecules also underwent oligosaccharide processing to yield a high-molecular-weight form of the protein, al-
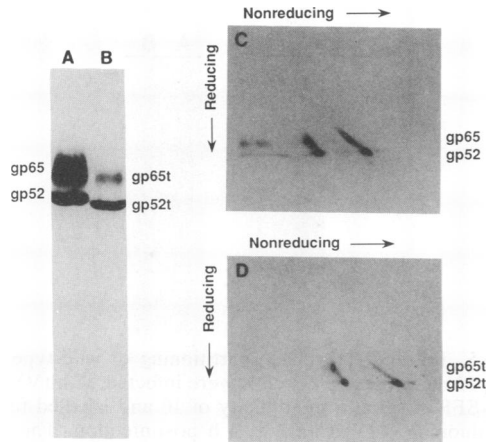
FIG. 2. Expression of SFFV gp52t molecules by recombinant vaccinia virus vectors. CV-1 cells were infected with rVV-SFFV (A) or rVV-SFFVt (B) at a multiplicity of 10 and labelled for 16 h with [^{3}H]glucosamine (100 μCi/ml) at 4 h postinfection. The cell lysates were subjected to radioimmunoprecipitation using goat anti-gp70 (R-MuLV) serum. (C and D) Two-dimensional reducing-nonreducing SDS-PAGE analyses of immunoprecipitates prepared from rVV-SFFV-infected (C) or rVV-SFFVt-infected (D) CV-1 cells.

though the extent of oligosaccharide processing was much lower than for wild-type gp52. The processed form of gp52t (gp65t) also displayed an electrophoretic mobility slightly faster than that of gp65 (Fig. 2, lane B). Labelling with [^{3}H]glucosamine demonstrates that gp52t is translocated across the rough endoplasmic reticulum (RER) membranes and undergoes glycosylation. The observed size decrease for gp52t is consistent with the expected size for gp52 molecules that lack transmembrane domain sequences.

SFFV gp52 has been shown to form disulfide-linked oligomers (10, 13, 37). We therefore examined the ability of gp52t molecules to form disulfide-linked oligomers by two-dimensional (reducing-nonreducing) SDS-PAGE. Under these conditions, disulfide-linked dimers and higher forms of both gp52 and gp65 were observed in rVV-SFFV-infected cells (Fig. 2C). Essentially similar results were observed in rVV-SFFVt-infected cells (Fig. 2D), suggesting that gp52t molecules also form disulfide-linked oligomers.

SFFV gp52t molecules are transport defective. It was previously shown that F-MCFV envelope proteins which lack the hydrophobic membrane-spanning sequences are efficiently secreted from cells (32). To investigate the effects of a similar deletion in the SFFV envelope gene upon localization of the protein product, we investigated the transport and secretion of the anchor-minus gp52t molecules. CV-1 cells were infected with rVV-SFFVt at a multiplicity of 10 and labelled with [^{3}H]glucosamine (100 μCi/ml) for 30 min at 6 h postinfection. The culture medium was replaced with DMEM containing 1% fetal calf serum. The culture supernatant and cell lysates were harvested at different intervals after labelling and analyzed by radioimmunoprecipitation. At all the time points tested, the majority of the label was associated with the gp52 form, which remained cell associated (Fig. 3). Small amounts of the processed (gp65t) form were observed in the cell lysates and in culture supernatants, consistent with the idea that a small proportion of these molecules exits the RER and is eventually secreted from the cell. Densitometric scanning of the autoradiograms indicates that the amount of gp65t secreted into the medium increases
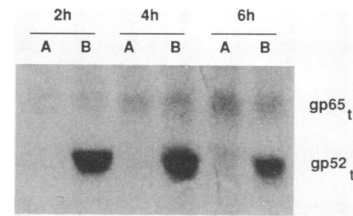

FIG. 3. Analysis of cell-associated and secreted forms of gp52t molecules. CV-1 cells infected with rVV-SFFVt at a multiplicity of 10 were labelled for 30 min with [^{3}H]glucosamine (100 μCi/ml) at 6 h postinfection. The radioactive medium was replaced with label-free DMEM containing 1% fetal calf serum. At 2, 4, and 6 h after the labelling period, culture supernatants and the cell lysates were harvested and subjected to radioimmunoprecipitation using goat anti-gp70 (R-MuLV) serum.

with time, but even after a prolonged chase (up to 6 h), more than 50% of the label remained cell associated. These findings are in sharp contrast to results with truncated anchor-minus F-MCFV envelope proteins, which are efficiently secreted from the cells within a 2-h chase period (32). Thus, these results indicate that the anchor-minus gp52t molecules retain the transport defect exhibited by wild-type gp52 molecules.

The intracellular levels of the processed forms of SFFV envelope proteins were considerably lower in rVV-SFFVt-infected than in rVV-SFFV-infected cells (Fig. 2). The processed forms of gp52 are readily detected in immunoprecipitates from cells labelled with radioactive sugar precursors but not with radioactive amino acids. We therefore compared the incorporation of [^{3}H]glucosamine into gp52 and gp65 species in rVV-SFFVt- and rVV-SFFV-infected cells to determine the relative efficiencies of processing and secretion of the wild-type and mutant envelope proteins. A significantly smaller proportion of the mutant gp52t than of wild-type gp52 was converted to the processed form (gp65t), suggesting that the mutant envelope proteins are processed less efficiently (Table 1). Furthermore, nearly the entire fraction of wild-type gp65 was cell associated, whereas up to one-third of the mutant gp65t was secreted into the culture medium, thus further reducing the intracellular levels of gp65t. The small proportion (~1%) of the wild-type gp65 that was secreted to the medium had a slightly faster electrophoretic mobility than did intracellular gp65, consistent with previous reports (10, 26), while no such difference was

TABLE 1. Quantitative analysis of intracellular and secreted forms of wild-type and mutant gp52 molecules

Virus	% of total[a]		Fraction of gp65 secreted[b]
	gp52	gp65	
rVV-SFFV	77.9	22.1	0.9
rVV-SFFVt	91.0	9.0	29.4

[a] Recombinant vaccinia virus-infected CV-1 cells were labelled with [^{3}H]glucosamine (100 μCi/ml) for 16 h. The culture supernatants and cell lysates were collected and subjected to immunoprecipitation with goat anti-gp70 (R-MuLV) serum. The autoradiograms were scanned with an LKB soft laser densitometric scanner connected to a microcomputer equipped with the Gelscan peak integrating program. The peak intensity values were used to calculate the amount of radioactivity associated with gp52 and gp65 (intracellular and secreted forms).

[b] Amount of radioactivity associated with the secreted form of gp65 (from immunoprecipitates of medium samples), expressed as a fraction of the total (intracellular plus secreted) radioactivity associated with gp65.
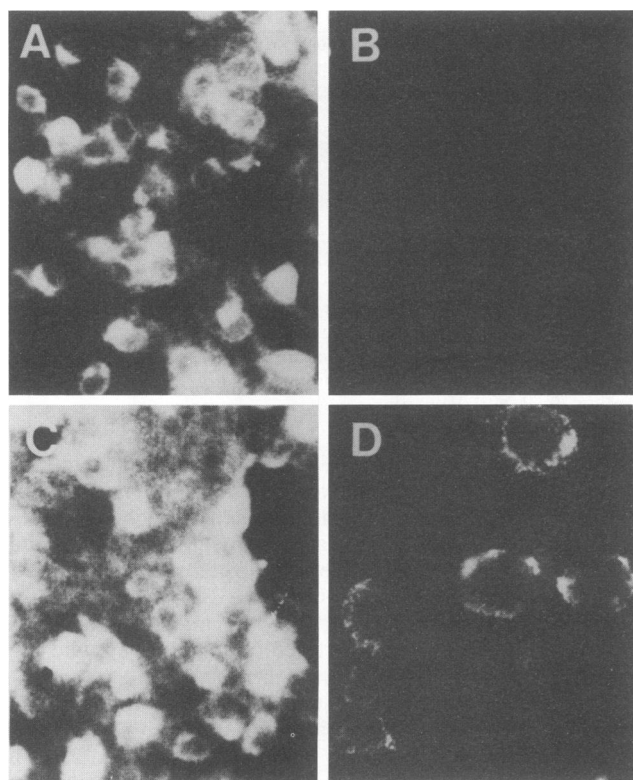
FIG. 4. Surface and intracellular localization of wild-type and truncated gp52 molecules. CV-1 cells were infected with rVV-SFFVt (A and B) or rVV-SFFV (C and D) at a multiplicity of 10. At 6 h postinfection, ethanol-acetic acid (95:5, vol/vol)-fixed (A and C) or unfixed (B and D) cells were reacted with goat-anti gp70 (R-MuLV) serum and fluorescein-conjugated rabbit anti-goat immunoglobulins. The cells were observed under a Nikon Optiphot fluorescence microscope.
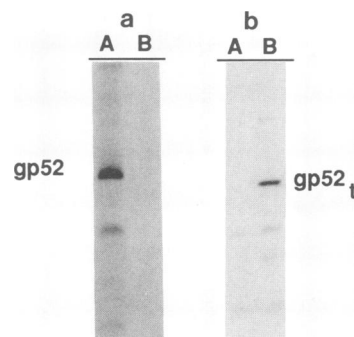

FIG. 5. Triton X-114 phase partitioning of wild-type and truncated gp52 molecules. CV-1 cells were infected with rVV-SFFV (a) or rVV-SFFVt (b) at a multiplicity of 10 and labelled for 2 h with [^{35}S]methionine (100 μCi/ml) at 4 h postinfection. The cells were lysed in cold 1% Triton X-114. The lysates were warmed to 37°C, and the detergent (lanes A) and aqueous (lanes B) phases were separated by centrifugation and analyzed by radioimmunoprecipitation using goat anti-gp70 (R-MuLV) serum.

evident between secreted and intracellular forms of gp65t. These results suggest that unlike with wild-type gp65, the secretion of mutant gp65t does not involve proteolytic processing, and occurs more efficiently.

Anchor-minus gp52t molecules are not associated with cell membranes. CV-1 cells infected with rVV-SFFV or rVV-SFFVt were analyzed by an indirect immunofluorescence assay using anti-gp70 serum to investigate the localization of the gp52t molecules. A strong intracellular fluorescence was observed in CV-1 cells infected with both rVV-SFFV and rVV-SFFVt (Fig. 4A and C). A low level of surface immunofluorescence was observed in CV-1 cells infected with rVV-SFFV (Fig. 4D), but no surface immunofluorescence was observed in cells infected with rVV-SFFVt (Fig. 4B). These results indicate that unlike SFFV gp65, the processed form of gp52t (gp65t) is not expressed on the cell membranes.

Upon solubilization in Triton X-114 and phase partitioning, membrane glycoproteins that carry hydrophobic membrane-anchoring domains partition into a separate detergent phase at temperatures above 20°C, while soluble and peripheral membrane glycoproteins partition into the aqueous phase (4). To investigate the possibility that the anchor-minus gp52 molecules remain membrane associated by some undefined hydrophobic sequences, we tested their partitioning into aqueous and detergent phases after solubilization by

Triton X-114. CV-1 cells were infected with rVV-SFFVt or rVV-SFFV at a multiplicity of 10, labelled at 3 h postinfection with [^{35}S]methionine (100 μCi/ml) for 2 h, and then lysed in 1% Triton X-114 at 4°C. The lysates were fractionated into detergent and aqueous phases by centrifugation after warming of the lysates to 37°C and analyzed by radioimmunoprecipitation with goat anti-gp70 serum. Nearly all of the gp52 molecules remained in the detergent phase in rVV-SFFV-infected cells (Fig. 5a), whereas the gp52t molecules partitioned into the aqueous fractions (Fig. 5b). This observation is consistent with the idea that truncated gp52 molecules are not membrane associated and exist within the content of the microsomes.

gp52t molecules do not associate with BiP. A family of closely related proteins, which includes the immunoglobulin heavy-chain-binding protein BiP, are resident proteins of the RER, and they have been suggested to play an important role in protein folding and transport (8, 9). These proteins are thought to associate with nascent proteins until they are properly folded and are exported to the Golgi complex from the RER. Additionally, they are thought to retain malfolded proteins and prevent their export along the exocytic pathway. In our previous studies, the transport-defective gp52 was not found to associate with BiP (13). To determine whether the mutations in gp52t molecules have altered their BiP-binding properties, and whether association with BiP is involved in the defective intracellular transport of SFFV gp52t molecules, rVV-SFFVt- or rVV-SFFV infected (multiplicity of 5) CV-1 cells were labelled with [^{35}S]methionine (100 μCi/ml) for 2 h, and the cell lysates were immunoprecipitated with a monoclonal antibody against BiP or with anti-gp70 serum. Anti-BiP antibody precipitated only BiP, either wild-type or truncated gp52 molecules. Likewise, immunoprecipitation with anti-gp70 serum precipitated only gp52-related molecules, not BiP (data not shown). Thus, neither wild-type nor mutant gp52 molecules were found to show any significant association with BiP.

Leukemogenicity of retroviral vectors expressing mutant SFFV envelope proteins. To determine the leukemogenicity of the anchor-minus gp52t molecules, we introduced the mutant envelope gene into an M-MuLV-based retroviral expression vector (pLXSN). The wild-type SFFV *env* gene was similarly cloned into pLXSN and used as a positive
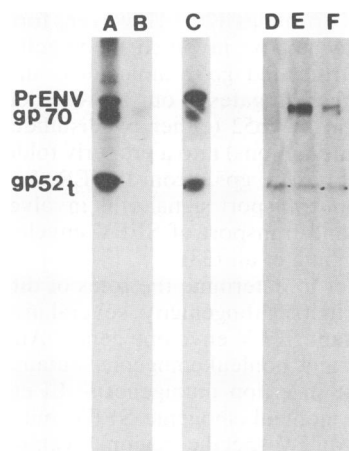
FIG. 6. Expression of gp52t molecules by recombinant retroviral vectors. NIH 3T3 cells cotransfected with pMOV-3 and pLXSN.SFFVt (lanes A and B) were labelled with [^{35}S]methionine for 4 h, and the cell lysates (lane A) and culture supernatants (lane B) were analyzed by immunoprecipitation with goat anti-gp70 serum. Expression of both MuLV (pre-Env [PrENV] and gp70) and SFFVt (gp52t) envelope glycoproteins are observed in transfected cells. The culture supernatants from these transfected cells were used as an inoculum to infect fresh NIH 3T3 cells, and the expression of MuLV and SFFVt genomes was monitored by radioimmunoprecipitation of [^{35}S]methionine-labelled (100 μCi/ml for 4 h) cell lysates (lane C). Lanes D to F show the profile of radioimmunoprecipitates of [^{35}S]methionine-labelled spleen cell suspensions prepared from M-MuLV (LXSN.SFFVt)-infected mice after 30 days postinfection.

control. Plasmid pMOV-3, which contains an integrated infectious proviral copy of the M-MuLV genome with flanking cellular sequences, was used as a cotransfectant in these studies to provide a helper virus for replication of the retroviral vectors. NIH 3T3 cells were electroporated with 10 μg of pLXSN.SFFVt or pLXSN.SFFV and 2 μg of an EcoRI fragment from pMOV-3. The cells were grown to confluence and passaged several times until all cells were uniformly infected with the input viruses. The expression of mutant SFFV and helper MuLV proteins in these cells was monitored by radioimmunoprecipitation. NIH 3T3 cells cotransfected with pLXSN.SFFVt and pMOV-3 were labelled with [^{35}S]methionine (100 μCi/ml) for 4 h, and the culture supernatants and cell lysates were immunoprecipitated with goat anti-gp70 serum. Both MuLV gp70 and SFFV gp52t were detected in the cell lysates (Fig. 6, lane A). Under these labelling conditions, little or no processing of gp52t into gp65t was observed, and gp52t was not detected in the culture supernatant (Fig. 6, lanes A and B). However, the processing and secretion of a small proportion of gp52t could be detected when the cells were labelled with [^{3}H]glucosamine (data not shown), and the extent of processing and secretion was similar to that observed in rVV-SFFVt-infected CV-1 cells. Infection of fresh NIH 3T3 cells with culture supernatants from pLXSN.SFFVt/pMOV-3-transfected cells also resulted in expression of both SFFV and MuLV genomes (Fig. 6, lane C), indicating that the mutant viral genome is efficiently packaged into MuLV pseudotypes.

Groups of adult NIH Swiss mice (strain CFW; Charles River) were inoculated intravenously with 0.2 ml of the culture supernatant and monitored for development of an erythroleukemia. At intervals after infection, mice were

TABLE 2. Leukemogenicity of retroviral vectors expressing wild-type or mutant SFFV envelope gene

Virus[a]	Days post-infection	Spleen wt (g)[b]
M-MuLV	15	0.15 (47), 0.16 (48), 0.18 (50)
	30	0.19 (49), 0.20 (48), 0.18 (47)
M-MuLV + pLXSN.SFFV	15	0.35 (54), 0.38 (49), 0.25 (48)
	30	1.36 (68), 1.95 (65), 2.15 (73)
	45	2.65 (73), 2.58 (70), 2.10 (69)
M-MuLV + pLXSN.SFFVt	15	0.15 (49), 0.14 (48), 0.20 (50)
	30	0.16 (48), 0.19 (47), 0.19 (48)
	45	0.17 (49), 0.20 (46), 0.19 (48)

[a] Samples (0.2 ml) of culture supernatants from NIH 3T3 cultures expressing the indicated viruses were inoculated intravenously into 4- to 6-week-old NIH Swiss mice (Charles River).

[b] Hematocrit values are given in parentheses.

sacrificed by cervical dislocation under anesthesia. Blood was collected by cardiac puncture, and the hematocrits were determined. The spleens were removed and weighed. Blood smears and impression smears from spleens were stained with Giemsa-Wright stain and examined microscopically for the presence of erythroblastoid Friend cells. The results are summarized in Table 2. Mice infected with M-MuLV alone did not show any signs of disease, while a marked splenomegaly, accompanied by polycythemia and the presence of large numbers of erythroblastoid cells in spleen and blood indicative of erythroleukemia, was readily observed in mice infected with M-MuLV pseudotypes of LXSN.SFFV (wild type). In contrast, mice infected with M-MuLV pseudotypes of the mutant SFFVt genome did not show any signs of erythroleukemic disease. The expression of MuLV and SFFV genomes in the infected animals was analyzed by radioimmunoprecipitation of [^{35}S]methionine-labelled (100 μCi/ml for 2 h) spleen cell suspensions prepared from mice sacrificed at 30 days postinfection, using goat anti-gp70 serum. The levels of expression of SFFV gp52 and MuLV gp70 varied in these animals (Fig. 6, lanes D to F); two of the three animals tested showed the presence of MuLV gp70, while SFFV gp52t was expressed in all the three animals. These results demonstrate that the absence of disease was not due to a lack of replication of mutant SFFV genomes in vivo.

DISCUSSION

SFFV gp52 is a recombinant, transport-defective envelope glycoprotein derived from the MuLV envelope proteins by a series of changes involving substitutions, deletions, and insertions. The precise structural features of SFFV gp52 that account for its defective intracellular transport and the cellular basis for this transport defect have not been clearly explained. We have previously observed that chimeric SFFV envelope glycoproteins, in which the membrane-spanning domain sequences were replaced with membrane-spanning and cytoplasmic domain sequences from MuLV envelope proteins, were also transport defective (31). Similarly, MuLV envelope proteins in which the transmembrane and cytoplasmic domains were replaced with the transmembrane domain of SFFV gp52 were found to be transport competent (14). Together, these results indicate that the absence of a cytoplasmic tail, or altered primary sequence of the transmembrane domain, does not account for the transport defect of SFFV gp52 and implicate changes in the extracellular domain of SFFV gp52 in its transport defect. In

this study, we show that the ectodomain of SFFV gp52, when expressed in the absence of any membrane-anchoring sequences, is not efficiently secreted from the cells but instead is largely retained inside the cell in a form that corresponds to the RER form of SFFV gp52. These results are consistent with the earlier findings and further suggest that the transport defect of the extracellular domain of gp52 is manifest even when it is expressed as a secretory protein devoid of membrane-anchoring sequences. These results implicate the changes found in the extracellular domain of gp52, i.e., deletion of a 195-amino-acid-long sequence, in the transport defect of SFFV gp52.

Using nonreducing-reducing two-dimensional gels, Gliniak and Kabat (10) reported that gp55 exists as both monomers and disulfide-linked dimers, while gp65 exists primarily as disulfide-linked dimers. Furthermore, only dimers of gp65 were found on the cell surface, while the secreted form of gp65 was found to exist as both monomers and dimers. On the basis of these results, they suggested that gp52 has a high propensity to form disulfide-linked complexes, and only properly folded dimers are export competent, while improperly folded proteins are retained in the ER. By similar approaches, Yang et al. (38) observed disulfide-linked dimers and trimers of both gp52 and gp65 from a variety of erythroleukemia cell lines. We have also observed disulfide-linked dimers and trimers of both gp52 and gp65 in cells infected with recombinant vaccinia virus vectors (13). The gp52t molecules and their processed forms also formed disulfide-linked dimers, trimers, and tetramers, although the higher-molecular-weight forms (trimers and tetramers of gp52t and gp65t) were less evident. Interestingly, Yang et al. (38) reported that gp52 from two nonpathogenic SFFV env mutants also formed disulfide-linked oligomers, although less efficiently than did wild-type gp52. We have not been able to distinguish between transport-defective and transport-competent subsets of gp52 dimers. Further studies are required to elucidate the relationships between different disulfide-linked oligomeric forms of gp52 and their biological significance.

BiP, a member of the 70-kDa heat shock protein family, is thought to act as a molecular chaperone in protein folding, and it retains malfolded proteins in the ER (8, 9). These proteins are thought to function as foldases and retain the proteins in the ER until they undergo proper folding; properly folded proteins are released for export, while malfolded proteins are retained within the ER. For example, influenza virus hemagglutinin associates with BiP soon after its synthesis, but this association is lost once the hemagglutinin molecules assemble into trimers (9). Mutants of influenza virus hemagglutinin which are defective in transport from the ER remain associated with BiP (9). Neither the wild-type nor truncated gp52 was found to associate with BiP, and BiP association does not appear to play a role in defective transport of SFFV gp52. It is possible that the lack of such association is responsible for misfolding and improper disulfide bonding of a large proportion of gp52 molecules, thus leading to their transport defect. Alternatively, BiP may not be involved in folding or retention of gp52. Examples of other transport-defective proteins which fail to associate with BiP have been documented; for example, the G protein of a temperature-sensitive mutant of vesicular stomatitis virus (VSV) was not found to aggregate with BiP, although wild-type VSV G protein transiently associates with BiP (8). Aberrant intermolecular disulfide bonding, leading to the accumulation of large protein complexes in the ER, has been shown to be responsible for defective transport of certain mutant VSV G proteins (20). However, formation of large aggregates may not be involved in defective transport of wild-type or truncated gp52 molecules, since we did not detect any such aggregates in our sucrose density gradients. Oligomerization of gp52 (either by disulfide linkage or by noncovalent interactions) into a properly folded form may be required for export of gp52 from the ER. It is possible that some additional transport signal(s) is involved in governing the retention and transport of SFFV envelope proteins, as suggested by Yang et al. (38).

In an attempt to determine the roles of different domains of SFFV gp52 in its pathogenicity, several investigators have generated mutant SFFV envelope genes. Among a series of leukemogenic and nonleukemogenic mutants generated by in-phase linker insertion mutagenesis, Li et al. (15) found that all of the nonleukemogenic SFFV mutants had insertions in the MCFV-specific region. Watanabe et al. (33) constructed mutant SFFV env genes in which the MCFV-specific sequences were replaced with ecotropic MuLV sequences and found these viruses to be nonleukemogenic. Thus, the available evidence indicates that the amino-terminal, MCFV-specific sequences of gp52 are required for its leukemogenicity. In other studies, a recombinant virus in which a restriction fragment of the SFFV env gene containing the 585-bp deletion was replaced with a corresponding fragment from the MCFV env gene was found to be nonpathogenic in adult mice (33). Upon passage of this virus in newborn mice, several leukemogenic variants of the virus emerged, and molecular analyses of these variants revealed the presence of distinct deletions (ranging from 312 to 590 bp) in each variant virus (33), suggesting that a deletion found in the SFFV env gene (in comparison with the MCFV env gene) is essential for leukemogenicity.

In comparison with the MuLV and MCFV envelope proteins, gp52 lacks any detectable cytoplasmic tail sequences and the membrane-spanning peptide has an altered primary sequence. We and others have shown that mutants of SFFV in which the transmembrane domain is replaced by transmembrane and cytoplasmic domains of MuLV are nonleukemogenic in adult mice (2, 31). Amanuma et al. (2) also observed the emergence of leukemogenic variants when the mutant virus was passaged in newborn mice, and analysis of the variant viruses revealed that they had regained a single base insertion. These studies indicate the importance of the frameshift-inducing single base insertion in the leukemogenicity of the SFFV envelope gene. It is presently not clear whether the changes in the primary sequence of the membrane-spanning peptide or the absence of cytoplasmic sequences accounts for the leukemogenicity of gp52. Machida et al. (22) have described a weakly pathogenic R-SFFV mutant that lacks the membrane anchor sequences and causes only a mild erythroblastosis in mice. A role for gp52 transmembrane domain sequences in disease production has also been suggested by other studies. Although both SFFV$_P$ and SFFV$_A$ can induce acute erythroleukemia, only cells infected with the SFFV$_P$ become erythropoietin independent for proliferation and differentiation. Studies on recombinant viruses made by replacing env gene fragments from SFFV$_A$ with sequences derived from SFFV$_P$ indicated that a 113-bp FokI-ClaI fragment, which encodes primarily the transmembrane domain of the envelope protein, determines erythropoietin-independent growth (5, 6). These results indicate that the transmembrane domain of SFFV gp52 plays a major role in the disease process. The lack of leukemogenicity of the SFFV gp52t molecules used in this study indicates that the transmembrane domain of gp52 may function both in devel-

opment of erythroleukemia and in determining the erythropoietin responsiveness of SFFV-infected erythroblasts. In addition to the loss of membrane anchor sequences, six adjacent residues, Glu-Gly-Leu-Phe-Asn-Arg-Ser, amino terminal to the membrane-anchoring sequences are replaced with an Asp residue in the mutant envelope gene (Fig. 1). It is therefore possible that this region of gp52 is functionally important, and the mutations in this region may be responsible, entirely or in part, for the lack of leukemogenicity of the mutant SFFV.

Studies with mutant SFFV genomes indicate that cell surface expression (or secretion) of the processed form of gp52 may be a prerequisite for leukemogenicity (2, 10, 15, 31). It is possible that a threshold concentration of the processed form is required for activity, in which case the reduced level of processing observed with the mutant gp52t may account for its lack of leukemogenicity. Furthermore, the secreted form of the mutant glycoprotein differs from the secreted form of the wild-type gp65 in its primary sequence due to the replacement of six residues amino terminal to the membrane with an extraneous amino acid residue, and these differences may also contribute to its nonleukemogenicity.

Recent studies indicate that SFFV gp52 binds directly to the murine erythropoietin receptor (EpoR) and mediates signal transduction (16, 29). Available evidence indicates that gp52-EpoR interactions occur intracellularly at the RER and affect receptor metabolism (39), and hence the ER may indeed be the site at which signal transduction occurs in SFFV-infected erythroblasts. Therefore, other possible explanations for the lack of leukemogenicity of the mutant gp52 molecules include (i) a lack of binding of truncated gp52 molecules to EpoR due to an altered conformation, (ii) requirement for membrane anchoring of gp52 to facilitate post-receptor binding events such as receptor clustering or endocytosis, or (iii) a direct involvement of the gp52 transmembrane domain in the signalling process by interaction either with EpoR or with other cellular factors after gp52 binds to EpoR. We currently favor the idea that the ectodomain of SFFV gp52 is involved in binding to EpoR and the transmembrane domain is involved in signalling. The differences in the biology of SFFV$_A$ and SFFV$_P$ gp52, which differ in their transmembrane regions, support such a hypothesis.

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