Erythropoietin Receptor (EpoR)-Dependent Mitogenicity of Spleen Focus-Forming Virus Correlates with Viral Pathogenicity and Processing of *env* Protein but Not with Formation of gp52-EpoR Complexes in the Endoplasmic Reticulum

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Received 6 July 1992/Accepted 16 November 1992

Recent evidence suggests that interactions between spleen focus-forming virus (SFFV) *env* products and the erythropoietin receptor (EpoR) are responsible for viral pathogenicity. Infection of factor-dependent cell lines expressing *epoR* (the cloned gene for EpoR) with SFFV_P is mitogenic, generating cell lines that are no longer dependent on added growth factor, and an immunoprecipitable complex between EpoR and immature *env* protein in the endoplasmic reticulum has been identified. The dependence of these *in vitro* activities on *env* protein processing and their relationship to pathogenicity of SFFV were explored by using glycosylation site mutants of SFFV *env*. Mutants carrying Asn→Asp mutations at each of the two consensus signals for N-linked glycosylation in the N-terminal domain of SFFV_{AP-L} *env* (gs1 and gs2), the gs1⁻2⁻ double mutant, and the gs0 quadruple mutant (mutated at all four signals utilized for N-linked glycosylation in SFFV_{AP-L} *env*) were made. The primary translation products (gp52) of single-site mutant *env*s were processed into more highly glycosylated forms, and the corresponding viruses induced splenomegaly in susceptible mice, whereas the gs1⁻2⁻ and gs0 proteins were not processed, and these viruses were not pathogenic. Unprocessed *env* proteins of both pathogenic and nonpathogenic mutants coprecipitated with EpoR. In the BaF3 cell assay for *epoR*-dependent mitogenicity, the pathogenic single mutants induced factor-independent growth efficiently whereas the nonpathogenic gs1⁻2⁻ and gs0 mutants did not. These data demonstrate that the ability of gp52 to form complexes with EpoR in the endoplasmic reticulum is not sufficient for either mitogenicity in cell culture or induction of splenomegaly in mice while supporting the hypothesis that pathogenicity and mitogenicity of SFFV both result from an interaction between EpoR and SFFV *env* protein.

Friend spleen focus-forming virus (SFFV), the pathogenic component of the Friend virus complex, induces erythroleukemia in mice (reviewed in reference 26). The disease induced by Friend virus complex has an early, preleukemic stage in which rapid polyclonal proliferation of erythroid precursor cells results in erythrocytosis and splenomegaly. The pathogenicity of SFFV has been shown to be a function of *env* (15), which encodes a defective viral envelope protein that appears to be derived from a replication-competent dualtropic (MCF) *env* (reviewed in references 9, 23, and 26). The primary translation product of wild-type SFFV *env*, located in the endoplasmic reticulum (ER), is a glycoprotein with a molecular mass of 52 kDa (gp52) that carries high-mannose N-linked glycans. A small fraction of gp52 is processed into a mature product, gp65 for the polycythemia-inducing SFFV_P isolates and gp60 for the anemia-inducing SFFV_A isolate. The maturation process involves transport through the Golgi apparatus, with conversion of most of the N-linked glycans to complex forms and the addition of O-linked glycans. The mature forms are found on the cell surface and are slowly yet efficiently released from cells. Genetic studies have demonstrated a correlation between pathogenicity and processing of SFFV *env* proteins to the cell surface and/or its secretion (1, 13, 16, 28).

Infection by SFFV interferes with the normal growth and

development of erythroid precursor cells (reviewed in reference 26). Erythroid burst-forming cells are elevated in animals infected with either SFFV_P or SFFV_A. For the SFFV_P isolates, these erythroid precursors can be cultured without added erythropoietin (Epo), which is required for growth of such cells from uninfected animals. Infection of erythroid precursors *in vitro* with SFFV_P also induces Epo-independent proliferation. Evidence for the involvement of the Epo receptor (EpoR) in SFFV-induced erythroid proliferation has come from recent experiments showing that coexpression of SFFV_P and recombinant *epoR* (a murine gene for a functional EpoR) causes growth factor-independent proliferation of the otherwise interleukin-3 (IL-3)-dependent cell lines BaF3 (14) and DA-3 (8). Direct evidence for an interaction between EpoR and SFFV *env* proteins was provided by the demonstration of complexes that precipitate with antibodies to either protein in 3T3 fibroblasts (14) and BaF3 cells (38) expressing both proteins. Evidence has also been presented for the presence of ternary complexes between Epo, EpoR, and processed *env* protein on the surface of Friend erythroleukemia cells (2). These studies suggest that an interaction between SFFV *env* proteins and EpoR is mitogenic *in vitro* and is responsible for the erythroid proliferation induced *in vivo*. However, it has not been definitively shown that the *in vitro* mitogenicity of SFFV is a function of *env* protein, and the nature and location of the putative mitogenic complex between EpoR and SFFV *env* protein has not been defined.

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TABLE 1. Mutagenic oligonucleotides

Glycosylation signal	Oligonucleotide sequence ^a							
gs1	Gln	Val	Phe	Asp	Val	Thr	Trp	
	CAG	GTC	TTC	<u>GAC</u>	GTC	ACT	TGG	AG
				<i>Bsa</i> HI				
gs2	Gly	Gln	Thr	Ala	Asp	Ala	Thr	
	GGA	CAA	ACC	<u>GCG</u>	GAT	GCT	ACC	
				<i>Ksp</i> I				
gs3 ^b	Tyr	Gln	Ala	Leu	Asp	Leu	Thr	Asn
	CC	TAC	CAA	GCT	<u>CTA</u>	GAT	CTC	ACC
				<i>Bgl</i> II				
gs4 ^b	Tyr	Phe	Asp	His	Thr			
	CT	TAT	TTT	<u>GAT</u>	CAT	ACC		
				<i>Bcl</i> I				

^a Mutant bases are underlined, and restriction sites associated with the mutations are italicized.

^b The sequence shown is the complement of the oligonucleotide used.

In this report, specific mutations of SFFV *env* were used to explore relationships among the ability of SFFV *env* proteins to be processed out of the ER, to form complexes with EpoR in the ER, to induce factor-independent growth in BaF3-EpoR cells, and to induce splenomegaly in mice. The results obtained indicate that the ability of gp52 to bind to EpoR in the ER of 3T3 cells is not sufficient for induction of factor-independent growth or splenomegaly. The correlation observed between processing of *env* products and biological activity is consistent with the hypothesis that activation of EpoR by processed forms of SFFV *env* protein is the basis of SFFV pathogenicity.

MATERIALS AND METHODS

Cell lines and viruses. Mouse NIH 3T3 fibroblast cells, the ecotropic packaging lines ψ_2 (17) and ψ_{CRE} (3), the amphotropic packaging lines PA317 (20) and ψ_{CRIP} (3), and pre-B-lymphoid BaF3 cells (18) and its Epo-responsive derivative BaF3-EpoR (14) were maintained as described below. SFFV_{AP-L} (27) is a recombinant virus between SFFV_A and SFFV_P that has biological and biochemical properties indistinguishable from those of SFFV_P. The helper virus for pathogenicity experiments was ecotropic Friend murine leukemia virus (F-MuLV) clone 57 (22). 3T3 cell lines expressing *epoR* were generated by infecting with pseudotyped pSFF-*epoR* retroviral vector prepared in ψ_2 cells (14).

Construction of mutants and transfection. Specific N-linked glycosylation signals (Asn-X-Ser/Thr) of SFFV *env* were modified by changing the Asn codons to Asp codons by oligonucleotide-directed site-specific mutagenesis, using the oligonucleotides described in Table 1. Mutations were isolated on phagemid subclones and enriched with the Kunkel method (12, 19), and fully sequenced fragments containing the mutations were cloned back into the complete viral genome plasmid. In the regions sequenced, the isolate of SFFV_{AP-L} used in these studies differed from the published sequences at a number of positions. In the sequences derived from SFFV_A (35), the published nucleotide at position 189, A, was found here to be G; the published nucleotides at positions 503 and 504, CA, were found here to be AG, resulting in a Pro→Gln coding difference. In the sequences derived from SFFV_P (36), the published nucleotide at position 986, C, was found here to be T, resulting in a Pro→Leu coding difference; the published nucleotide at position 945, T, was found here to be C; the published nucleotide at

position 948, C, was found here to be T. Mutations that eliminate glycosylation signals, and plasmids or viruses carrying these mutations, are designated gsX⁻, where X is the sequential number of the glycosylation signal from the N terminus. The gs0 mutant carries mutations that eliminate all four utilized glycosylation signals in SFFV_{AP-L} *env*. The resulting plasmids were transfected into a mixture of ψ_2 and PA317 or ψ_{CRE} and ψ_{CRIP} packaging cells by calcium phosphate precipitation (CellPfect; Pharmacia). Infectious pseudotypes containing the SFFV genomes obtained from these cocultures between 1 and 4 weeks posttransfection were used to prepare 3T3 and BaF3-EpoR cell lines expressing SFFV *env*.

Analysis of protein expression. Radioimmunoprecipitation assays (RIPA) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed as previously described (25) except for the addition of a preclearing ultracentrifugation step for coprecipitation experiments as described elsewhere (14). Sequential immunoprecipitations with anti (α)-gp70 followed by α -EpoR antibodies were performed as described previously (14). The following antibodies were used: hyperimmune goat sera to Rauscher MuLV gp70 (SU) and to BV2 MuLV gp70 from Microbiological Associates, rat monoclonal antibody 7C10 (35a) specific for SUs of MCF MuLVs and SFFV *env* products, and two hyperimmune rabbit sera to the cloned murine *epoR* product raised against peptides matching either the N terminus (14) or C terminus (38) of EpoR. Deglycosylations were performed with peptide N-glycosidase F (PNGase F) as previously described (25).

Determination of pathogenicity. 3T3 cell lines expressing SFFV were superinfected with F-MuLV to generate stocks of Friend virus complex containing infectious SFFV pseudotypes and replication-competent helper virus. Relative levels of expression of F-MuLV and SFFV *env* proteins were monitored by RIPA. Culture supernatants (0.1 ml) from cultures that were producing similar amounts of SFFV and helper virus proteins were injected intraperitoneally into 4- to 6-week-old HSD:ND4 mice. Infected mice were sacrificed 2 and 6 weeks postinfection, and spleen weights were determined.

In vitro mitogenicity assay. BaF3-EpoR cells were infected with SFFV pseudotypes from packaging line cocultures and grown in IL-3-containing medium for 2 days. Cultures were adjusted to 50% infected cells (as determined by immunofluorescence with 7C10, which is specific for SFFV *env* proteins in this context) by diluting with uninfected BaF3-EpoR cells and were then inoculated into multititer plates at various cell densities in medium without growth factor. Wells were scored for cell growth after 2 weeks.

RESULTS

Processing of SFFV *env* mutants. Asn→Asp mutations at predicted N-linked glycosylation sites were introduced into SFFV *env* to generate proteins defective in processing and pathogenicity. To characterize the products of the *env* mutants, cells expressing wild-type SFFV and the gs1⁻, gs2⁻, gs1⁻2⁻, and gs0 mutants were each examined by RIPA and SDS-PAGE. The data shown here are from cell lines that were superinfected with F-MuLV; similar processing of SFFV *env* products was observed in the absence of helper virus. In Fig. 1A, immunoprecipitates of cell lysates labeled with ³⁵S-amino acids are shown. In each of the cell lines, the primary translation product of SFFV *env* was seen. Relative to the wild-type gp52, the single-mutant *env* prod-

FIG. 1. Characterization of mutant SFFV *env* products. 3T3 cell lines expressing wild-type and various mutant SFFVs that had been superinfected with F-MuLV were metabolically labeled and analyzed by RIPA. (A) Cells were labeled for 1.5 h with a mixture of [³⁵S]methionine and [³⁵S]cysteine, and cell lysates were immunoprecipitated with 7C10, a monoclonal antibody that reacts with SFFV but not F-MuLV *env* products. Untreated lysates (left panel) and samples that had been digested with PNGase F (Ngly) prior to electrophoresis (right panel) were compared. (B) Cells were labeled for 3 h with [³H]glucosamine and immunoprecipitated with hyperimmune α -BV₂ gp70 serum, which recognizes both SFFV (gp52 and gp65) and F-MuLV *env* products. Lanes are the same as in panel A. Lanes: A, wild type; B, *gs1*⁻; C, *gs2*⁻; D, *gs1*⁻²; E, *gs0*.

ucts appeared to be 2 to 3 kDa smaller, the double mutant product appeared to be approximately 5 kDa smaller, and the *gs0* product appeared to be approximately 10 kDa smaller (left panel). These data were consistent with the expected loss of one, two, or four N-linked glycans, respectively. For convenience, all of these proteins will be referred to as gp52, regardless of size or glycan content and all processed forms will be called gp65. After digestion with PNGase F to remove N-linked glycans, the primary translation products of each of these *envs* comigrated with untreated *gs0* protein (right panel). These data demonstrated that the altered mobilities of the mutant proteins were due to differences in N-linked glycosylation and that the *gs0* product did not carry any N-linked glycans.

Detection of gp65 is difficult when amino acid radiolabel is used because only a small fraction of SFFV *env* product undergoes processing and because the gp65 band is very diffuse as a result of extensive glycan heterogeneity. gp65 is readily detected with glucosamine radiolabel as a result of the incorporation of additional label into O-linked glycans and modified N-linked glycans (24). With use of [³H]glucosamine label, processing of wild-type gp52 to gp65 was seen, as was processing of the *gs1*⁻ and *gs2*⁻ *env* products, although less label was associated with the processed mutant proteins (Fig. 1B). The size increase between gp52 and gp65 was close to normal for these mutants, indicating that the amount of carbohydrate added per mutant molecule during processing was similar to the amount added to wild-type protein. Thus, the lower level of label in processed protein reflected a large decrease in the efficiency with which these mutant gp52s were processed to gp65. Since larger forms were not detected for the *gs1*⁻² mutant and no SFFV *env*

product was detected for the *gs0* mutant with use of [³H]glucosamine label, O-linked glycans were not added to either of these mutant proteins. These observations indicated that these mutant *env* proteins were not transported into the Golgi apparatus compartment where O-linked glycosylation occurs. Thus, there was a stringent block to processing for the *gs1*⁻² and *gs0* proteins.

Pathogenicity of SFFV *env* mutants. The ability of the SFFV *env* mutant proteins to induce splenomegaly was evaluated by injecting complexes of F-MuLV pseudotypes of SFFV and helper virus intraperitoneally into susceptible mice. Spleen weights, determined at 2 and 6 weeks postinfection, are presented in Table 2. As expected, infection with F-MuLV helper alone resulted in a mild increase in spleen weight that disappeared by 6 weeks. This effect is believed to be a normal proliferative response to the mild hemolytic anemia caused by F-MuLV infection (29). By 2 weeks, infection with wild-type SFFV caused massive splenomegaly. The *gs1*⁻ and *gs2*⁻ mutants each induced splenomegaly in five of eight mice, and the enlarged spleens induced by these mutants were similar in size to those induced by wild-type SFFV. These results indicated that the *gs1*⁻ and *gs2*⁻ mutants were pathogenic, albeit slightly attenuated for induction of splenomegaly. In contrast, mice infected with the *gs1*⁻² and *gs0* mutants were indistinguishable at 2 weeks from those infected with only F-MuLV helper virus. At 6 weeks, one animal infected with each of the *gs1*⁻² and *gs0* mutants had an enlarged spleen. Examination of the *env* proteins synthesized in primary cell cultures from these enlarged spleens showed that these cells did not express the input SFFV *env* mutant proteins but did express larger *env*-related proteins that were likely to have been the prod-

TABLE 2. Induction of splenomegaly by SFFV Mutants

Virus	Spleen wt (g) of infected HSD:ND4 mice	
	2 wk postinfection	6 wk postinfection
None	0.09, 0.18	
F-MuLV alone	0.14, 0.15, 0.15, 0.21, 0.24, 0.26, 0.36, 0.37	0.11, 0.14, 0.17, 0.20
Wild-type + F-MuLV	0.65, 0.90, 1.44, 1.50, 2.09, 2.14, 2.27	
<i>gs1</i> ⁻ + F-MuLV	0.26, 0.31, 0.42, 1.23, 1.25, 1.50, 1.75, 2.18	
<i>gs2</i> ⁻ + F-MuLV	0.27, 0.30, 0.40, 0.92, 0.98, 1.45, 1.58, 1.65	
<i>gs1</i> ⁻² + F-MuLV	0.14, 0.18, 0.24, 0.24, 0.25	0.11, 1.09
<i>gs0</i> + F-MuLV	0.11, 0.16, 0.18, 0.24, 0.34	0.10, 0.12, 0.13, 0.98

FIG. 2. Characterization of SFFV revertant *env* proteins. Cells were labeled with [35 S]methionine for 1 h and precipitated with hyperimmune α -Rauscher gp70 serum, which recognizes both SFFV and F-MuLV *env* products. Lanes: A, wild-type SFFV-infected 3T3 cells; B, $gs1^{-2-}$ -infected 3T3 cells; C, spleen cells from the diseased animal infected with the $gs1^{-2-}$ mutant; D, $gs0$ -infected 3T3 cells; E, spleen cells from the diseased animal infected with the $gs0$ mutant. The tick mark indicates wild-type gp52, open arrows indicate the $gs1^{-2-}$ mutant protein and its putative revertant, and closed arrows indicate the $gs0$ protein and its putative recombinant derivative.

ucts of recombinant or revertant *envs* that had regained pathogenicity (Fig. 2). The absence of splenomegaly at 2 weeks and the absence of input SFFV *env* mutant proteins in the rare enlarged spleens found 6 weeks postinfection demonstrated that the $gs1^{-2-}$ and $gs0$ mutants were not pathogenic.

Formation of complexes between mutant gp52s and EpoR.

To determine whether the glycosylation site mutant SFFV proteins were able to bind to EpoR in the ER, 3T3 cell lines expressing both SFFV *env* and *epoR* were analyzed for the presence of precipitable complexes. Figure 3 presents RIPA data demonstrating complex formation between EpoR and gp52. Cell lysates were immunoprecipitated with antibodies against either *env* protein or EpoR. The migration positions of the gp52 bands (Fig. 3A, lane A for wild type) and the doublet of 64 and 66 kDa, seen for recombinant EpoR (Fig. 3B, lane F) are indicated. Coprecipitation was seen when both EpoR and wild-type gp52 were present (Fig. 3A and B, lanes A), as previously reported (14). The α -gp70 monoclonal antibody does not precipitate EpoR in the absence of SFFV *env* protein (Fig. 3A, lane F), and the α -EpoR antisera do not precipitate gp52 in the absence of EpoR (Fig. 3B, lane G). α -gp70 antibody precipitated the EpoR doublet, and the α -EpoR antisera precipitated the primary translation product of *env* from lysates of 3T3 cells coexpressing each of the SFFV mutants and EpoR, including the nonpathogenic $gs1^{-2-}$ and $gs0$ mutants. The α -EpoR sera reproducibly

TABLE 3. Induction of Factor-Independent Growth in BaF3/EpoR Cells

SFFV mutant	Fraction of wells showing factor-independent growth at indicated inoculation density (cells/well)			
	10^1	10^2	10^3	10^4
Wild type	2/24	8/24	16/16	16/16
$gs1^{-}$	0/24	3/24	11/16	16/16
$gs2^{-}$	0/24	4/24	12/15	16/16
$gs1^{-2-}$	0/24	0/24	0/16	0/16
$gs0$	0/24	0/24	0/16	0/16

coprecipitated larger amounts of the mutant gp52s than of wild-type gp52 for reasons that are not clear at this time. The identification of the coprecipitated 64- and 66-kDa doublet as EpoR was confirmed for the $gs1^{-}$ - $gs2^{-}$ mutant by reprecipitating the proteins present in the α -gp70 precipitate with α -EpoR antiserum (Fig. 3A, lane D'). Similar analysis of the α -gp70 precipitate from cells expressing the SFFV mutant in the absence of EpoR did not detect the EpoR bands (Fig. 3A, lane G').

In vitro mitogenicity of mutant SFFV *envs*. The mitogenic activity of the mutant SFFV *envs* was tested in the BaF3-EpoR cell line (14). BaF3 cells require IL-3 for growth, and the expression of recombinant *epoR* in these cells (BaF3-EpoR cells) allows Epo to substitute for IL-3. When BaF3-EpoR cells are infected with wild-type SFFV, they acquire the ability to grow in the absence of any growth factors. The SFFV *env* mutants were introduced into BaF3-EpoR cells to determine whether they were also able to induce factor-independent growth. BaF3-EpoR cells growing in IL-3-supplemented medium were infected with pseudotyped SFFV virus, and the cultures were diluted into factor-free medium and inoculated into 96-well plates at various cell densities. Data from a representative experiment are presented in Table 3. Wild-type SFFV induced factor-independent growth efficiently, with the number of infected cells per well needed to produce factor-independent growth varying from fewer than 10 to approximately 100 in different experiments. The basis for the requirement for multiple infected cells per well to generate factor-independent growth in these experiments is unclear. It may simply be a stochastic effect, or it may result from more explicit causes. There may be a cell density effect; alternatively, the expression level of *env* or *epoR* may be important and variable, or a different relevant property of the cells may vary within these cultures.

FIG. 3. Identification of gp52-EpoR complexes by coprecipitation. 3T3 cells coexpressing *epoR* and each of the SFFV mutants and wild type, along with control cells, were labeled for 2 h with a mixture of [35 S]methionine and [35 S]cysteine, and cell lysates were immunoprecipitated. (A) For lanes A to G, the α -gp70 monoclonal antibody 7C10 was used. For lanes D' and G', cell lysates immunoprecipitated with α -BV $_2$ gp70 serum were solubilized and reprecipitated with α -N-terminal EpoR serum. Lanes: A, wild-type SFFV and *epoR*; B, $gs1^{-}$ SFFV and *epoR*; C, $gs2^{-}$ SFFV and *epoR*; D and D', $gs1^{-2-}$ SFFV and *epoR*; E, $gs0$ SFFV and *epoR*; F, ϕ_2 cells expressing pSFF-*epoR* vector; G and G', $gs1^{-2-}$ SFFV. (B) Cell lysates were immunoprecipitated with a mixture of α -N-terminal and α -C-terminal EpoR sera. Lanes are as in panel A.

The pathogenic single site mutants were effective in this mitogenicity assay, requiring only 5- to 50-fold more infected cells per well to generate cell proliferation than did wild-type SFFV. Mitogenicity was not detected for the nonpathogenic *gs1*⁻² and *gs0* mutants in this experiment. These mutants did show very low levels of induction of factor independence in other experiments in which larger numbers of cells were inoculated in each well (33). The *gs1*⁻² mutant required approximately 10³-fold more cells per well than did wild type, and the *gs0* mutant was even less effective. Even these very inefficient effects presumably reflect an impact of the SFFV infection, since uninfected controls showed absolutely no growth. Thus, the efficiency with which various mutants induced factor-independent growth in BaF3-EpoR cells varied over a wide range, and this variation correlated with the pathogenicity of these mutants.

DISCUSSION

Recent studies demonstrating a mitogenic interaction between wild-type SFFV_P and *epoR* in lymphoid cells and the formation of a complex between the primary translation products of *epoR* and SFFV *env* in the ER led to the suggestion that interactions between SFFV *env* products and EpoR are the basis of SFFV pathogenicity (14). It has been suggested that gp52-EpoR complexes located in the ER may be responsible for SFFV disease induction (14, 30, 38). However, genetic data indicate a correlation between SFFV pathogenicity and *env* products that are processed to the cell surface and secreted (1, 13, 16, 28). The coimmunoprecipitation assay employed in the studies cited above used amino acid-labeled protein and would not have detected complexes between EpoR and the small amount of gp65, the processed form of *env* protein, present in infected cells. Complexes between EpoR and gp65 were identified in cross-linking experiments using ¹²⁵I-Epo to probe the cell surface (2). Thus, the nature and location of putative mitogenic complexes between EpoR and SFFV *env* protein remained unresolved. The present study used SFFV *env* mutants, some of which are not processed and are nonpathogenic, to determine the role of *env* protein in the in vitro mitogenicity of SFFV and to explore whether formation of complexes between EpoR and the unprocessed *env* protein gp52 can account for SFFV mitogenicity.

The mutations that were introduced perturbed *env* protein processing by eliminating the Asn attachment sites for specific N-linked glycans. In other systems, including ecotropic MuLV, such mutations have been shown to affect various processing steps in the maturation of membrane proteins (5, 7, 10, 21). Mutants eliminating either the first or second N-linked glycan from SFFV *env* were processed to gp65 at reduced levels, while simultaneous elimination of both of these sites abolished processing completely. Consistent with reports on other SFFV *env* mutants, the mutant *env* proteins that were not processed were unable to induce splenomegaly in mice. The single mutants remained pathogenic despite the decreased extent of *env* processing, suggesting that the amount of processing that occurs for wild-type SFFV_{AP-L} *env* product exceeds the amount required for pathogenicity.

The ability of mutant SFFV *envs* to induce factor-independent growth in BaF3-EpoR cells correlated with induction of splenomegaly, suggesting that these in vivo and in vitro activities share an underlying mechanism. The demonstration that the in vitro mitogenicity of SFFV in BaF3-EpoR cells can be influenced by mutations in *env* establishes that

the mitogenic effect of SFFV in this system is a function of *env* protein. These data support the hypothesis that a mitogenic interaction between SFFV *env* protein and EpoR is responsible for the erythroblast proliferation induced by SFFV in susceptible mice.

The molecular details of the mitogenic interaction between *env* and *epoR* have not been delineated. All SFFV *env* proteins examined here, including the processing-defective nonpathogenic proteins, formed complexes with EpoR in the ER of 3T3 cells. Thus, it is clear that certain mutant gp52 proteins can form complexes with EpoR that fail to transduce a mitogenic signal. The most straightforward interpretation of this result is that the ER is not the site of the mitogenic interaction. The previous data establishing a correlation between processing of gp52 and pathogenicity and its extension to in vitro mitogenicity in this report suggest that an interaction between gp65 and EpoR either within the Golgi apparatus or on the cell surface is essential for mitogenicity. Dimerization appears to be a necessary and perhaps the critical step in activation of the human growth hormone receptor, another member of the receptor superfamily to which EpoR belongs (4, 32), and a recent study shows that a mutation that causes dimerization of EpoR results in its constitutive activation (34). Several studies have shown that gp65 exists predominantly as a disulfide-linked dimer (6, 11, 31, 37). Thus, the activation of EpoR by SFFV *env* may be the result of EpoR dimerization induced by its binding to dimeric gp65.

The possibility remains that complexes between wild-type gp52 and EpoR in the ER are in fact mitogenic, but that the complexes formed with nonpathogenic mutant gp52s are in some way defective. These defective complexes might result from aberrant binding that is not mitogenic, or the glycosylation mutants might be deficient in a step required for mitogenicity that is separable from EpoR binding. This step would have to be tightly linked to the ability of gp52 to be processed to gp65, since analyses of a number of other types of mutation, as well as those reported here, establish the close correlation between processing and pathogenicity. Further work with these and additional glycosylation mutants is under way to determine more precisely the nature and location of the mitogenic interaction between SFFV *env* and *epoR*.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant CA-42129.

We thank Karl Drlica and Ellen Murphy for helpful comments on the manuscript.

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