Cell Surface Activation of the Erythropoietin Receptor by Friend Spleen Focus-Forming Virus gp55

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The leukemogenic membrane glycoprotein gp55, encoded by Friend spleen focus-forming virus (SFFV), induces erythroid cell proliferation through its interaction with the erythropoietin receptor (EPO-R). There are two forms of gp55 in SFFV-infected cells: an intracellular form (more than 95% of the total protein), which is localized within the endoplasmic reticulum (ER) membranes, and a cell surface form (about 3 to 5%). Because both forms of the viral proteins bind to EPO-R, it is not clear whether the viral protein induces mitogenesis intracellularly or at the cell surface. To address this question, we constructed an EPO-R mutant that contained a 6-amino-acid (DEKKMP) C-terminus ER retention signal. Biochemical and functional analyses with this mutant indicated that it was completely retained in the ER and not expressed at the cell surface. Further analysis showed that the mutant, like the wild-type EPO-R, interacted with SFFV gp55. However, this apparent intracellular interaction between the two proteins failed to induce growth factor-independent proliferation of Ba/F3 cells. Furthermore, spontaneous variants of the ER-retained EPO-R selected on the basis of their ability to induce cell proliferation when coexpressed with gp55 were exclusively expressed at the cell surface. Thus, our results support the hypothesis that the mitogenic activation of the EPO-R by gp55 requires the interaction of the two proteins at the cell surface.

Friend spleen focus-forming virus (SFFV) induces an acute erythroleukemia in susceptible mice (12, 28, 40; see reference 17 for a review). The disease involves a multistage process (3). The early, preleukemia stage, which occurs a few days postinfection, is characterized by a prolonged, polyclonal proliferation of the infected erythroprecursor cells, primarily at the BFU-E or CFU-E stage (15, 16). The uncontrolled erythroid proliferation eventually leads to the late stage of the disease, about 3 weeks postinfection, due to monoclonal or oligoclonal expansion of true leukemia cells with additional cytogenetic changes, such as the activation of the *Spi-1* oncogene (29) and mutations in the *p53* tumor suppressor gene (30).

Genetic analyses have shown that the membrane glycoprotein, gp55, encoded by the SFFV *env* gene, is responsible for initiation of the disease: mutations introduced into the *env* sequence often resulted in viruses unable to induce erythroleukemia in mice (21, 22, 24, 25, 37), and pathogenic viruses derived from some of these mutants always contained reversions in the *env* sequence (21, 22). Recent studies have indicated that initiation of the SFFV-induced erythroleukemia is due to an interaction between gp55 and the erythropoietin receptor (EPO-R) in the infected cells (23). The mitogenic activity of the gp55–EPO-R interaction can be demonstrated in a novel *in vitro* system involving an interleukin-3 (IL-3)-dependent hematopoietic cell line, Ba/F3 (27). Ba/F3 cells can be converted to EPO dependence when the EPO-R cDNA is introduced, and they become growth factor independent when gp55 is coexpressed (23). Furthermore, the mitogenicity of gp55 in erythroid cells can also be demonstrated by its ability to abrogate the EPO dependence of an erythroid cell line (35).

The majority of gp55, which contains four N-linked oligosaccharides, is retained intracellularly in association with the

endoplasmic reticulum (ER) membranes. Only about 3 to 5% is further processed in the Golgi apparatus, with conversion of the N-linked glycans to complex forms and the addition of O-linked glycans (10, 33, 34, 36, 38). The mature protein, called gp55^P (also called gp65 because of its slightly larger size), is then transported to cell surface as oligomeric forms (13, 14, 18). Direct and indirect evidence has indicated that both the intracellular and cell surface forms of gp55 bind to EPO-R (5, 11, 23). However, it is not clear which form is critical for activating EPO-R to trigger the prolonged erythroid proliferation. While the intracellular binding may represent an interesting “short-circuit” mechanism, which would be consistent with the subcellular distribution of gp55, previous studies of SFFV mutants have indicated that cell surface expression of gp55 may be critical for its leukemogenicity (22). Further evidence supporting this idea came from recent studies of gp55 mutants defective in glycosylation and/or processing. Some of these mutants bound to EPO-R intracellularly, but they failed to induce cell proliferation in the Ba/F3 system and leukemia *in vivo* (11, 19a, 41). Further studies to determine the subcellular location for the activation of EPO-R by gp55 may contribute to the understanding of the mechanism of SFFV-induced erythroleukemogenesis.

Proteins that are normally transported to the plasma membranes or secreted may be retained in the ER by the addition of small peptide signal sequences at the C terminus (26, 32). Because the C-terminal sequence of EPO-R is dispensable and large deletions and/or additions can be introduced into this region without affecting its activation by gp55 or EPO (9, 31, 45), it is possible that the addition of a retention signal to the C terminus retains the EPO-R protein in the ER without substantially altering its structure. The analysis of such a retention mutant may provide some useful information as to whether the activation of EPO-R by gp55 occurs intracellularly or at the cell surface. A mutant was made by adding a 6-amino acid (aa) DEKKMP ER retention signal sequence of the adenovirus E3/19K protein (32) to the C terminus of EPO-R.

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Characterization of the mutant and several spontaneous revertants derived from it revealed a strict correlation between the cell surface expression of EPO-R and its mitogenic activation by SFFV gp55.

MATERIALS AND METHODS

Cell growth and virus infection. Mouse NIH 3T3 fibroblast cells were maintained in Dulbecco modified Eagle medium (DMEM) plus 7.5% calf serum. The IL-3-dependent pro-B-lymphoid Ba/F3 cells were grown in RPMI medium supplemented with 7.5% calf serum and 10% WEHI-3 supernatant (as a source of IL-3). For growth analysis of the various Ba/F3 lines, cells were harvested from IL-3-containing cultures and washed three times with RPMI medium without supplements, and aliquots of about 2×10^4 cells were resuspended in 0.1 ml of RPMI medium containing 10% fetal bovine serum, with or without EPO or IL-3, and placed into 96-well microtiter plates. After 48 h of incubation at 37°C, cells were stained with equal volume of 0.4% trypan blue, and viable cells were counted with a hemocytometer.

For virus infection, about 5×10^5 cells were mixed with 1 ml of various supernatants collected from virus-producing fibroblast packaging cells immediately before use. Polybrene (8 µg/ml; Sigma) was added to facilitate the infection. After 3 to 4 h of incubation at 37°C, 2 ml of fresh IL-3-containing medium was added, and the cultures were kept for 48 h before they were used for growth factor independence analysis or for cloning by limiting dilutions. All infections were done with virus produced from fibroblast packaging cells transfected with plasmid pL2-6k, which contains the proviral DNA of the Lilly-Steeves polycythemia SFFV strain (22). For selection of growth factor independence, cells were washed three times with RPMI medium before they were transferred to 0.1 ml of medium containing 10% fetal bovine serum without any added growth factors, at densities of 10, 100, or 1,000 cells per well in 96-well microtiter plates. After 2 weeks of incubation at 37°C, the numbers of wells with positive cell growth were scored and cells were transferred to larger cultures for further analysis. For isolation of the SFFV-infected 4-10 cell lines, at 48 h after infection, cells were placed in 96-well plates under limiting-dilution conditions in IL-3-containing medium. Two weeks later, individual clones were grown to obtain enough cells for Western immunoblot analysis using anti-gp55 monoclonal antibody 7C10 (43). Of about 100 clones screened, 3 expressed gp55, and they were used for the analyses of growth factor independence and the interaction between gp55 and EPO-Rcd in the combined immunoprecipitation-Western blot assay.

Plasmid construction and DNA sequence analysis. The *neo* gene was inserted into the *gag* sequence between nucleotides 1905 and 3325 (6) of plasmid pL2-6k (22) to construct a new plasmid, pSFneo. Separately, a 46-base (CGATCGATC ACGGCATTTCTTCTCATCGGAGCAGGCCACTATGCC) oligonucleotide primer complementary to the coding sequences for the C terminus of EPO-R and including the DEKKMP ER retention signal, a stop codon, and a *Cla*I cloning site and a 23-base (AGCTCCTTCCCTGAGGATCCACC) primer (called ER-Bam) containing nucleotides 983 to 1006 of the EPO-R cDNA sequence (8) with an internal *Bam*HI site were used in PCR with EPO-R cDNA as the template. The 650-bp PCR fragment was cut with *Bam*HI and *Cla*I and used to replace the corresponding sequence in the retroviral vector plasmid that contained the wild-type EPO-R cDNA (23) and the *neo* gene to create the plasmid construct (called pSFnERcd) for EPO-Rcd. After its sequence was verified, pSFnERcd was transfected into a mixture of Ψ -cre and Ψ -crip packaging cells with ecotropic and amphotropic host ranges, respectively, and virus containing the EPO-Rcd cDNA was obtained from the culture supernatant after 2 weeks of cocultivation as described previously (4, 23).

For sequence analysis of the various revertants of EPO-Rcd, genomic DNA was extracted from about 5×10^5 cells of the growth factor-independent Ba/F3 lines that expressed revertants of EPO-Rcd. About 50 ng of the DNA was then used as a template in PCR with the ER-Bam primer and a 24-base (GGCATC CCAGCTGGCCTTTCTACC) primer complementary to the sequence immediately downstream of the *Eco*RI site in the pSFneo sequence. In all cases, a single fragment of about 650 bp was obtained. Such fragments were cut with *Bam*HI and *Eco*RI (in the retroviral vector sequence) and cloned into the pBSK⁺ vector (Stratagene) for direct DNA sequence analysis using Sequenase (United States Biochemical) and the T3 or T7 promoter primer according to the manufacturer's recommendation.

¹²⁵I-EPO binding assay. Duplicates of about 2.5×10^6 cells were washed two times in DMEM containing 1% bovine serum albumin (BSA; Fisher Biotech), resuspended in 0.1 ml of the same medium, and incubated with 600 pM ¹²⁵I-EPO (Amersham) at room temperature for 1 h with vigorous shaking. At the end of incubation, cells were washed three times with DMEM-BSA and lysed with 1 ml of 1 N NaOH. The radioactivity of the cell lysates was then counted with a Beckman gamma counter as described previously (8).

Other methods. For Western blot analysis, about 5×10^5 cells were lysed with 30 µl of lysis buffer (10 mM Tris [pH 7.4], 0.5% Nonidet P-40, 0.02% Na₃N₃), and the supernatants were collected after a brief centrifugation and mixed with 15 µl of sodium dodecyl sulfate (SDS) sample buffer (180 mM Tris [pH 6.8], 3% SDS, 30% glycerol, 3% 2-mercaptoethanol, 0.3% bromophenol blue). The protein samples were boiled before loading onto SDS-polyacrylamide gels. After electrophoresis, the gel-resolved proteins were transferred onto nitrocellulose mem-

branes and incubated with various antibodies at a dilution of 1:1,000 (for rabbit or rat anti-EPO-R serum) or 1:5 (for supernatant containing the anti-gp55 monoclonal antibody 7C10). The corresponding alkaline phosphatase-conjugated secondary antibodies and the substrates for the colorimetric reaction (Promega Biotech) were used according to the manufacturer's recommendation.

For endoglycosidase H (endo H) digestion of EPO-R, about 5×10^6 of the EPO-R-expressing Ba/F (Ba/F-ER) cells or 4-10 cells expressing the wild-type EPO-R or EPO-Rcd, respectively, were lysed with 1 ml of immunoprecipitation buffer (20 mM Tris [pH 7.4], 0.5% Nonidet P-40, 0.5 M NaCl, 1 mM EDTA) and microcentrifuged, and the supernatants were incubated with 10 µl of preimmune serum on ice for 30 min. About 100 µl of 10% fixed *Staphylococcus aureus* cell suspension (Boehringer Mannheim) was then added, and after a brief microcentrifugation, the precleared supernatants were collected and incubated with 5 µl of anti-EPO-R (23) and 50 µl of protein A-agarose (Bethesda Research Laboratories) at 4°C for 4 h. The pellets were then microcentrifuged, washed several times with immunoprecipitation buffer and once with 10 mM Tris buffer (pH 7.4), and heated in endo H digestion buffer (10 mM Tris [pH 7.4], 1% SDS) at 95°C for 10 min. Half of each sample was digested with endo H (0.1 U/ml; Boehringer Mannheim) at 37°C for 2 h. The digested and undigested samples were then mixed with SDS sample buffer, heated, and loaded onto an SDS-12.5% polyacrylamide gel. After electrophoresis, the EPO-R proteins were analyzed by Western blotting using a rat serum against an EPO-R fusion protein that contained the maltose-binding domain.

For analysis of the gp55-EPO-Rcd interaction, proteins extracted from about 5×10^6 cells were first precipitated with a goat anti-Rauscher murine leukemia virus gp70 antiserum (National Cancer Institute) which cross-reacted with SFFV gp55 (23). The precipitated proteins were then dissolved in SDS sample buffer and subjected to anti-EPO-R Western blot analysis. The double-immunoprecipitation assay to detect the gp55-EPO-Rcd interaction was done by a method described previously (23), using several fibroblast lines that coexpressed gp55 and EPO-Rcd.

RESULTS

Construction of an ER-retained mutant of EPO-R. By site-directed mutagenesis using synthetic oligonucleotides and PCR, the DEKKMP ER retention sequence of the adenoviral E3/19K protein (32) was added to the C terminus of EPO-R. After the sequence of the resulting EPO-R mutant (called EPO-Rcd) was verified, it was cloned into a *neo* gene-containing retroviral vector, pSFneo (see Materials and Methods), and the construct was transfected into the packaging cells as described previously (4, 23). Virus produced was then used to infect Ba/F3 cells, and infected cells expressing EPO-Rcd (called Ba/F-ERcd cells) were obtained through G418 selection. Individual clones of Ba/F-ERcd cells were then obtained through limiting dilutions. Western blot analysis using an antiserum against the N-terminal peptide of EPO-R (23) showed that all of the 10 individual clones analyzed expressed EPO-Rcd (data not shown). Line 4-10, which had the highest level of EPO-Rcd expression, and six other clones were further characterized.

First, we analyzed the intracellular processing and the possible ER retention of the EPO-Rcd protein. The wild-type EPO-R contains an N-linked glycosylation site (8). In the Ba/F-ER cells, there are three forms of the EPO-R protein: a 62-kDa nonglycosylated form, a 64-kDa glycosylated and endo H-sensitive form, and a 66-kDa endo H-resistant form. The 64-kDa form is presumably localized in the ER, while the 66-kDa form is processed in the Golgi apparatus and transported to the cell surface (44). The 64- and 66-kDa forms are the predominant forms of EPO-R in Ba/F3 cells, and they closely comigrate during SDS-polyacrylamide gel electrophoresis (44) (Fig. 1A, lane 2). After endo H digestion, the 64-kDa but not the 66-kDa form is converted to the 62-kDa form (Fig. 1A, lane 1). When EPO-Rcd was analyzed, it was found that the protein was mostly in the endo H-sensitive, 64-kDa form (Fig. 1A, lane 4), which was completely converted to the 62-kDa form by endo H (Fig. 1A, lane 3), indicating that the receptor protein was successfully retained in the ER.

Next, we used ¹²⁵I-EPO to analyze cell surface expression of EPO-Rcd. While labeled EPO bound to the Ba/F-ER cells

FIG. 1. EPO-Rcd processing and its association with gp55. (A) Endo H digestion of EPO-R and EPO-Rcd. Wild-type EPO-R (lanes 1 and 2) and EPO-Rcd (lanes 3 and 4) extracted from Ba/F-ER cells or 4-10 cells, respectively, were first immunoprecipitated with a rabbit anti-EPO-R serum (23) and then either digested with endo H (lanes 1 and 3) or not treated (lanes 2 and 4) before being subjected to Western blot analysis using a rat antiserum against EPO-R. (B) Association of EPO-Rcd with gp55. Proteins extracted from about 5×10^6 uninfected cells (lanes 1) or from SFFV-infected 4-10 cells (lanes 2) were first immunoprecipitated with anti-gp55 and then subjected to anti-EPO-R Western blot analysis. In lanes 3 and lane 4, proteins extracted from about 5×10^5 uninfected and SFFV-infected 4-10 cells, respectively, were loaded directly to the gel for the same Western blot analysis.

expressing the wild-type EPO-R efficiently, it did not bind to the 4-10 cells beyond the background level (Table 1, experiment A), indicating that the EPO-Rcd protein was not expressed at the cell surface. Similar results were obtained for all of the other six Ba/F-ERcd clones examined (data not shown).

To further examine the cell surface expression of EPO-Rcd, the growth of the Ba/F-ERcd cells in EPO-containing medium was analyzed. As shown in Fig. 2, while the Ba/F-ER cells could be grown in culture containing as little as 0.1 U of EPO per ml, the 4-10 cells could not be grown in EPO-containing medium, even at an EPO concentration of 1 U/ml. Similar results were obtained for six other Ba/F-ERcd lines (data not shown). These results confirmed that the EPO-Rcd protein was not expressed at the cell surface.

SFFV gp55 binds to EPO-Rcd intracellularly. To examine whether SFFV gp55 could bind to the ER-retained EPO-Rcd protein, we used a combined immunoprecipitation-Western blot assay as follows. Proteins extracted from the uninfected cell line or one of the SFFV-infected 4-10 cell lines that expressed gp55 (see Materials and Methods) were first immunoprecipitated with an antiserum against Rauscher murine leukemia virus gp70, which cross-reacts with SFFV gp55 (23). The proteins were then dissociated from the precipitates by heating

FIG. 2. Growth of Ba/F3 cells expressing EPO-Rcd. Triplicates of 2×10^4 cells were grown in 0.1 ml of RPMI medium with 10% WEHI-3 supernatant (as a source of IL-3) and various amounts of EPO as indicated, or without any added growth factors, for 48 h. Data shown represent averages of cell counts from the triplicates and their standard deviations.

in the presence of 1% SDS, loaded onto an SDS-polyacrylamide gel, and subjected to anti-EPO-R Western blot analysis. As shown in Fig. 1B, lane 2, EPO-Rcd protein was coprecipitated by anti-gp55, indicating that it interacted with gp55. The EPO-Rcd protein from uninfected cells was not detected by the same procedure (Fig. 1B, lane 1), indicating that the observed gp55-EPO-Rcd interaction in the infected cells was specific. A similar result was obtained in the double-immunoprecipitation assay described previously (23) for detection of the gp55-wild-type EPO-R interaction (data not shown). Thus, our results showed that the intracellularly retained EPO-Rcd, like the wild-type EPO-R, interacted specifically with gp55.

Mitogenicity of the intracellular interaction between gp55 and EPO-Rcd. To examine whether the intracellular interaction between gp55 and EPO-Rcd could generate a mitogenic signal for the Ba/F3 cells, we infected about 5×10^5 of the 4-10 cells with SFFV and grew them in RPMI medium without added growth factors for 2 weeks. While normally SFFV infection could convert about 1 to 3% of the Ba/F-ER cells to growth factor independence (the low infectibility of the Ba/F3 cells, as reported previously [20, 27], was probably due to interference by the endogenous *env*-related glycoprotein [19]), the SFFV-infected 4-10 cells failed to grow in the absence of added growth factors (Table 2). Similar results were obtained after SFFV infection of six other clones of Ba/F-ERcd cells. The inability of SFFV to induce factor-independent growth was not due to a lowered infectibility of these cells because

TABLE 1. Analysis of cell surface expression of EPO-R

Cell line	¹²⁵ I-EPO binding ^a (avg $\pm$ SD [$n = 2$])
Expt A	
Ba/F3	431 $\pm$ 2.1
Ba/F-ER	3,302 $\pm$ 49
4-10	456 $\pm$ 104
Expt B	
SF/Ba/F-ER ^b	2,404 $\pm$ 8.5
Revertants ^c	
R1	1,863 $\pm$ 62
R2	4,405 $\pm$ 46
R3	4,109 $\pm$ 258
R4	5,434 $\pm$ 124
R5	1,746 $\pm$ 87

^a Binding of ¹²⁵I-EPO (600 pM) to duplicates of 2.5×10^6 cells.

^b An SFFV-infected Ba/F-ER line.

^c Five SFFV-infected, growth factor-independent Ba/F-ERcd lines expressing revertants of EPO-Rcd.

TABLE 2. Growth factor independence of cells after SFFV infection

SFFV-infected cells	No. of wells with factor-independent cell growth ^a (from 32 wells examined)		
	10 ^{3b}	10 ²	10 ¹
Ba/F-ER	32	27	4
4-10	0	0	0
Ba/F3	0	0	0

^a Aliquots of 10, 100, and 1,000 cells were placed into 32 wells of a 96-well plate in 0.1 ml of RPMI medium containing 10% fetal bovine serum, 48 h after SFFV infection. After incubation for 2 weeks at 37°C, cell growth in individual wells was examined.

^b Initial cell number per well.

FIG. 3. Western blot analyses of the revertants of EPO-Rcd. Proteins extracted from five growth factor-independent Ba/F3 clones expressing revertants of EPO-Rcd (R1 to R5) and from 4-10, Ba/F3, or Ba/F-ER cells were analyzed by Western blotting using an antiserum against the N-terminal peptide of EPO-R (23) (A), an antiserum against the C-terminal peptide of EPO-R (44) (B), and the monoclonal anti-gp55 antibody 7C10 (43) (C).

Western blot analysis of the individual clones, grown in IL-3-containing medium after SFFV infection, revealed that about 3% of the 4-10 cells were infected with SFFV (see Materials and Methods). Although gp55 interacted strongly with EPO-Rcd (Fig. 1B) in the infected cells, the cells remained strictly dependent on IL-3 (data not shown). Therefore, the intracellular interaction between gp55 and EPO-Rcd was not sufficient to provide a mitogenic signal for the Ba/F3 cells.

Cell surface expression of the mitogenic revertants of EPO-Rcd. Although we were unable to isolate growth factor-independent cells from the individual clones of the Ba/F-ERcd cells after they were infected with SFFV, we could occasionally obtain factor-independent lines by infecting uncloned Ba/F-ERcd cells with SFFV. However, the frequency for obtaining such lines after SFFV infection was less than 1 in every 10^4 cells and at least 100-fold lower than that for the Ba/F-ER cells (data not shown). We obtained about 20 such lines from three separate infections.

When cell surface expression of EPO-R in these cells was analyzed by 125 I-EPO binding, it was found without exception that all 20 clones expressed high levels of EPO-R at the cell surface (Table 1, experiment B, revertants R1 through R5, and data not shown), indicating that the EPO-R proteins expressed in these cells may be functional revertants derived from EPO-Rcd. This conclusion was supported by the results from Western blot analyses. First, when an antiserum against the N-terminal peptide sequence of EPO-R (23) was used, it was found that EPO-Rs expressed in these cells were either smaller (Fig. 3A, R2, R3, and R4) or slightly larger (Fig. 3A; compare results for R1 and R5 with that for 4-10) than EPO-Rcd. Second, an antiserum against the C-terminal sequence of EPO-R (44) and reactive against EPO-Rcd could not recognize the EPO-Rs from any of these cells (Fig. 3B and data not shown), indicating that the EPO-Rs expressed in these cells contained different C-terminal sequences.

To further examine the structures of the receptor proteins, we cloned the C-terminal region of the EPO-R genes from R1, R2, R3, and R5 cells by PCR. DNA sequence analysis showed that all of the EPO-R genes analyzed still contained the 18-bp DEKKMP-coding sequence at the C terminus. However, they all had a single-base-pair insertion or deletion upstream at different positions in the C-terminal region, resulting in reading frame shifts and/or premature termination of the encoded EPO-Rs and the elimination of the ER retention signal. A summary of these results is shown in Table 3. The sizes of the encoded proteins and the fact that they all had C termini different from that of the wild-type EPO-R correlated well with the results from Western blot analysis (Fig. 3A and B). For

example, the EPO-R genes from both R1 and R5 contained an identical 1-bp deletion at nucleotide 1438. This caused a shift in the reading frame which extended into the 3' noncoding region of the cDNA and part of the retroviral vector sequences, resulting in a 521-aa receptor protein with a deletion of 37 aa from the C terminus of the wild-type sequence and an addition of 75 aa from the new reading frame.

gp55 was expressed in all of the 20 growth factor-independent Ba/F-ERcd clones isolated after SFFV infection (Fig. 3C and data not shown). Considering the low infectibility of the Ba/F-ERcd cells, the universal expression of gp55 and the previous observation that nearly the entire cytoplasmic domain of EPO-R could be deleted without affecting the receptor binding by gp55 (9) indicated that the interaction between gp55 and the EPO-R revertants was responsible for inducing the growth factor independence of these cells. The exclusive cell surface expression of the revertant EPO-Rs (Table 1 and data not shown) further suggested that the activation of EPO-R by gp55 occurs at the cell surface.

DISCUSSION

Previous experiments showed that SFFV gp55 bound to EPO-R both in fibroblast cells that expressed the two proteins and in erythroleukemia cells transformed by the Friend virus (23). The biological activity of this interaction was demonstrated through a novel in vitro mitogenicity assay using an IL-3-dependent pro-B-cell line, Ba/F3, such that the absolute dependence of this line on IL-3 could be abrogated when both gp55 and EPO-R were expressed (23). The mitogenic activity of gp55 in erythroid cells was also demonstrated by its ability to

TABLE 3. Sequence analysis of the mitogenic revertants of EPO-Rcd

EPO-R protein	DNA sequence change	Length of encoded polypeptide (aa)	No. of aa changed	
			Deletion	Addition
Wild type		483		
EPO-Rcd	18-bp addition ^a	489	0	6
Revertant				
R1	G-1438 deletion ^b	521	37	75 ^c
R2	G-1041 deletion	315	169	1
R3	C-1244 insertion	383	101	1
R5	G-1438 deletion	521	37	75

^a Coding sequence for DEKKMP at C terminus.

^b Position of nucleotide according to D'Andrea et al. (7).

^c Reading frame for both R1 and R5 extended into the pSFF vector (4).

abrogate EPO dependence of an erythroleukemia cell line (35). Subsequent experiments have confirmed both the physical interaction between the two proteins (5, 9, 11, 39, 41, 44, 47) and the relevance of the Ba/F3 cell proliferation assay to the leukemogenicity of SFFV (11, 19, 41).

One major question concerning the mechanism of SFFV-induced erythroleukemogenesis is whether the mitogenic interaction between gp55 and EPO-R occurs at the cell surface or intracellularly. Because initial experiments detected a strong interaction between EPO-R and the intracellular form of gp55 (23) and because it was shown that the majority of the viral glycoprotein was localized within the ER membranes (10, 36, 38), it was suggested that gp55 might activate EPO-R intracellularly (23, 44). However, analyses of various SFFV mutants showed that cell surface expression of gp55 might be critical for the initiation of mitogenesis or leukemogenesis (11, 22, 41), and recent experiments using an indirect method have also demonstrated that gp55^P indeed binds to EPO-R at the cell surface (5, 11). Nevertheless, because of the low quantity of gp55^P and the lack of a sensitive assay to directly detect the protein at the cell surface, it has been difficult to ascertain whether there is leakage of cell surface expression for the various gp55 mutants.

In contrast to the difficulties related to the study of the cell surface expression of gp55, the transport of EPO-R to the plasma membranes is relatively efficient and cell surface expression of the protein can be readily measured by the ¹²⁵I-EPO binding assay (8). Furthermore, the biological activity of the cell surface receptor can be measured by a sensitive cell proliferation assay using Ba/F-ER cells (23) which respond to EPO at picomolar ranges (9, 44). Because it was shown that the C terminus of the EPO-R protein is dispensable (see below), we decided to make an ER-retained mutant by adding the DEKKMP retention signal of the adenovirus E3/19K protein (32) to the C terminus of EPO-R and use this mutant (EPO-Rcd) to address the question of whether the mitogenic activation of EPO-R by gp55 occurs within the ER membranes or at the cell surface. The retention signal completely retained EPO-Rcd in the ER, and no cell surface expression of the receptor was detected, even in cells that expressed a high level of the protein intracellularly (Fig. 1A and Table 1). Further characterization showed that the mutant, like the wild-type EPO-R, specifically interacted with gp55 (Fig. 1B). This apparent intracellular interaction, however, failed to promote growth factor-independent proliferation of the Ba/F3 cells (Table 2). Although the possibility remains that the mitogenic activation of EPO-R by gp55 occurs at a post-ER compartment such as the Golgi apparatus, the fact that all 20 spontaneous revertants of EPO-Rcd, selected on the basis of their mitogenicity when coexpressed with gp55, were found exclusively expressed at the cell surface (Table 1 and data not shown) suggests that the activation of EPO-R by gp55 occurs at the cell surface.

It has been shown that extensive C-terminal deletions (up to 180 aa) and/or additions of apparently random sequences (up to 23 aa) do not affect the activation of EPO-R (9, 31, 45). It is thus unlikely that a small DEKKMP addition to the C terminus would result in a drastic disturbance in either the overall structure or the proper folding of the EPO-R protein. This view was further supported by our findings that EPO-Rcd specifically interacted with gp55 (Fig. 1B) and that C-terminal deletions of up to 169 aa and/or additions of up to 75 aa did not affect the mitogenic activation of the revertants derived from EPO-Rcd (Table 3). Thus, the failure of the gp55-EPO-Rcd complex to induce mitogenesis is most likely due to its intracellular localization, not the loss of function of EPO-Rcd.

Mutations in the WSXWS motif of EPO-R also abolished

receptor processing and its transport to the cell surface (46). Interestingly, such EPO-R mutants could not be activated by gp55. However, the WSXWS motif is highly conserved among members of the cytokine receptor superfamily, which includes EPO-R (1), and it may form part of the ligand-binding site (2). Thus, mutations in the motif, unlike that in the C-terminal region, may directly affect the function of the receptor. Indeed, a mutation in the WSXWS sequence, but not a C-terminal deletion of 42 aa and an addition of 2 aa, prevented the constitutive activation of an EPO-R mutant (42).

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REFERENCES

1. Bazan, J. F. 1989. A novel family of growth factor receptors: a common binding domain in the growth hormone, prolactin, erythropoietin and IL-6 receptors, and the p75 IL-2 receptor β -chain. *Biochem. Biophys. Res. Commun.* **164**:788–793.
2. Bazan, J. F. 1990. Structural design and molecular evolution of a cytokine receptor superfamily. *Proc. Natl. Acad. Sci. USA* **87**:6934–6938.
3. Ben-David, Y., and A. Bernstein. 1991. Friend viral-induced erythroleukemia and the multistage nature of cancer. *Cell* **66**:831–834.
4. Bestwick, R., S. Kozak, and D. Kabat. 1988. Overcoming interference to retroviral superinfection results in amplified expression of cloned genes. *Proc. Natl. Acad. Sci. USA* **85**:5404–5408.
5. Casadevall, N., C. Lacombe, O. Muller, S. Gisselbrecht, and P. Mayeux. 1991. Multimeric structure of the membrane erythropoietin receptor of murine erythroleukemia cells (Friend cells). *J. Biol. Chem.* **266**:16015–16020.
6. Clark, S., 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.
7. D'Andrea, A., G. Fasman, and H. Lodish. 1989. Erythropoietin receptor and interleukin-2 β chain: a new receptor family. *Cell* **58**:1023–1024.
8. D'Andrea, A., H. Lodish, and G. Wong. 1989. Expression cloning of the murine erythropoietin receptor. *Cell* **57**:277–285.
9. D'Andrea, A., A. Yoshimura, H. Yousoufian, L. Zon, A.-W. Koo, and H. Lodish. 1991. The cytoplasmic region of the erythropoietin receptor contains nonoverlapping positive and negative growth-regulatory domains. *Mol. Cell. Biol.* **11**:1980–1987.
10. Dresler, S., M. Ruta, M. J. Murray, and D. Kabat. 1979. Glycoprotein encoded by the Friend spleen focus-forming virus. *J. Virol.* **30**:564–575.
11. Ferro, F., S. Kozak, M. Hoatlin, and D. Kabat. 1993. Cell surface site for mitogenic interaction of erythropoietin receptors with the membrane glycoprotein encoded by Friend erythroleukemia virus. *J. Biol. Chem.* **268**:5741–5747.
12. Friend, C. 1957. Cell-free transmission in adult Swiss mice of a disease having the character of a leukemia. *J. Exp. Med.* **105**:307–318.
13. Gliniak, B., 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.
14. Gliniak, B., S. Kozak, R. Jones, and D. Kabat. 1991. Disulfide bonding controls processing of retroviral envelope glycoproteins. *J. Biol. Chem.* **266**:22991–22997.
15. Hankins, D., T. Kost, M. J. Koury, and S. Krantz. 1978. Erythroid bursts produced by Friend leukemia virus *in vitro*. *Nature (London)* **276**:506–508.
16. Hankins, D., and D. Troxler. 1980. Polycythemia- and anemia-inducing erythroleukemia viruses exhibit differential erythroid transforming effects *in vitro*. *Cell* **22**:693–699.
17. Kabat, D. 1989. Molecular biology of Friend viral erythroleukemia. *Curr. Top. Microbiol. Immunol.* **148**:1–42.
18. Kilpatrick, D., R. Srinivas, and R. Compans. 1989. The spleen focus-forming virus envelope glycoprotein is defective in oligomerization. *J. Biol. Chem.* **264**:10732–10737.
19. Kozak, S., M. Hoatlin, F. Ferro, M. Majumdar, R. Geib, M. Fox, and D. Kabat. 1993. A Friend virus mutant that overcomes Fv-2rr host resistance encodes a small glycoprotein that dimerizes, is processed to cell surfaces, and specifically activates erythropoietin receptors. *J. Virol.* **67**:2611–2620.
- 19a. Li, J.-P. Unpublished data.
20. Li, J.-P., and D. Baltimore. 1991. Mechanism of leukemogenesis induced by the mink cell focus-forming murine leukemia viruses. *J. Virol.* **65**:2408–2414.

21. Li, J.-P., R. K. Bestwick, C. Machida, and D. Kabat. 1986. Role of a membrane glycoprotein in Friend virus erythroleukemia: nucleotide sequences of nonleukemogenic mutant and spontaneous revertant viruses. *J. Virol.* **57**: 534–538.
22. 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.
23. Li, J.-P., A. D'Andrea, H. 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.
24. Linemeyer, D., S. Ruscetti, E. Scolnick, L. Evans, and P. Duesberg. 1981. Biological activity of the spleen focus-forming virus is encoded by a molecularly cloned subgenomic fragment of the spleen focus-forming virus DNA. *Proc. Natl. Acad. Sci. USA* **78**:1401–1405.
25. Machida, C., R. Bestwick, and D. Kabat. 1985. A weakly pathogenic Rauscher spleen focus-forming virus mutant that lacks the carboxyl-terminal membrane anchor of its envelope glycoprotein. *J. Virol.* **53**:990–993.
26. Mallabiabarrena, A., M. Fresno, and B. Alarcon. 1992. An endoplasmic reticulum retention signal in the CD3e chain of the T-cell receptor. *Nature (London)* **357**:593–596.
27. Mathey-Prevot, B., G. Nabel, R. Palacios, and D. Baltimore. 1986. Abelson virus abrogation of interleukin-3 dependence in a lymphoid cell line. *Mol. Cell. Biol.* **6**:4133–4135.
28. Mirand, E., R. Steeves, L. Avila, and J. Grace. 1968. Spleen focus formation by polycythemic strains of Friend leukemia virus. *Proc. Soc. Exp. Biol. Med.* **127**:900–904.
29. Moreau-Gachelin, F., A. Tavitian, and P. Tambourin. 1988. Spi-1 is a putative oncogene in virally induced murine erythroleukemias. *Nature (London)* **331**:277–280.
30. Mowat, M., A. Cheng, N. Kimura, A. Bernstein, and S. Benchimol. 1985. Rearrangements of the cellular p53 gene in erythroleukemic cells transformed by Friend virus. *Nature (London)* **314**:633–636.
31. Nakamura, Y., N. Komatsu, and H. Nakauchi. 1992. A truncated erythropoietin receptor that fails to prevent programmed cell death of erythroid cells. *Science* **257**:1138–1141.
32. Nilsson, T., M. Jackson, and P. Peterson. 1989. Short cytoplasmic sequence serves as retention signals for transmembrane proteins in the endoplasmic reticulum. *Cell* **58**:707–718.
33. Pinter, A. 1988. O-linked glycosylation of retroviral envelope gene products. *J. Virol.* **62**:1016–1021.
34. Pinter, A., and W. Honnen. 1985. The mature form of the Friend spleen focus-forming virus envelope protein, gp65, is efficiently secreted from cells. *Virology* **143**:646–650.
35. Ruscetti, S., N. Janesch, A. Chakraborti, S. Sawyer, and D. Hankins. 1990. Friend spleen focus-forming virus induces factor independence in an erythropoietin-dependent erythroleukemia cell line. *J. Virol.* **64**:1057–1062.
36. Ruscetti, S., D. Linemeyer, J. Feild, D. Troxler, and E. Scolnick. 1979. Characterization of a protein found in cells infected with 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.
37. Ruta, M., R. Bestwick, C. Machida, and D. Kabat. 1983. Loss of leukemogenicity caused by mutations in the membrane glycoprotein structural gene of Friend spleen focus-forming virus. *Proc. Natl. Acad. Sci. USA* **80**:4704–4708.
38. Ruta, M., and D. Kabat. 1980. Plasma membrane glycoproteins encoded by cloned Rauscher and Friend spleen focus-forming viruses. *J. Virol.* **35**:844–853.
39. Showers, S., J. DeNartino, Y. Saito, and A. D'Andrea. 1993. Fusion of the erythropoietin receptor and the Friend spleen focus-forming virus gp55 glycoprotein transforms a factor-dependent hematopoietic cell line. *Mol. Cell. Biol.* **13**:739–748.
40. Steeves, R., E. Mirand, A. Bulba, and P. Trudul. 1970. Spleen foci and polycythemia in C57BL mice infected with host-adapted Friend leukemia virus. *Int. J. Cancer* **5**:346–356.
41. Wang, Y., S. Kayman, J.-P. Li, and A. Pinter. 1993. In vitro mitogenicity of spleen focus-forming virus in the presence of erythropoietin receptor (EPO-R) correlates with viral pathogenicity and processing of *env* protein, but not with formation of gp52-EPO-R complexes in the endoplasmic reticulum. *J. Virol.* **67**:1322–1373.
42. Watowich, S., A. Yoshimura, G. Longmore, D. Hilton, Y. Yoshimura, and H. Lodish. 1992. Homodimerization and constitutive activation of the erythropoietin receptor. *Proc. Natl. Acad. Sci. USA* **89**:2140–2144.
43. Wolff, L., R. Koller, and S. Ruscetti. 1982. Monoclonal antibody to spleen focus-forming virus-encoded gp52 provides a probe for the amino-terminal region of retroviral envelope proteins that confers dual tropism and xenotropism. *J. Virol.* **43**:472–481.
44. Yoshimura, A., A. D'Andrea, and H. 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.
45. Yoshimura, A., G. Longmore, and H. Lodish. 1990. Point mutation in the exoplasmic domain of the erythropoietin receptor resulting in hormone-independent activation and tumorigenicity. *Nature (London)* **348**:647–649.
46. Yoshimura, A., T. Zimmers, D. Neumann, G. Longmore, Y. Yoshimura, and H. Lodish. 1992. Mutations in the Trp-Ser-X-Trp-Ser motif of the erythropoietin receptor abolished processing, ligand binding, and activation of the receptor. *J. Biol. Chem.* **267**:11619–11625.
47. Zon, L., J.-F. Moreau, J.-W. Koo, B. Mathey-Prevot, and A. D'Andrea. 1992. The erythropoietin receptor transmembrane region is necessary for activation by the Friend virus spleen focus-forming virus gp55 glycoprotein. *Mol. Cell. Biol.* **12**:2949–2957.