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Both the Changes of Six Amino Acids and the C-Terminal Truncation Caused by a One-Base Insertion in the Defective *env* Gene of Friend Spleen Focus-Forming Virus Significantly Affect the Pathogenic Activity of the Encoded Leukemogenic Membrane Glycoprotein (gp55)

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Friend spleen focus-forming virus (F-SFFV) causes acute erythroleukemia in mice and encodes in its defective *env* gene an Env-like membrane glycoprotein (gp55). The F-SFFV *env* gene has three characteristic structures compared with that of ecotropic murine leukemia viruses (MuLVs): substitution by the polytropic MuLV *env* sequence, a 585-bp deletion, and a 1-bp insertion. All of these characteristic structures are essential for the leukemogenic potential of gp55 of polycythemia-inducing isolates of F-SFFV (F-SFFVp). The 1-bp insertion causes changes of six amino acids and truncation by 34 amino acids at the C terminus. In this study, we constructed 12 mutant F-SFFV genomes starting from the wild-type F-SFFVp and examined the effect of the C-terminal truncation and the six altered amino acids on the pathogenic activity of gp55. The results indicated that at least 18 to 24 amino acids must be deleted from the C terminus for the *env* product to be pathogenically active. We also found that the six altered amino acids contributed significantly to the pathogenic activity of gp55. Analyses of the cellular processing of these mutant gp55s supported a correlation between the pathogenic activity of gp55 and its efficiency in overall cellular processing.

Friend spleen focus-forming virus (F-SFFV) is a replication-defective murine type C retrovirus which causes acute erythroleukemia of a multistage nature in adult mice of susceptible strains when injected as a virus complex with an ecotropic helper virus (reviewed in references 3 and 18). F-SFFV is unusual among acutely transforming retroviruses in that it does not contain a v-*onc* sequence in its genome, but the modified *env* gene without cell-derived sequences is responsible for at least the early stage of leukemogenesis (reviewed in reference 8). The modified *env* gene of F-SFFV encodes an Env-like membrane glycoprotein called gp55. In the early stage of leukemogenesis caused by polycythemia-inducing isolates of F-SFFV (F-SFFVp), the early committed erythroid precursor cells proliferate and differentiate abnormally, resulting in massive hepatosplenomegaly and severe polycythemia. The gp55 of F-SFFVp can specifically bind to and activate the murine erythropoietin receptor (EPO-R), causing the factor-independent growth of interleukin-3-dependent lymphoid cell lines forced to express the EPO-R (12, 30).

Compared with the *env* gene of ecotropic murine leukemia viruses such as Friend murine leukemia virus (F-MuLV), the *env* sequence of F-SFFVp has several structural differences. The F-SFFVp *env*, from the 5′ end, has a substitution by the polytropic MuLV *env* sequence, a 585-bp deletion, a 6-bp duplication, and a 1-bp insertion (1, 4, 28). We previously showed that all of these structural characteristics were essential for the pathogenic activity of gp55 (2, 23, 24). The 1-bp insertion causes a frameshift of translation that results in changes of six amino acids and truncation by 34 amino acids at the C terminus. The latter implies that gp55 has no cytoplasmic domain. The importance of the 1-bp insertion for pathogenic activity was demonstrated by using a mutant F-SFFV in which a viral DNA fragment of the wild-type F-SFFVp containing the 6-bp duplication and the 1-bp insertion had been replaced by that of the F-MuLV that does not contain these characteristic structures and obtaining revertant F-SFFVs which regained the 1-bp insertion (2).

Here, we further analyzed the relationship between the amino acid sequence of gp55 at its C terminus and its pathogenic potential in vivo. We constructed mutant F-SFFV genomes by site-specific mutagenesis to obtain information about the importance of the six altered amino acids and the degree of truncation at the C terminus. We also examined the pathogenicity of other mutant F-SFFVs, in each of which a viral DNA sequence encoding three consecutive amino acids in or near the transmembrane region of gp55 was deleted. We analyzed the cellular processing of these mutant gp55s to determine whether it is affected by the mutations and is linked to pathogenic activity.

MATERIALS AND METHODS

Construction of mutant F-SFFV genome DNAs. We constructed mutant genome DNAs by oligonucleotide-directed site-specific mutagenesis (10). A viral DNA fragment of the wild-type F-SFFVp excised by *Eco*RI and *Kpn*I, which corresponds to the C-terminal half of gp55 and the U3 portion of the 3′ long terminal repeat, was first subcloned into the M13tg130 phage vector. Using the single-stranded DNA derived from this recombinant phage and one of the

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oligonucleotides described below, we prepared mutant genome DNAs. The oligonucleotides used were: 1, 5′GCAGGGTCCAAAAAGCAGAATTA3′; 2, 5′AATCGATTAGAATGCA3′; 3, 5′CAAATTGTCATAATCGA3′; 4, 5′TCC TGTCTTAAACAAAT3′; 5, 5′CTACTGATTACCTGTCT3′; 6, 5′GAGTCAG TTATAAAGCC3′; 7, 5′ATTCTAGTTATTTTAGC3′; 8, 5′GTTAAACGATC CTCCTTGGCTCGA3′; 9, 5′GGGGGATCTGTTTTCGAACCATCC3′; 10, 5′ GGTAAACCAGGGAAACGATCCTTC3′; 11, 5′TATCAACGTGGTGGATC TGTTAAA3′; 12, 5′GATGGTGGATATAAACCAGGGGGA3′; and 13, 5′AA ACCCCATGATCAACGTGGTAAA3′.

Oligonucleotides 1 to 7 and 8 to 13 were used to construct the E and S mutant genomes and the D mutant genomes, respectively. The mutated DNA fragments were digested with *Eco*RV, and the resultant fragments replaced the corresponding fragment of the wild-type F-SFFVp genome DNA. Finally, the full-size colinear F-SFFV genome DNAs were reconstructed by the procedure described before (24). Plasmid pMSF4 (1) was used as the wild-type F-SFFVp genome DNA. This plasmid contained the biologically active F-SFFV genome of the polycythemia-inducing isolate.

Cell culture and DNA transfection. NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. A recombinant plasmid harboring the mutant F-SFFV genome DNA was cotransfected into NIH 3T3 cells by the calcium phosphate precipitation method (26) with the selectable marker plasmid pSV2neo (20). Transfected cells with growth resistance to G418 (1 mg/ml) were selected.

Immunoblotting. The immunoblotting procedures used to detect Env proteins present in NIH 3T3 cells and spleen cells were described before (2). Goat anti-Rauscher-MuLV gp70 serum (Microbiological Associates) and ^{125}I-protein A (Amersham) were used. To quantify Env proteins, portions of the blotted membrane filter corresponding to each Env protein band were excised, and the bound ^{125}I-protein A radioactivity was counted.

In vivo experiments. F-MuLV clone 57 (14) was used as a helper virus to rescue F-SFFVs. NIH 3T3 cells were infected by the virus in the presence of Polybrene (4 μg/ml). Plasmid pULSF (24) served as a molecular clone for producing the wild-type F-SFFVp. Male DBA/2J mice (6 to 8 weeks of age) (Charles River Japan, Inc.) were intravenously injected with a virus sample (0.2 ml) via the tail vein. At the indicated times, splenic enlargement and hematocrit values were determined.

[^{3}H]glucosamine labeling of cells and immunoprecipitation. NIH 3T3 cells infected with a wild-type or mutant F-SFFV and F-MuLV were metabolically labeled with [^{3}H]glucosamine, and then the culture supernatant and the cleared cell lysate were immunoprecipitated as described before (2). [^{3}H]glucosamine (specific activity, 1.57 TBq/mmol) was purchased from Du Pont-New England Nuclear Corp.

RESULTS

Construction of mutant F-SFFV genomes. We constructed two series of mutant F-SFFV genomes, named E and S. The 1-bp insertion in the wild-type F-SFFVp *env* was first removed to construct an E34 mutant genome. The E34 mutant *env* encodes six C-terminal amino acids different from those encoded by the wild-type F-SFFVp *env* and 34 additional amino acids at the C terminus (Fig. 1). The sequence of these 6 plus 34 amino acids is identical to that encoded by the F-MuLV *env* gene (13). By introducing a nonsense mutation at various sites in the sequence of the E34 mutant *env* encoding the additional 34 amino acids, we obtained five other mutant genomes, E3, E7, E10, E17, and E27. Each of these mutant *env* genes encodes a different number of additional amino acids at the C terminus (Fig. 1). The E34 mutant is similar to but distinct from the previously reported mutant (2), which was constructed by exchanging DNA fragments instead of by site-specific mutagenesis.

For the S series mutants, the first nucleotide (T) of the termination codon (TAA) for gp55 in the wild-type F-SFFVp *env* was removed, resulting in the S34 mutant. In this mutant, the frameshift of translation caused by the 1-bp insertion was corrected by the removal of this nucleotide, resulting in the addition of a sequence of 34 amino acids at the C terminus which is identical to that encoded by the F-MuLV *env* gene (Fig. 1). As in the E series mutants, a nonsense mutation was introduced into different sites in the sequence of the S34 mutant *env* encoding the additional 34 amino acids, resulting in five additional mutant genomes, S3, S7, S10, S17, and S27 (Fig. 1). The peptides of the additional amino acids at the C terminus encoded by the *env* genes of these 12 mutants are probably located in the cytoplasm.

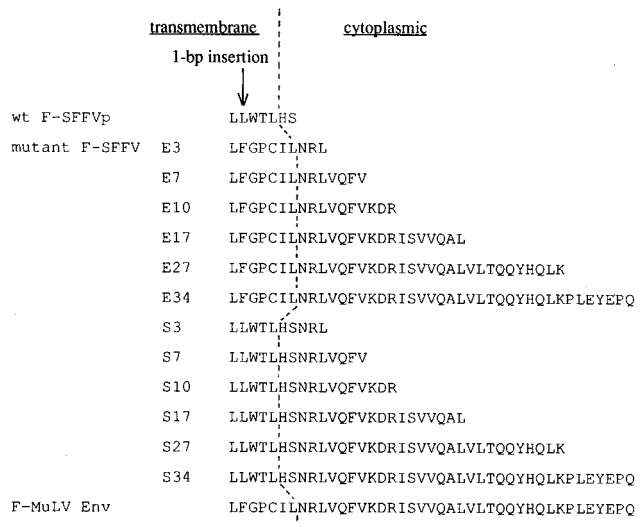

FIG. 1. C-terminal amino acid sequences of the *env* products encoded by the E and S series mutant F-SFFV genomes. For comparison, those of the wild-type (wt) F-SFFVp gp55 (1) and the F-MuLV Env (13) are also shown. Standard one-letter amino acid codes are used. Presumed transmembrane regions and the site of the 1-bp insertion are indicated.

Pathogenicity of mutant F-SFFVs. Each full-size, mutant F-SFFV genome DNA and the wild-type F-SFFVp DNA were transfected into NIH 3T3 cells, and cell clones expressing the F-SFFV *env* product were selected. To determine the pathogenic activity of the mutant F-SFFVs, each mutant virus was rescued from the transfected NIH 3T3 cell clones by coinfection with F-MuLV. Fresh NIH 3T3 cells were then infected with the mutant virus complex as well as the wild-type F-SFFVp-F-MuLV complex, and the amounts of the Env products (i.e., gp55 of F-SFFV and gp70 of F-MuLV) in these cells were estimated by immunoblotting analysis of whole-cell lysates (data not shown). Assuming that the amount of the Env product was proportional to the titer of each virus used for infection, the titers of F-SFFV and F-MuLV were adjusted so that each virus complex contained fixed levels of F-SFFV and F-MuLV titers. After further dilution, the virus complex was intravenously injected into adult DBA/2 mice.

The wild-type F-SFFVp–F-MuLV complex, used as a positive control, caused rapid splenomegaly and severe polycythemia (Table 1). Some of the S series mutants, i.e., S3, S7, and S10, were similar in pathogenic activity to the wild-type F-SFFVp. On the other hand, the mutants S17, S27, and S34 were not pathogenic. Among the E series mutants, E3, E7, and E10 were weakly pathogenic. These mutants induced less profound splenomegaly (i.e., about one-third of that caused by the wild type) and did not cause polycythemia, and the splenomegaly was transient. Profound splenomegaly was occasionally induced in mice injected with the E3, E7, or E10 mutant (data not shown). In these spleens, however, gp55 was of the same molecular mass as that of gp55 encoded by the wild-type F-SFFVp, indicating the presence of a revertant F-SFFV derived from the mutant. The E17, E27, and E34 mutants were not pathogenic. In summary, adding more than 17 amino acids at the C terminus abolished the pathogenic activity of gp55, and the sequence of the C-terminal six amino acids of gp55 was important for pathogenic activity, as revealed by comparing the result, for example, of the S3 mutant with that of the E3 mutant.

TABLE 1. Pathogenicity of the E and S series mutant F-SFFVs in adult mice[a]

F-SFFV	Day postinfection	Spleen wt, g (hematocrit)
Wild type	10	0.48 (49), 0.32 (48)
	18	2.2 (66), 1.9 (65)
Mutants		
E3	9	0.11 (45), 0.13 (52)
	20	0.57 (45), 0.46 (46)
	27	0.60 (41), 0.79 (42)
	36	0.27 (48)
E7	9	0.11 (44), 0.12 (53)
	20	0.46 (45), 0.40 (49)
	27	0.46 (45), 0.57 (43)
	36	0.42 (40)
E10	9	0.09 (45), 0.10 (47)
	20	0.42 (49), 0.34 (44)
	27	0.65 (44), 0.31 (46)
	36	0.17 (47), 0.17 (45)
E17	10	0.10 (46), 0.12 (46)
	17	0.16 (47), 0.12 (50)
	36	0.08 (48), 0.08 (49)
E27	10	0.13 (47), 0.10 (47)
	17	0.13 (47), 0.11 (47)
	36	0.09 (48), 0.08 (49)
E34	9	0.11 (49), 0.10 (48)
	20	0.10 (51), 0.10 (48)
	36	0.08 (48), 0.10 (58)
S3	9	0.20 (53), 0.12 (52)
	20	1.96 (62), 1.46 (72)
	27	2.68 (74), 3.29 (66)
S7	9	0.10 (49), 0.07 (49)
	20	2.02 (64), 1.77 (62)
	27	2.19 (70), 2.64 (72)
	36	2.95 (71), 3.05 (75)
S10	9	0.14 (54), 0.16 (49)
	20	0.66 (56), 0.98 (58)
	27	2.16 (69), 1.70 (69)
	36	2.09 (74), 1.74 (78)
S17	9	0.08 (47), 0.09 (48)
	18	0.20 (46), 0.08 (49)
	35	0.14 (50)
S27	9	0.08 (48), 0.08 (49)
	18	0.10 (47), 0.10 (46)
	35	0.07 (48)
S34	9	0.10 (51), 0.11 (53)
	20	0.09 (48), 0.12 (53)
	36	0.09 (50), 0.10 (49)

[a] Adult DBA/2J mice (male, 6 to 8 weeks of age) were intravenously injected with the F-SFFV–F-MuLV complex (0.2 ml) via the tail vein. At the indicated times, splenic enlargement and hematocrit values were determined. Duplicate samples were taken in most cases, and both values are given.

Analysis of cellular processing of mutant gp55s. A question arose as to which biochemical properties of gp55 were affected by the C-terminal six amino acids and by the additional amino acids at the C terminus. There are three known distinct molecular species of the wild-type F-SFFVp gp55 during biosynthesis and subsequent cellular processing, namely, gp55, gp55^P, and gp55^s. gp55, gp55^P, and gp55^s are located in the rough endoplasmic reticulum membrane, the plasma membrane, and the cell culture supernatant, respectively (15, 17, 19). These molecules can be conveniently identified and roughly quantified by biosynthetic labeling of cells with [^{3}H]glucosamine and subsequent radioimmunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15).

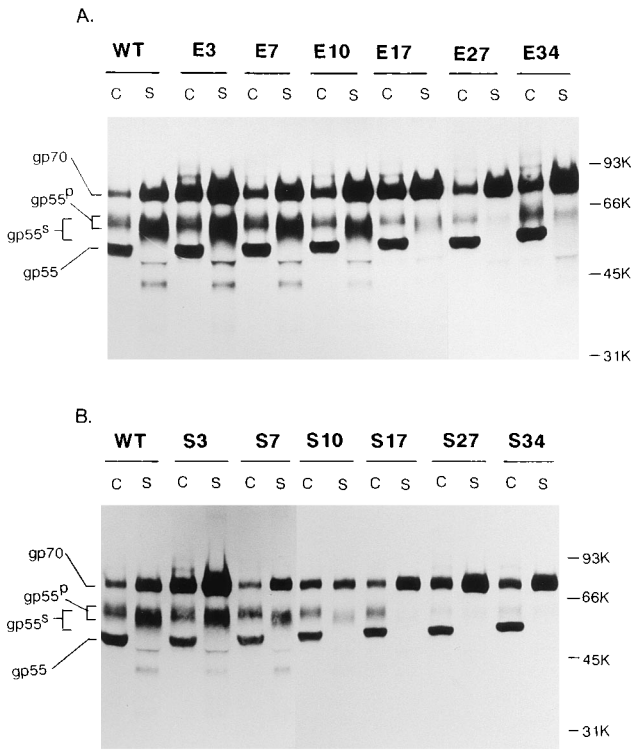

FIG. 2. Cellular processing of gp55s of the wild-type (WT) and the E and S series mutant F-SFFVs. NIH 3T3 cells infected with an F-SFFV–F-MuLV complex were metabolically labeled with [^{3}H]glucosamine. The cells (C) and the concentrated culture supernatant (S) were solubilized with a detergent, and then the cleared lysates were immunoprecipitated with goat anti-Rauscher-MuLV gp70 serum, resolved by SDS-PAGE (8% polyacrylamide), and visualized by fluorography. (A) E series mutants. (B) S series mutants. Molecular mass standards are indicated (in kilodaltons) on the right.

NIH 3T3 cells infected with a virus complex containing one of the mutant F-SFFVs and F-MuLV were metabolically labeled with [^{3}H]glucosamine (final concentration, 3.7 MBq/ml) for 19 h. Labeled cells and the concentrated culture supernatant were solubilized with 1% Nonidet P-40, and then the cleared lysates were immunoprecipitated with the anti-MuLV gp70 antibody, resolved by SDS-PAGE, and visualized by fluorography. The amounts of these lysates used for immunoprecipitation were adjusted so that they corresponded to the same number of cells for each virus complex infection. The results are shown in Fig. 2. The relative amount of gp55^P, in general, appeared to decrease when the number of additional amino acids at the C terminus increased. The relative amount of gp55^s similarly decreased. Similar results were obtained with the mutant gp55s of both the E and S series. The results shown in Fig. 2 suggest that the cellular processing of gp55 to gp55^P and gp55^s is less efficient when gp55 has more additional amino acids at its C terminus. It should be mentioned that the gp55^P equivalents produced by the mutant F-SFFVs are not yet confirmed to be located in the plasma membrane.

TABLE 2. Quantitation of relative amounts of $gp55^{p}$ and $gp55^{s}$ produced by E and S series mutant F-SFFVs[a]

F-SFFV	$gp55^{p}/gp55$	$gp55^{s}/gp55$
WT	1	1
E3	0.56	0.62
E7	0.71	0.94
E10	0.56	0.37
E17	0.27	0.18
E27	0.46	0.12
E34	0.49	0.11
S3	1.56	0.73
S7	1.68	0.73
S10	0.65	0.28
S17	0.52	0.08
S27	0.28	0.11
S34	0.40	0.11

[a] Portions of the dried SDS gels corresponding to the bands of gp55, $gp55^{p}$, and $gp55^{s}$, shown in Fig. 2, were excised, and ^{3}H radioactivity was measured. The ratio of the counts of $gp55^{p}$ to those of gp55 ($gp55^{p}/gp55$) was calculated for each mutant gp55 and normalized to the value obtained for the wild type (WT). Similar calculation and normalization were performed for $gp55^{s}$ ($gp55^{s}/gp55$).

Quantitative analysis of relative amounts of $gp55^{p}$ and $gp55^{s}$. To quantitatively express the results shown in Fig. 2, portions of the dried SDS gel corresponding to each radioactively labeled protein band were excised after fluorography, and the amount of incorporated radioactivity was measured. The ratio of the counts of $gp55^{p}$ to those of gp55 ($gp55^{p}/gp55$) and the ratio of the counts of $gp55^{s}$ to those of gp55 ($gp55^{s}/gp55$) were calculated. These values were then normalized to the values for the wild-type gp55 (Table 2). Table 2 shows that the normalized $gp55^{p}/gp55$ values for both the E and S series mutants are below 1, except for the S3 and S7 mutants, and tend to become smaller as the number of additional amino acids at the C terminus increases, although the decrease is not very consistent, especially in the E series mutants. On the other hand, the normalized $gp55^{s}/gp55$ values for both series of mutants are more closely related to the C-terminal structure of gp55. These values significantly and consistently become smaller as the number of the additional amino acids at the C terminus increases. Since, under the experimental conditions used, $gp55^{s}$ accumulates outside the cells as a dead-end product after cellular processing of gp55, its level should be an indication of the efficiency of overall cellular processing of each mutant gp55.

Studies on mutant gp55s with three consecutive amino acids in or near the transmembrane region deleted. Figure 2 shows that the molecular mass of $gp55^{s}$ is slightly smaller than that of $gp55^{p}$ in cells expressing wild-type or mutant gp55, which is consistent with other reports (6, 16) and suggests that proteolytic cleavage of transmembrane hydrophobic amino acids from the C terminus is responsible for the conversion of $gp55^{p}$ to $gp55^{s}$. To examine the importance of $gp55^{s}$ per se for the pathogenicity of F-SFFVp, we constructed six other mutant F-SFFV genomes, designated D1 to D6. A sequence encoding three consecutive amino acids of the wild-type gp55 was deleted to make these mutant F-SFFV genomes. These deletions were located at the N-terminal side of or in the transmembrane region, as shown in Fig. 3, so that one of the deletions may cause a loss of the proteolytic cleavage site responsible for the conversion of $gp55^{p}$ to $gp55^{s}$.

Cellular processing of the gp55s encoded by these mutant F-SFFVs was analyzed by the same procedure as that used for the E and S series mutant gp55s. The results of the radioimmunoprecipitation are shown in Fig. 4. All of the mutant gp55s were processed to $gp55^{p}$ and $gp55^{s}$ almost as efficiently as the wild-type gp55, indicating that we did not obtain the expected mutant. Either the proteolytic cleavage site for the conversion of $gp55^{p}$ to $gp55^{s}$ is located outside the region that was covered by the mutants, or there are multiple cleavage sites.

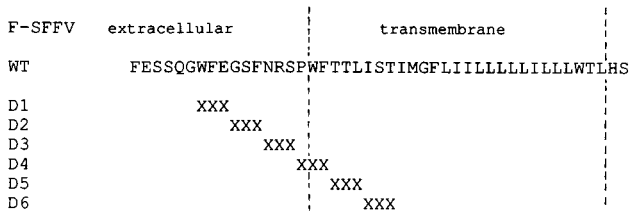

FIG. 3. Sites of deletions in F-SFFV mutants D1 to D6. In each mutant, three consecutive amino acids, indicated by XXX, are deleted from the sequence of the wild-type (WT) F-SFFVp gp55. A presumed transmembrane region is indicated. Standard one-letter amino acid codes are used.

Table 3 shows the pathogenicity of these mutant F-SFFVs in adult DBA/2 mice. The D1, D2, and D4 mutants were similar in pathogenicity (i.e., caused splenomegaly and polycythemia) to the wild-type F-SFFVp, whereas D3 was slightly less and D5 and D6 were weakly pathogenic. Both the D5 and D6 mutants had a three-amino-acid deletion in the transmembrane region of gp55.

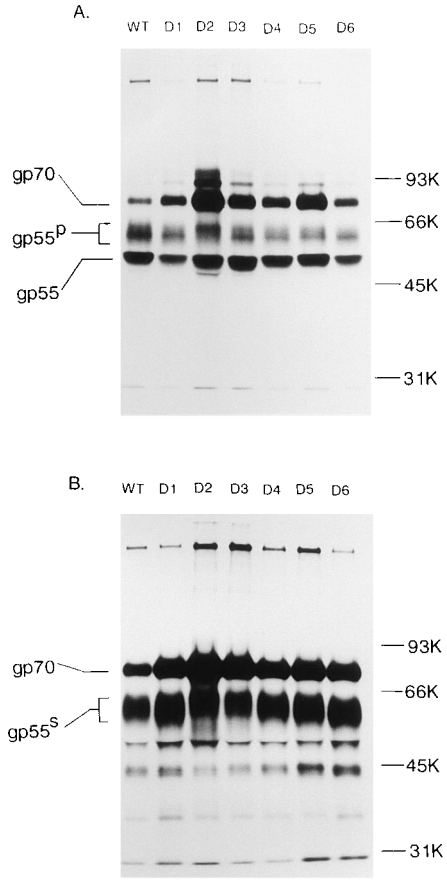

FIG. 4. Cellular processing of gp55s of the wild-type (WT) and the D1 to D6 mutant F-SFFVs. The methods were the same as described in the legend to Fig. 2. (A) Cells. (B) Concentrated culture supernatants. Molecular mass standards are indicated (in kilodaltons) on the right.

TABLE 3. Pathogenicity of D mutant F-SFFVs in adult mice[a]

F-SFFV	Day postinfection	Spleen wt, g (hematocrit)
Wild type	8	0.30 (46), 0.31 (48)
	14	1.86 (60), 1.82 (61)
	21	3.04 (67), 3.17 (62)
D1	8	0.20 (46), 0.17 (46)
	14	2.03 (58), 2.26 (58)
	21	2.86 (63), 3.16 (67)
	28	2.22 (73)
D2	8	0.36 (47), 0.44 (48)
	14	1.79 (62), 1.88 (58)
	21	3.46 (69), 3.33 (64)
	28	2.78 (70), 2.45 (72)
D3	8	0.09 (45), 0.11 (50)
	14	0.73 (50), 0.64 (51)
	21	1.71 (61), 2.17 (65)
	28	1.70 (68), 2.07 (69)
D4	8	0.14 (48), 0.10 (50)
	14	2.01 (54), 1.82 (55)
	21	2.90 (61), 2.31 (59)
	28	2.30 (72)
D5	8	0.06 (46), 0.06 (47)
	14	0.31 (48), 0.43 (48)
	21	0.20 (47), 1.04 (46)
	28	0.80 (51), 0.23 (48)
D6	8	0.17 (49), 0.10 (50)
	14	0.83 (50), 0.72 (51)
	21	0.83 (55), 1.01 (59)
	28	0.47 (55), 0.44 (54)

[a] Adult DBA/2J mice (male, 6 to 8 weeks of age) were intravenously injected with the F-SFFV–F-MuLV complex (0.2 ml) via the tail vein. At the indicated times, splenic enlargement and hematocrit values were determined. Duplicate samples were taken in most cases, and both values are given.

DISCUSSION

We analyzed the pathogenicity of various mutant F-SFFVs, each of which encoded a mutant gp55. We categorized the mutant F-SFFVs into three groups, depending on the domain of gp55 affected by the mutation: those with alterations in (i) the C-terminal cytoplasmic region, (ii) the transmembrane region, and (iii) the extracellular domain (but close to the transmembrane region). We also analyzed the cellular processing of these mutant gp55s. The results are consistent with those reported earlier (11, 22), suggesting that cellular processing of gp55 is linked to its pathogenic activity.

The defective *env* gene of F-SFFV encoding gp55 has a 1-bp insertion near the 3′ end. We previously presented evidence of an essential role for this insertion in the pathogenic activity of gp55 (2). The 1-bp insertion causes both changes of six amino acids and truncation by 34 amino acids at the C terminus compared with the sequence of the normal Env protein. Here, we demonstrated that both of these structural changes can affect the pathogenic activity of gp55. In both the E and S series mutants, the degree of C-terminal truncation correlated with pathogenic activity, and the S series mutants with changes of six amino acids were more pathogenic than the E series mutants without these changes. We examined whether the protein structural changes caused by the 1-bp insertion were related to the cellular processing of gp55. Pathogenically active mutant gp55s having a highly truncated C terminus were more efficiently processed than the pathogenically inactive mutant gp55s having a slightly truncated or an untruncated C terminus, as revealed by the relative amounts of $gp55^s$ (Fig. 2 and Table 2). On the other hand, judging from the relative amounts of $gp55^s$, changes of six amino acids had no relation to the efficiency of the cellular processing of gp55.

We could not find a good correlation between the relative amount of $gp55^p$ and the degree of C-terminal truncation, especially in the case of the E series mutant gp55s. Normalized $gp55^p$/gp55 values were about 0.5 with most of the E series mutant gp55s irrespective of the degree of C-terminal truncation. Previously, we reported (2) the construction of a mutant F-SFFV which was similar to the E34 mutant but was constructed by exchange of DNA fragments instead of by site-specific mutagenesis. Because of the method of construction, this F-SFFV mutant had changes of three amino acids and deletion of two amino acids in the extracellular and transmembrane regions of gp55 in addition to the changes caused by removal of the 1-bp insertion. Nevertheless, the cellular processing of gp55 of this mutant was similar to that of the E34 mutant in that the relative amount of $gp55^s$ but not that of $gp55^p$ was greatly reduced.

Two mutant gp55s (those encoded by the D5 and D6 mutants) had a mutation in the transmembrane region, and they showed weaker pathogenic activity than those encoded by the wild type and the other D mutants (Table 3). The D5 and D6 mutant gp55s were not significantly different in efficiency of cellular processing from the wild-type and the other D mutant gp55s (Fig. 4).

It is not yet clear whether the conversion of $gp55^p$ to $gp55^s$ is indispensable for the pathogenic activity of gp55. We tried to construct a mutant gp55 that does not convert $gp55^p$ to $gp55^s$, but we could not. Further analysis with other mutants is necessary to solve this problem.

Although the available evidence indicates a link between the cellular processing and the pathogenic activity of gp55 and is consistent with the idea that gp55 is pathogenically active only when it is processed at least to the plasma membrane, it could be a mere coincidence, and other possibilities cannot be excluded. For example, if gp55 has an extended C-terminal sequence, it may affect the efficiency of producing a specific homodimer of gp55 (7, 29), which could be essential for activation of the EPO-R (25). It is also possible that upon binding of gp55 to the EPO-R, the extended cytoplasmic sequence of gp55 may cause steric hindrance, which could interfere with the association of the EPO-R and signal-transducing tyrosine kinases, such as JAK2 (27). We did not obtain evidence showing a link between the mutations in the transmembrane region of gp55 (i.e., changes of the C-terminal six amino acids and the three-amino-acid deletion in the D5 and D6 mutants) which affected the pathogenic activity of gp55 and its cellular processing. If a sequence-specific interaction between the transmembrane regions of gp55 and the EPO-R is assumed, an alteration of the sequence of gp55 in that region should affect the ability of gp55 to activate the EPO-R and the pathogenic activity in vivo. It was reported (9, 31) that the specific amino acid sequence of the transmembrane region of EPO-R was necessary for activation by gp55.

gp55 has three major structural differences compared with the Env protein of ecotropic MuLV, namely, substitution by the polytropic MuLV *env* sequence, a 585-bp deletion, and a 1-bp insertion. The polytropic *env* sequence would be important for gp55 binding to the extracellular domain of the EPO-R, and the 585-bp deletion may grossly affect the configuration of the extracellular domain of gp55 to result in the juxtaposition of gp55 relative to the EPO-R for specific inter-

action to occur (31). The 585-bp deletion, however, causes a defect in gp55 cellular processing (21, 23). The 1-bp insertion seemed to be essential to compensate for this defect in cellular processing. The structural alteration caused by the 1-bp insertion favors more efficient cellular processing of gp55.

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REFERENCES

1. **Amanuma, H., A. Katori, M. Obata, N. Sagata, and Y. Ikawa.** 1983. Complete nucleotide sequence of the gene for the specific glycoprotein (gp55) of Friend spleen focus-forming virus. Proc. Natl. Acad. Sci. USA **80:**3913–3917.
2. **Amanuma, H., N. Watanabe, M. Nishi, and Y. Ikawa.** 1989. Requirement of the single base insertion at the 3′ end of the *env*-related gene of Friend spleen focus-forming virus for pathogenic activity and its effect on localization of the glycoprotein product (gp55). J. Virol. **63:**4824–4833.
3. **Ben-David, Y., and A. Bernstein.** 1991. Friend virus-induced erythroleukemia and the multistage nature of cancer. Cell **66:**831–834.
4. **Clark, S. P., and T. W. Mak.** 1983. Complete nucleotide sequence of an infectious clone of Friend spleen focus-forming provirus: gp55 is an envelope fusion glycoprotein. Proc. Natl. Acad. Sci. USA **80:**5037–5041.
5. **D'Andrea, A. D., H. F. Lodish, and G. G. Wong.** 1989. Expression cloning of the murine erythropoietin receptor. Cell **57:**277–285.
6. **Gliniak, B. C., and D. Kabat.** 1989. Leukemogenic membrane glycoprotein encoded by Friend spleen focus-forming virus: transport to cell surfaces and shedding are controlled by disulfide-bonded dimerization and by cleavage of a hydrophobic membrane anchor. J. Virol. **63:**3561–3568.
7. **Gliniak, B. C., S. L. Kozak, R. T. Jones, and D. Kabat.** 1991. Disulfide bonding controls the processing of retroviral envelope glycoproteins. J. Biol. Chem. **266:**22991–22997.
8. **Kabat, D.** 1989. Molecular biology of Friend viral erythroleukemia. Curr. Top. Microbiol. Immunol. **148:**1–42.
9. **Kishi, A., T. Chiba, M. Sugiyama, M. Machide, Y. Nagata, H. Amanuma, H. Taira, T. Katsumata, and K. Todokoro.** 1993. Erythropoietin receptor binds to Friend virus gp55 through other membrane components. Biochem. Biophys. Res. Commun. **192:**1131–1138.
10. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
11. **Li, J.-P., R. K. Bestwick, C. Spiro, and D. Kabat.** 1987. The membrane glycoprotein of Friend spleen focus-forming virus: evidence that the cell surface component is required for pathogenesis and that it binds to a receptor. J. Virol. **61:**2782–2792.
12. **Li, J.-P., A. D. D'Andrea, H. F. Lodish, and D. Baltimore.** 1990. Activation of cell growth by binding of Friend spleen focus-forming virus gp55 glycoprotein to the erythropoietin receptor. Nature (London) **343:**762–764.
13. **Obata, M., H. Amanuma, Y. Harada, N. Sagata, and Y. Ikawa.** 1984. *env*-related leukemogenic genes (gp55 genes) of two closely related polycythemic strains of Friend spleen focus-forming virus possess different recombination points with an endogenous mink cell focus-forming virus *env* gene. Virology **136:**435–438.
14. **Oliff, A. I., G. L. Hager, E. H. Chang, E. M. Scolnick, H. W. Chan, and D. R. Lowy.** 1980. Transfection of molecularly cloned Friend murine leukemia virus DNA yields a highly leukemogenic helper-independent type C virus. J. Virol. **33:**475–486.
15. **Pinter, A., and W. J. Honnen.** 1985. The mature form of the Friend spleen focus-forming virus envelope protein, gp65, is efficiently secreted from cells. Virology **143:**646–650.
16. **Pinter, A., and W. J. Honnen.** 1989. Biochemical characterization of cell-associated and extracellular products of the Friend spleen focus-forming virus *env* gene. Virology **173:**136–143.
17. **Ruscetti, S. K., D. Linemeyer, J. Feild, D. Troxler, and E. M. Scolnick.** 1979. Characterization of a protein found in cells infected with the spleen focus-forming virus that shares immunological cross-reactivity with the gp70 found in mink cell focus-inducing virus particles. J. Virol. **30:**787–798.
18. **Ruscetti, S., and L. Wolff.** 1984. Spleen focus-forming virus: relationship of an altered envelope gene to the development of a rapid erythroleukemia. Curr. Top. Microbiol. Immunol. **112:**21–44.
19. **Ruta, M., S. Clarke, B. Boswell, and D. Kabat.** 1982. Heterogeneous metabolism and subcellular localization of a potentially leukemogenic membrane glycoprotein encoded by Friend erythroleukemia virus. J. Biol. Chem. **257:**126–134.
20. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1:**327–341.
21. **Srinivas, R. V., S. P. Tucker, D. R. Kilpatrick, and R. W. Compans.** 1992. A 585-bp deletion found in the spleen focus-forming virus (SFFV) *env* gene is responsible for the defective intracellular transport of SFFV gp52. Virology **188:**181–192.
22. **Wang, Y., S. C. Kayman, J.-P. Li, and A. Pinter.** 1993. 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. J. Virol. **67:**1322–1327.
23. **Watanabe, N., M. Nishi, Y. Ikawa, and H. Amanuma.** 1990. A deletion in the Friend spleen focus-forming virus *env* gene is necessary for its product (gp55) to be leukemogenic. J. Virol. **64:**2678–2686.
24. **Watanabe, N., M. Nishi, Y. Ikawa, and H. Amanuma.** 1991. Conversion of Friend mink cell focus-forming virus to Friend spleen focus-forming virus by modification of the 3′ half of the *env* gene. J. Virol. **65:**132–137.
25. **Watowich, S. S., A. Yoshimura, G. D. Longmore, D. J. Hilton, Y. Yoshimura, and H. F. Lodish.** 1992. Homodimerization and constitutive activation of the erythropoietin receptor. Proc. Natl. Acad. Sci. USA **89:**2140–2144.
26. **Wigler, M., A. Pellicer, S. Silverstein, and R. Axel.** 1978. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. Cell **14:**725–731.
27. **Witthuhn, B. A., F. W. Quelle, O. Silvennoinen, T. Yi, B. Tang, O. Miura, and J. N. Ihle.** 1993. JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell **74:**227–236.
28. **Wolff, L., E. Scolnick, and S. Ruscetti.** 1983. Envelope gene of the Friend spleen focus-forming virus: deletion and insertion in 3′ gp70/p15E-encoding region have resulted in unique features in the primary structure of its protein product. Proc. Natl. Acad. Sci. USA **80:**4718–4722.
29. **Yang, Y., A. Tojo, N. Watanabe, and H. Amanuma.** 1990. Oligomerization of Friend spleen focus-forming virus (SFFV) *env* glycoproteins. Virology **177:**312–316.
30. **Yoshimura, A., A. D. D'Andrea, and H. F. Lodish.** 1990. Friend spleen focus-forming virus glycoprotein gp55 interacts with the erythropoietin receptor in the endoplasmic reticulum and affects receptor metabolism. Proc. Natl. Acad. Sci. USA **87:**4139–4143.
31. **Zon, L. I., J.-F. Moreau, J.-W. Koo, B. Mathey-Prevot, and A. D. D'Andrea.** 1992. The erythropoietin receptor transmembrane region is necessary for activation by the Friend spleen focus-forming virus gp55 glycoprotein. Mol. Cell. Biol. **12:**2949–2957.