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Role of the Transmembrane Sequence of Spleen Focus-Forming Virus gp55 in Erythroleukemogenesis

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The membrane glycoprotein encoded by the *env* gene of either the polycythemia- or anemia-inducing spleen focus-forming virus (SFFVp or SFFVa, respectively) is responsible for the induction of erythroleukemia in mice. It has been shown that the SFFVp glycoprotein, gp55, interacts with the erythropoietin receptor (EPO-R) and promotes EPO-independent proliferation of an EPO-R-expressing hematopoietic cell line, Ba/F3 (Li *et al., Nature* 343:762, 1990). We show here that when residues within the transmembrane (TM) sequence of an SFFVp gp55 are altered based on the sequences of the anemia-inducing gp55s by a methionine-to-isoleucine (M-I) substitution, a di-leucine deletion (dLL), or both, the resulting mutants display an attenuated phenotype that resembles an SFFVa: they induce milder erythroproliferative disease without polycythemia *in vivo* and are unable to promote EPO-independent cell proliferation *in vitro.* The dLL mutation directly interferes with EPO-R binding by decreasing the affinity of gp55 for the receptor. On the other hand, the M-I mutation hampers the full mitogenic activation of EPO-R while having no effect on receptor binding and asserts a dominant negative effect over the wild-type SFFVp gp55. Two other sequence changes within the TM sequence did not affect the biological activities of the SFFVp gp55. These results indicate that the TM sequence of the SFFV *env* glycoprotein plays a prominent role in SFFV-induced erythroleukemogenesis through its influence on the mitogenic activation of EPO-R.

INTRODUCTION

Several different strains of Friend spleen focus-forming virus (F-SFFV) and one Rauscher SFFV (R-SFFV) have been isolated (Axelrod and Steeves, 1964; Friend, 1957; Lilly and Steeves, 1973; Mirand *et al.,* 1968; Rauscher, 1962). All cause an acute erythroleukemia that is manifested by a prolonged proliferation of erythroprecursor cells in susceptible adult mice. However, disease induced by the anemia-inducing SFFV (SFFVa) strains (including the original F-SFFVa and R-SFFV) is accompanied by a normal to slightly anemic condition, whereas that induced by the polycythemia-inducing SFFV (SFFVp) strains, which are all derived from the F-SFFVa, develops more rapidly with an overproduction of erythrocytes, resulting in a polycythemic condition (Axelrod and Steeves, 1964; Lilly and Steeves, 1973; Mirand *et al.,* 1968). In addition, erythroid precursors infected by the two types of viruses also differ in their requirements for erythropoietin (EPO) during proliferation and differentiation *in vitro*, whereas the SFFVp-infected colony-forming unit-erythroid (CFU-E) or burst-forming unit-erythroid (BFU-E) cells are capable of forming hemoglobinized colonies in the absence of EPO, the formation of similar erythroid colonies from SFFVa-infected cells requires the addition of EPO (Hankins *et al.,* 1978; Hankins and Troxler, 1980).

Previous experiments have shown that the membrane glycoprotein, gp55, encoded by the env gene is highly conserved among different SFFV strains and is responsible for causing the erythroleukemia (Amanuma *et al.,* 1983; Bestwick *et al*., 1984; Clark and Mak, 1983; Linemeyer *et al.,* 1981, 1982). The molecular mechanism for SFFV-induced leukemogenesis was first suggested by the findings that gp55 from one of the SFFVp strains was able to interact with the receptor for EPO (EPO-R) (Li *et al.,* 1990) and that this interaction was capable of converting either an IL-3-dependent hematopoietic cell line, Ba/F3 (Li *et al.,* 1990), or an EPO-dependent erythroleukemia line (Ruscetti *et al.,* 1990) to growth factor independence. Further studies have shown that the mitogenic signal is activated only when both gp55 (Ferro *et al.,* 1993; Wang *et al.,* 1993) and EPO-R (Li *et al.,* 1995) are expressed at the cell surface. In addition, studies have established that cell surface expression of gp55 is critical for its leukemogenicity in vivo (Li *et al.,* 1987; Wang *et al.,* 1993). These results indicate that gp55 activates a mitogenic signaling pathway through its interaction with EPO-R at the cell surface, resulting in uncontrolled erythroid proliferation that ultimately leads to leukemia. However, it is not clear whether gp55 encoded by the SFFVa strains interacts with EPO-R and induces erythroleukemia through a similar mechanism.

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A. Wildtype
                                  390    396/397
                                   ↓      ↓↓
F-SFFVp(1)  -NRSPWFTTLISAIMGSLIILLLLLLILLIWTLYS-COOH
F-SFFVp(2)  -:::::::::::T:::L:::::::::::L:::H:
F-SFFVp(3)  -:::::::::::T:::F:::::::::::L:::H:

F-SFFVa     -:::P::::::V:T:I::::::--:I::::F:::H:
R-SFFV      -:::T::::::FT:I:P:::--::::::F:::H:

B. TM mutations

SFa         -:::T::::::FT:I:P:::--::::::F:::H:
MI          -:::::::::::::I:::::::::::::::::::
dLL         -:::::::::::::::::::--:::::::::::::
MIdLL       -:::::::::::::I:::::--:::::::::::::
IF          -:::::::::::::::::::::::::F:::::
YH          -:::::::::::::::::::::::::::::H:

C. Revertants

dLL-R1      -:::::::::::::::::::::::::::::::::
dLL-R2      -:::::::::::::::::::::::::::::::::
MI-R1       -:::::::::::::::::::::::::::::::::
MI-R2       -:::::::::::::::::::::::::::::::::
MIdLL-R1    -:::::::::::::::::::::::::::::::::
MIdLL-R2    -:::::::::::::::::::::::::::::::::
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FIG. 1. A comparison of the gp55 TM sequences. Sequences of various TM mutants (B) and revertants (C) are aligned with that of the wild-type SFFVs (A) including F-SFFVp(1) (Wolff *et al.*, 1983), F-SFFVp(2) (Clark and Mak, 1983), F-SFFVp(3) (Amanuma *et al.,* 1983), F-SFFVa (Wolff *et al.,* 1985), and R-SFFV (Bestwick *et al.,* 1984). Amino acid residues at positions 390, 396, and 397 are shown in bold. Identical residues (:) and corresponding residues that are absent (-) are shown.

It has been shown that the transmembrane (TM) sequence of gp55 may determine the anemia- or polycythemia-inducing phenotype of SFFVs (Chung *et al.*, 1987, 1989) and that mutations within the TM sequence of an SFFVp gp55 may directly affect its leukemogenicity (Watanabe *et al.,* 1995). To further study the potential role that gp55 TM sequence may play in the SFFV-induced leukemogenesis, we introduced several mutations into the TM sequence of an SFFVp gp55 based on the SFFVa sequences. Our results show that the function of the SFFVp gp55 can be severely affected by mutations in the TM sequence.

RESULTS

Construction of the gp55 TM mutants

A comparison of the TM sequences from different SFFV strains, as noted previously (Bestwick *et al.*, 1984; Wolff *et al.,* 1985), shows that two characteristics consistently present in the SFFVp but not in the SFFVa sequences may be responsible for the conversion of the anemia- to the polycythemia-inducing phenotype (Fig. 1A): an I-to-M substitution at position 390 and a dileucine (dLL) duplication at positions 396 and 397. In addition, a phenylalanine residue (F) at position 404 of the SFFVa sequences is changed to either an I or L in the SFFVp sequences. To study the possible effects of these sequence differences on the pathogenicity of the SFFV, we changed the coding sequence of an SFFVp gp55 [shown as F-SFFVp(1) in Fig. 1A] and made several mutant constructs (Fig. 1B): TM-SFa, with the entire TM sequence derived from anemia-inducing R-SFFV gp55; TM-MI, with an M-to-I substitution at position 390; TM-dLL, with a deletion of the dLL residues at positions 396 and 397; TM-MIdLL, with both the MI and dLL mutations; and TM-IF, with an I-to-F change at position 404. In addition, mutant TM-YH, with a Y-to-H change at position 408, was made. Because the other two SFFVp gp55 sequences contained an H residue at position 408 (Fig. 1A), the Y-to-H mutation was not expected to affect the activity of the SFFVp gp55.

Normally, a small fraction of the intracellular gp55 is further processed into a slightly larger form, gp55^{p}, in the Golgi apparatus and transported to the cell surface (Dresler *et al.,* 1979; Wang *et al.,* 1993) where it binds to EPO-R (Casadevall *et al.,* 1991; Ferro *et al.,* 1993) and exerts its biological activity (Li *et al.,* 1987, 1995; Wang *et al.,* 1993). To make certain that mutations in the TM sequence did not affect the processing of gp55 and its transport to cell surface, we next examined the processing of the TM mutants. Because the expression of the amphotropic MuLV gp70 in either the fibroblast packaging (Bestwick *et al.*, 1988) or the Ba/F3 (Ferro *et al.,* 1993) cells may interfere our analysis, we examined gp55 processing in the human 293T cells transiently expressing the TM mutants. As shown in Fig. 2, gp55^{p} was found from cells expressing TM-YH (lane 3), TM-MI (lane 4), TM-dLL (lane 5), and TM-MIdLL (lane 6), indicating that mutations in the TM sequence did not significantly affect gp55 processing and transport to the cell surface. Although the amount of the processed form for these mutants varied slightly in comparison with that of SFFVp (lane 8), no consistent difference in gp55^{p} was observed for any of these mutants (data not shown). The processed TM-SFa was reproducibly less (lane 7) than that of the SFFVp gp55 (lane 8) but was comparable with the

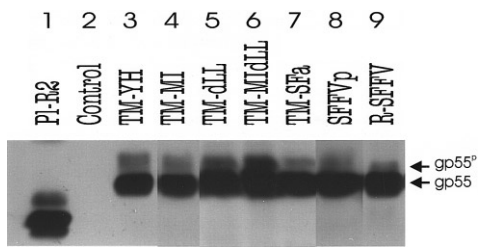

FIG. 2. Analysis of the TM mutant gp55^{p} expression. Proteins extracted from ~10^5 293T cells transfected with a control plasmid (lane 2), or plasmids containing the proviral sequences of TM-YH (lane 3), TM-MI (lane 4), TM-dLL (lane 5), TM-MIdLL (lane 6), TM-SFa (lane 7), SFFVp (lane 8), or R-SFFV (lane 9) were analyzed in Western blotting using anti-gp55 monoclonal and horse radish peroxidase-conjugated rabbit anti-rat antibodies (Sigma) in a standard ECL method. Cells expressing an internal truncated gp55 encoded by a mitogenic SFFVp variant (Fang *et al.,* unpublished data), PL-R2 (lane 1) were also included as a control.

TABLE 1

Pathogenesis of SFFV TM Mutants

Virus	Time	Spleen weight (g/hematocrit)	Average
Uninfected	Week 2	N.D.	
	Week 3	0.16 (42%), 0.15 (46%), 0.13 (48%), 0.14 (48%), 0.19 (48%)	0.15 (46%)
R-MuLV	Week 2	0.10 (45%), 0.12 (46%), 0.14 (50%), 0.14 (50%), 0.12 (52%)	0.12 (48%)
	Week 3	0.21 (41%), 0.17 (42%), 0.26 (40%), 0.18 (43%), 0.19 (46%), 0.18 (48%)	0.20 (43%)
SFFVp	Week 2	1.13 (55%), 0.59 (64%), 1.26 (67%), 0.21 (54%), 0.21 (51%), 0.63 (66%)	0.67 (60%)
	Week 3	4.03 (90%), 2.98 (85%), 1.21 (60%), 3.66 (72%), 2.52 (80%)	2.88 (77%)
R-SFFV	Week 2	0.14 (51%), 0.13 (56%), 0.23 (55%), 0.11 (54%), 0.36 (59%)	0.19 (55%)
	Week 3	0.70 (48%), 0.74 (54%), 0.52 (48%), 0.37 (58%) (two died)	0.58 (52%)
TM-SFa	Week 2	0.50 (55%), 0.54 (55%), 0.58 (58%), 0.47 (56%), 0.23 (54%), 0.19 (53%)	0.42 (55%)
	Week 3	0.30 (46%), 0.39 (51%), 0.60 (41%), 1.10 (50%), 0.22 (47%), 0.32 (46%), 0.17 (41%), 0.71 (41%)	0.48 (45%)
TM-MI	Week 2	0.88 (49%), 0.51 (53%), 0.50 (51%), 0.43 (58%), 0.59 (45%), 0.60 (47%)	0.59 (51%)
	Week 3	0.71 (50%), 0.52 (49%), 0.47 (57%), 0.40 (46%), 0.72 (53%), 1.26 (49%), 0.26 (51%)	0.62 (51%)
TM-dLL	Week 2	0.17 (53%), 0.14 (56%), 0.16 (54%), 0.20 (50%)	0.17 (53%)
	Week 3	0.28 (47%), 0.81 (50%), 0.27 (47%), 0.38 (49%), 0.37 (45%), 0.18 (44%)	0.38 (47%)
TM-MIdLL	Week 2	0.39 (52%), 0.22 (51%), 0.19 (54%), 0.48 (54%), 0.19 (60%), 0.26 (60%)	0.29 (55%)
	Week 3	0.30 (50%), 0.66 (48%), 0.27 (48%), 0.60 (41%), 0.32 (45%), 0.31 (45%), 0.39 (49%)	0.41 (47%)
TM-YH	Week 2	1.98 (68%), 1.37 (45%), 1.80 (67%), 1.52 (69%), 2.35 (63%), 0.5 (62%)	1.59 (62%)
	Week 3	2.03 (80%), 1.60 (55%), 2.59 (82%), 1.76 (85%), 1.74 (78%)	1.94 (76%)
TM-IF	Week 2	1.62 (55%), 1.85 (52%), 0.68 (60%), 1.53 (65%), 1.01 (55%)	1.34 (59%)
	Week 3	4.09 (82%), 0.61 (78%), 1.34 (62%), 3.04 (80%), 2.11 (54%), 1.66 (70%)	2.14 (71%)

Note. N.D. = not determined.

processed SFFVa (R-SFFV) gp55 (lane 9). This result is consistent with the previous observation that the SFFVa gp55 is less efficiently processed (Ruscetti *et al.*, 1981) and suggests that TM sequence characteristics other than I^{390} or the lack of $L^{396}L^{397}$ may influence SFFVa gp55 processing.

Pathogenesis of the TM mutant SFFVs

We next examined the leukemogenicity of the TM mutants. NIH Swiss mice infected with the mutant viruses were killed 2 or 3 weeks after infection, and their spleen weight and hematocrit were measured. As shown in Table 1, TM-SFa, TM-MI, TM-dLL, and TM-MIdLL mutants, like the anemia-inducing R-SFFV, had properties similar to that of an SFFVa: they induced a relatively milder splenomegaly and the infected mice had an apparently normal hematocrit. In contrast, mutants TM-YH and TM-IF had properties similar to that of the SFFVp and induced a more severe splenomegaly as well as polycythemia. These results indicate that both the I-to-M substitution at position 390 and the dLL duplication at positions 396 and 397, but not the F-to-I sequence change at position 404, contribute to the increased leukemogenicity and the ability to induce polycythemia. The Y-to-H mutation at position 408, as expected, had no effect on the biological activities of the SFFVp gp55.

Mitogenesis of the gp55 TM mutants in the Ba/F-ER cells

To examine whether the gp55 TM mutants could promote EPO-independent proliferation, the EPO-R expressing Ba/F3 (called Ba/F-ER) cells infected with each of the *neo*-containing mutant viruses (see Materials and Methods) were first selected by growing the cultures in medium containing G-418 and EPO to avoid possible variations in virus titers. Analysis of the individual clones isolated from the G-418-resistant cell populations after infections showed that 100% of the clones were infected with the viruses and expressed gp55 (data not shown). The abilities of the gp55 TM mutants to promote EPO-independent cell proliferation were then analyzed by growing the infected Ba/F-ER cells at different densities in medium with or without added EPO. As shown in Table 2, TM mutants with an apparent SFFVa(R-SFFV) phenotype including TM-SFa, TM-MI, TM-dLL, and TM-MIdLL formed EPO-independent Ba/F-ER clones at frequencies at least 10- to 100-fold lower than those infected with the SFFVp-like YH and IF viruses indicating that the mitogenic activation of EPO-R by gp55 was also affected by the M-I and/or dLL mutations. Furthermore, the appearance of the rare EPO-independent clones from the SFFVa-like virus-infected Ba/F-ER cells (Table 2) was due to the emergence of revertants rather than a low mitogenic activity of the gp55 mutants, as DNA sequence analysis of the *env* genes cloned from several of the EPO-independent lines derived from the TM-MI, TM-dLL ,or TM-MIdLL virus-infected Ba/F-ER cells showed that all had reverted to the SFFVp wild-type at positions 390, 396, and 397 in the TM sequence (Fig. 1C). Taken together, these results indicate that the SFFVa-like gp55s are incapable of inducing EPO-independent proliferation of the Ba/F-ER cells and suggest that both the I-to-M

TABLE 2

EPO-Independent Growth of Ba/F-ER Cells Infected with the TM Mutants

	Fraction of wells with cell growth in medium					
	Without EPO[a]			With EPO (0.2 u/ml)		
	Inoculation density (cells/well)					
Virus	10^4	10^3	10^2	10^3	10^2	10^1
Mock	0/16	0/16	0/16	16/16	15/16	1/16
SFFVp	16/16	16/16	4/16	16/16	16/16	4/16
R-SFFV	2/16	0/16	0/16	16/16	11/16	0/16
TM-SFa	10/16	2/16	0/16	16/16	15/16	5/16
TM-YH	16/16	16/16	5/16	16/16	16/16	1/16
TM-IF	16/16	14/16	3/16	N.D.	N.D.	N.D.
TM-MI	15/16	2/16	0/16	16/16	16/16	2/16
TM-dLL	5/16	1/16	0/16	16/16	14/16	1/16
TM-MIdLL	1/16	0/16	0/16	16/16	11/16	4/16

Note. N.D., not determined.

[a] Sixteen wells of 96-well plates were seeded with cells at the indicated densities, and positive cell growth was scored 2 weeks later.

substitution at position 390 and the dLL duplication at positions 396 and 397 in TM sequence contributed to the increased mitogenicity of the SFFVp gp55.

Formation of erythroid colonies from the SFFV TM mutant infected progenitors

One prominent feature of the SFFVp-infected erythroid progenitors is that they are capable of forming EPO-independent CFU-E colonies *in vitro*. In contrast, the SFFVa-infected erythroid progenitors still depends on the presence of EPO for the maximal formation of such colonies (Horoszewicz *et al.*, 1975; Liao and Axelrad, 1975; Steinheider *et al.*, 1979). To analyze the EPO requirement of the SFFV TM mutant-infected erythroid progenitors, spleen cell suspensions made from mice infected with various SFFV viruses were placed in methylcellulose medium for 48 h in the presence or absence of EPO and CFU-E colony formation was determined. As shown in Table 3, while erythroid progenitors infected with the SFFVp or TM-YH mutant viruses were capable of forming large numbers of EPO-independent CFU-E colonies, those infected with the SFFVa-like viruses, including TM-MI, TM-dLL, TM-MIdLL, and TM-SFa, formed colonies that remained EPO dependent. These data suggest that the low mitogenicity of gp55 is also responsible for the inability of the SFFVa-like viruses to promote EPO-independent growth of the erythroid progenitors *in vitro*.

Interaction between the gp55 TM mutants and EPO-R

It has been shown that the SFFVp gp55 binds to EPO-R and that such interaction may be critical for the SFFV-induced erythroid proliferation (Casadevall *et al.,* 1991; Ferro *et al.,* 1993; Li *et al.,* 1990). We therefore examined EPO-R binding by the various gp55 TM mutants in several Ba/F-ER cell lines coexpressing EPO-R (Fig. 3A) and gp55 (Fig. 3B), using a combined anti-gp55 immunoprecipitation (IP)/anti-EPO-R Western blot analysis (Li *et al.,* 1995). As shown in Fig. 3C, all of the gp55 mutants without the $L^{396}L^{397}$ duplication in the TM sequence including TM-dLL (lane 5), TM-MIdLL (lane 6), and TM-SFa (lane 7), like the R-SFFV gp55 (lane 8), had a lower affinity for EPO-R than the wild-type SFFVp gp55 (lane 2) or the TM-YH mutant (lane 3). In addition, mitogenic revertants derived from the SFFVa-like mutants isolated from the growth factor-independent Ba/F-ER lines (Table 2) that regained the dLL duplication interacted strongly with EPO-R (data not shown). These data suggest that the dLL duplication found in all three of the SFFVp se-

TABLE 3

CFU-E in Spleen of Mice Infected with the SFFV TM Mutants

	CFU-E per 10^5 spleen cells (mean ± SD)[a]	
Virus	−EPO	+EPO[b]
R-MuLV	7 ± 4	61 ± 24
SFFVp	2514 ± 31	2387 ± 267
R-SFFV	7 ± 12	57 ± 6
TM-YH	1807 ± 210	2040 ± 227
TM-MI	163 ± 106	1925 ± 135
TM-dLL	45 ± 27	960 ± 20
TM-MIdLL	30 ± 10	1026 ± 311
TM-SFa	57 ± 42	607 ± 152

[a] Values were calculated based on the results of triplicate cultures.

[b] EPO was added at 0.5 u/ml.

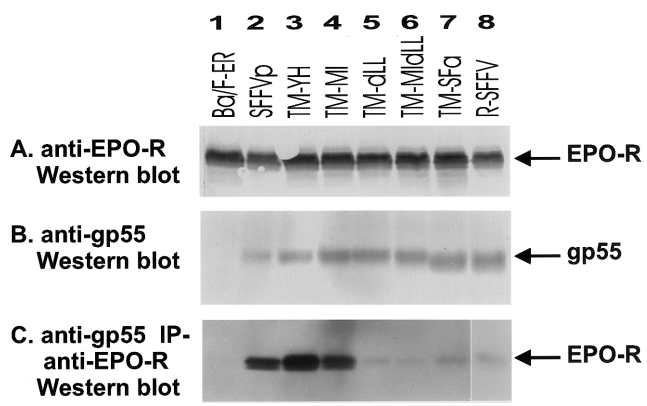

FIG. 3. Analysis of the gp55-EPO-R interaction. Proteins extracted from equal numbers of uninfected (lane 1) or SFFVp (lane 2), TM-YH (lane 3), TM-MI (lane 4), TM-dLL (lane 5), TM-MIdLL (lane 6), TM-SFa (lane 7), or R-SFFV (lane 8) virus-infected Ba/F-ER cells were either analyzed by anti-EPO-R (A) or anti-gp55 (B) Western blotting or they were first precipitated with anti-gp55 monoclonal antibodies, and the coprecipitated EPO-R was then analyzed by anti-EPO-R Western blotting (C).

TABLE 4

Dominant Negative Effect of the TM-MI Mutant

	Factor-independent growth[a] after superinfection with			
	SFFVp		SF/IL-3	
Ba/F-ER line	10^3	10^2	10^3	10^2
Uninfected				
Line 1	9/24	2/24	16/24	13/24
Line 2	15/24	4/24	24/24	24/24
TM-SFa/BE				
Line 1	8/24	3/24	24/24	24/24
TM-MI/BE				
Line 1	0/24	0/24	17/24	7/24
Line 2	0/24	0/24	13/24	5/24
TM-dLL/BE				
Line 1	11/24	2/24	23/24	14/24
Line 2	7/24	0/24	15/24	3/24

[a] Twenty-four wells of a 96-well plate were seeded with the Ba/F-ER lines at the indicated inoculation densities (cells/well), and cell growth was scored 2 weeks later.

quences (Fig. 1A) may increase the affinity of gp55 for EPO-R, resulting in a more potent viral protein that is capable of inducing EPO-independent erythroproliferation *in vitro* and a more rapid erythroproliferative disease *in vivo*.

Interestingly, the M^{390}-to-I^{390} substitution did not affect the apparent affinity of gp55 for EPO-R (Fig. 3, lane 4). One possibility is that the MI mutation may instead interfere with the full mitogenic activation of EPO-R by gp55 at a postbinding step. If so, one prediction would be that such a mutation may have a dominant negative effect over the wild-type SFFVp gp55. To examine this possibility, we superinfected several of the TM mutant-infected Ba/F-ER clones with the wild-type SFFVp and analyzed the growth of these cells. As shown in Table 4, while Ba/F-ER clones infected with any of the other SFFVa-like TM mutants were converted by SFFVp to EPO independence at similar frequencies as the uninfected Ba/F-ER clones, no EPO-independent growth was observed for the two TM-MI expressing clones after SFFVp superinfection. This was not due to a decreased infectibility of the TM-MI virus-infected clones as superinfection with another retrovirus carrying the cDNA for IL-3 efficiently converted these cells to growth-factor independence (Table 4). Thus these results suggest that the TM-MI mutant, through its interaction with EPO-R, exerts a dominant negative effect over the wild-type SFFVp gp55.

DISCUSSION

The two types of SFFVs exhibit both common and distinct characteristics. *In vivo*, both induce a prolonged proliferation of erythroid precursor cells that eventually leads to erythroleukemia (Axelrod and Steeves, 1964; Friend, 1957; Lilly and Steeves, 1973; Mirand *et al.,* 1968; Rauscher, 1962). However, the proliferative disease induced by SFFVp develops rapidly and is accompanied by polycythemia, while that induced by the SFFVa (R-SFFV) develops slower with the infected animals having a hematocrit ranging from normal to slightly anemic (Table 1). In addition, erythroid precursors infected with SFFVa form 5- to 10-fold fewer EPO-independent B-FUE or CFU-E colonies than those infected with SFFVp (Hankins and Troxler, 1980; Horoszewicz *et al.,* 1975; Liao and Axelrad, 1975; Steinheider *et al.,* 1979, and see Table 3). This difference in EPO dependence of the infected erythroid cells was reflected in our TM mutant studies: only mutants with the phenotype of a fully pathogenic SFFVp were able to induce EPO-independent growth of either the Ba/F-ER (Table 2) or the CFU-E cells (Table 3) *in vitro.* These results are consistent with the hypothesis that the mitogenic activity of gp55 is a major determinant for the SFFV-induced erythroleukemia. Furthermore, they suggest that the high mitogenicity of the SFFVp gp55 may also be responsible for the induction of polycythemia, possibly through promoting the proliferation of the more mature erythroblasts *in vivo*.

Previous studies suggest that the mitogenic interaction between the SFFVp gp55 and EPO-R is responsible for the virus-induced erythroid proliferation (Casadevall *et al.,* 1991; Ferro *et al.,* 1993; Li *et al.,* 1995; 1990). Our results from the gp55 TM mutant studies strongly support this hypothesis. First, all gp55 mutants without the $L^{396}L^{397}$ duplication, including TM-dLL, TM-MIdLL, and TM-SFa, had a reduced affinity for EPO-R (Fig. 3). This low affinity correlated with their inability to promote EPO-independent Ba/F-ER cell proliferation *in vitro* (Tables 2) and an attenuation in their leukemogenicity *in vivo* (Table 1). Second, mitogenic revertants derived from some of the TM mutants isolated from the EPO-independent Ba/F-ER lines strongly interacted with EPO-R (data not shown), and sequence analysis of several such revertants showed that they all contained the $L^{396}L^{397}$ duplication (Fig. 1C). Third, the TM-MI mutant, through its strong binding to EPO-R (Fig. 3C, lane 4), exerted a dominant negative effect and abrogated the mitogenic activation of EPO-R by the wild-type SFFVp gp55 (Table 4). Taken together, these results suggest that a full mitogenic activation of EPO-R by gp55 is required for the SFFVp-induced EPO-independent erythroproliferation.

Cell surface expression of the SFFVp gp55 has been demonstrated to be critical for its mitogenic activity *in vitro* (Ferro *et al.,* 1993; Li *et al.,* 1990, 1995; Wang *et al.,* 1993) and leukemogenicity in vivo (Li *et al.,* 1987; Wang *et al.,* 1993). Our finding that the TM-MI, TM-dLL, and TM-MIdLL gp55s were efficiently processed (Fig. 2) suggests that the attenuated phenotype of these mutants results from a reduced ability to bind and/or activate EPO-R, not

from a lack of gp55 cell surface expression. The fact that the TM-MI mutant exerts a dominant negative effect over the wild-type SFFVp gp55 (Table 4) and that a normal level of EPO-Rs as measured by radiolabeled EPO binding (data not shown) or by the ability to sustain growth in EPO medium (Table 2, and data not shown) is found at the surface of the TM-MI mutant-infected Ba/F-ER cells is consistent with a proper processing and cell surface interaction of this mutant with EPO-R.

Previous observations have shown that the same gp55 sequence elements that affect the induction of both EPO-independent erythroid proliferation in vitro (Chung *et al.,* 1989) and erythroleukemia *in vivo* (Watanabe *et al.,* 1995) may overlap with those that determine the anemia- or polycythemia-inducing phenotype of SFFVs (Chung *et al*., 1987, 1989). Our results characterize the relative importance of the differences between the transmembrane sequences of SFFVa (R-SFFV) and SFFVp gp55s. Specifically, we have demonstrated that either the dLL or MI mutation within the TM sequence generates an attenuated phenotype resembles that of R-SFFV. Furthermore, our findings that both the dLL and MI mutations interfere with the mitogenic activation of EPO-R by the SFFVp gp55 (Table 2) either at the binding (Fig. 3) or a postbinding step (Fig. 3 and Table 4), respectively, and the fact that the gp55s of the two SFFVs are otherwise highly homologous (Wolff *et al.,* 1985) suggest that both the M^{390} and $LL^{396/397}$ sequences may have been selected during the evolution from the original F-SFFVa to the mitogenically more active SFFVp viruses. This is further supported by our findings that both of these mutations quickly revert to the SFFVp sequence (Fig. 1C) either in the Ba/F-ER cells (Table 2) or in mice (data not shown). Given the ability ofan SFFVa to induce erythroproliferation *in vivo*, theweaker interaction between the SFFVa gp55 and EPO-R (Fig. 3), although not sufficient to completely abrogate the requirement for EPO, may increase the sensitivity to the hormone *in vivo* (Hankins and Troxler, 1980), ultimately leading to erythroleukemia.

While it is clear that both the M^{390} and $LL^{396/397}$ sequences of gp55 contribute to the abilities of an SFFVp to promote EPO-independent erythroproliferation *in vitro* and to induce polycythemia *in vivo*, it is not known whether the induction of the mild anemia by the SFFVa viruses is solely determined by the lack of these sequence characteristics. This is complicated by the fact that other factors such as the monoclonal/oligoclonal expansion of erythroleukemia cells that crowd out the normal bone marrow erythroblasts during the late stage of the disease (Kabat, 1989) and the blocking of terminal differentiation in the infected erythroblasts by either Spi-1 (Schuetze *et al*., 1993) or Stat5 (Fang *et al.,* 1998) may potentially lead to an anemic condition. Thus it is possible that such an anemic condition is associated with both the SFFVa- and SFFVp-induced diseases during the late stage but is normally obscured by the concomitant induction of polycythemia in the SFFVp-infected mice. Consistent with this hypothesis, it has been shown that when virus replication is limited due to the absence of a helper, which may reduce the number of the proliferating erythroblasts undergoing terminal differentiation, the disease induced by an SFFVp is accompanied by a severe anemia (Spiro *et al.,* 1988).

The fact that the dLL mutation interferes with EPO-R binding by the SFFVp gp55 further suggests that the TM sequences of the two proteins may be in direct contact with each other. Alternatively, the TM mutation may affect the overall structure and/or the proper membrane anchorage of gp55, therefore interferring with EPO-R binding indirectly. Our data cannot affirm these possibilities, and we are conducting experiments to further study the nature of the gp55–EPO-R interaction by using these and other gp55 mutants.

MATERIALS AND METHODS

Cell growth and virus infection

Mouse NIH 3T3 fibroblasts or the human 293T cells were maintained in DME medium plus 7.5% of calf serum or 10% FCS, respectively. The Ba/F-ER cells (Li *et al.,* 1995) were grown in RPMI medium supplemented with 7.5% calf serum and 10% of WEHI-3 supernatant (as a source of IL-3) or 0.2 U/ml EPO. For virus infection, $\sim 5 \times 10^5$ Ba/F-ER cells were mixed with EPO (0.2 U/ml) and 2 ml of various supernatants collected from virus-producing $\psi 2$ fibroblast packaging cells (Mann *et al.,* 1983) immediately before use. In addition, Polybrene (8 μg/ml, Sigma) was added to facilitate infection. After 4 h of incubation at 37°C, 2 ml of fresh medium containing 0.2 U/ml EPO was added, and the cultures were kept for 24 h before they were transferred to G-418 (1 mg/ml)-containing medium, and the virus-infected (drug-resistant) cells were obtained 1 week later. For growth analysis of the virus-infected cells, they were harvested from cultures in logarithmic growth, washed three times with RPMI medium without supplements, resuspended at different densities in aliquots of 0.1 ml RPMI medium containing 10% fetal bovine serum, and placed into 96-well microtiter plates as described previously (Li and Baltimore, 1991; Li *et al.,* 1990). After 2 weeks of incubation, wells with positive growth were scored, and cells were transferred to larger cultures for further analysis.

For analysis of the possible dominant negative effect of the TM mutants, individual Ba/F-ER lines infected with various mutants were first superinfected with SFFVp or SF/IL-3 (see below). After incubation in IL-3-containing medium for 24 h, they were switched to RPMI medium without added growth factors and placed at different densities in 96-well plates. After 2 weeks of incubation at 37°C, the numbers of wells with positive cell growth were scored.

TM mutant plasmids construction and revertant cloning

To facilitate gp55 TM mutant construction, we first engineered an *Xho*I and a *Cla*I site at NT1282 or NT1459 of the *env* sequence, respectively, in plasmid pSFneo (Li *et al.,* 1995) containing the proviral DNA of the Lilly-Steeves SFFVp strain (Lilly and Steeves, 1973) and the G-418 resistance marker. The *Xho*I insertion at NT1282 within the gp55-coding sequence did not affect the function of the SFFVp gp55 (Li *et al.,* 1987). To obtain DNA fragments containing the coding sequences for each of the TM mutant constructs, a 5′ oligonucleotide primer (AGCATGGCTCGAGCCAAATT) based on the env sequence at NT1282 and the *Xho*I site and one of the 3′ primers based on the coding sequences for the mutant constructs (except SFa) and a *Cla*I site were then used to amplify the plasmid containing the SFFVp sequence in PCR. For mutant SFa construction or some of the TM revertant cloning from the growth factor independent Ba/F-ER lines, the same 5′ primer and another 3′ primer based on sequence downstream of *env* and the *Cla*I site were used to amplify plasmid pBC-10, which contained the proviral DNA of R-SFFV (Bestwick *et al*., 1984) or genomic DNA extracted from the Ba/F-ER lines, respectively. All PCR DNA fragments were then ligated with plasmid pBSK$^+$ (Stratagene) for DNA sequence analysis. After sequence verification, the mutant DNA fragments were cloned into pSFneo (Li *et al.,* 1995). Plasmids containing the proviral sequences of the mutants were then transfected into the $\psi 2$ (Mann *et al*., 1983) or $\psi 2$/PA317 1:1 mixed (Wang *et al.,* 1993) fibroblast packaging cells or 293T using the standard calcium precipitation method.

The coding sequence for mouse IL-3 was obtained using primers [GAATTCTC-AATCAGTGGCCGGGATAC-CCA and CTCGAGACATTCCACGGTTCCACG-GTTAG, based on the published sequence (Cambell *et al.,* 1985)] and cDNA templates prepared from the WEHI-3 cells in PCR. The PCR clone was sequence verified and cloned into an SFFV-based retroviral expression vector (Bestwick *et al.*, 1988). The resulting plasmid was transfected into fibroblast packaging cells and virus (called SF/IL-3) produced was used to infect the Ba/F-ER lines.

Analysis of leukemogenicity

Supernatants from the fibroblast packaging cell cultures containing wild-type or TM-mutant SFFVs of similar titers were mixed with Rauscher murine leukemia virus (R-MuLV) helper, and 0.5 ml of the virus preparations was injected into the tail veins of 4- to 6-week-old female NIH Swiss mice. Control mice were either uninfected or infected with R-MuLV alone. Splenomegaly and hematocrit of the infected mice were examined after 14 or 21 days by previously described methods (Li *et al.,* 1986).

CFU-E colony assay

The CFU-E assay was based on protocols described previously (Ahlers *et al.,* 1994; Horoszewicz *et al.*, 1975; Liao and Axelrad, 1975; zharvSteinheider *et al*., 1979). Briefly, splenocytes obtained from mice infected with various SFFVs for 3 weeks were homogenized and dissociated into a single cell suspension. The red blood cells were then lysed by the NH_4Cl buffer (0.83% NH_4Cl, 0.1% $NaHCO_3$, and 10 μM EDTA). Nucleated cell numbers were then determined using a hemocytometer. Cell numbers were adjusted to 5×10^4 or 5×10^5 cells/ml in 0.5 ml of IMDM/10% FBS with or without EPO (0.5 U/ml) and mixed with 1.5 ml of the EPO-minus CFU-E methylcellulose medium (Stem Cell Technologies). Triplicates of cell mixtures were placed in 12-well plates (0.5 ml/well) and incubated for 2 days before the CFU-E colonies were scored.

Immunoprecipitation and Western blot analyses

The gp55^p expression was analyzed in Western blotting using equal amounts of proteins extracted from the human 293T cells 2 days after they were transfected with various SFFV proviral plasmids and the anti-gp55 monoclonal antibody, 7C10 (Li *et al.,* 1995). Western blot analyses for the expression of EPO-R or gp55 in the Ba/F-ER cells were carried out as described previously (Li *et al.,* 1995). Analysis of the interaction between gp55 and EPO-R was carried out using a modified version of a previously described immunoprecipitation–Western blot method (Li *et al.,* 1995). Briefly, $\sim 10^6$ of Ba/F-ER cells expressing various gp55s at a similar level were lysed with 0.5 ml of the culture supernatant containing the rat anti-gp55 monoclonal, 7C10, 1% NP-40, and 0.04% NaN_3, and supernatants collected after a brief microcentrifugation were mixed with 50 μl of 5 M NaCl and 50 μl of anti-rat IgG agarose (Sigma) and kept at 4°C overnight. Proteins in the precipitates were then dissolved in SDS-sample buffer and subjected to anti-EPO-R Western blot analysis, using rabbit anti-EPO-R (Li *et al.,* 1990) and horseradish peroxidase-conjugated Protein A (Sigma) in a standard ECL reaction.

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