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Deletions in One Domain of the Friend Virus-Encoded Membrane Glycoprotein Overcome Host Range Restrictions for Erythroleukemia†

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Although the Friend virus-encoded membrane glycoprotein (gp55) activates erythropoietin receptors (EpoR) to cause erythroblastosis only in certain inbred strains of mice but not in other species, mutant viruses can overcome aspects of mouse resistance. Thus, mice homozygous for the resistance allele of the *Fv-2* gene are unaffected by gp55 but are susceptible to mutant glycoproteins that have partial deletions in their ecotropic domains. These and other results have suggested that proteins coded for by polymorphic *Fv-2* alleles might directly or indirectly interact with EpoR and that changes in gp55 can overcome this defense. A new viral mutant with an exceptionally large deletion in its ecotropic domain is now also shown to overcome *Fv-2*^{rr} resistance. In all cases, the glycoproteins that activate EpoR are processed to cell surfaces as disulfide-bonded dimers. To initiate analysis of nonmurine resistances, we expressed human EpoR and mouse EpoR in the interleukin 3-dependent mouse cell line BaF3 and compared the abilities of Friend virus-encoded glycoproteins to convert these cells to growth factor independence. Human EpoR was activated in these cells by erythropoietin but was resistant to gp55. However, human EpoR was efficiently activated in these cells by the same viral mutants that overcome *Fv-2*^{rr} resistance in mice. By construction and analysis of human-mouse EpoR chimeras, we obtained evidence that the cytosolic domain of human EpoR contributes to its resistance to gp55 and that this resistance is mediated by accessory cellular factors. Aspects of host resistance in both murine and nonmurine species are targeted specifically against the ecotropic domain of gp55.

Friend spleen focus-forming virus (SFFV), the replication-defective component of the viral complex, encodes a membrane glycoprotein (gp55) that associates with erythropoietin receptors (EpoR) to cause erythroblast proliferation in susceptible strains of mice (5, 18, 28, 33, 63, 64). Although gp55 acts as a viral-encoded hemopoietin mimic, gp55 and erythropoietin (Epo) are unrelated structurally and bind to nearby but nonoverlapping sites on the 66,000-M_r EpoR protein (5, 18).

Sequence studies have shown that gp55 is a recombinant-type *env* glycoprotein with an amino-terminal dual-tropic domain followed by an ecotropic domain and by a carboxyl-terminal hydrophobic membrane anchor (1, 7, 20, 25, 48, 61). Although it occurs principally as a monomer in the rough endoplasmic reticulum, a small proportion (ca. 5%) forms a disulfide-bonded dimer that is processed to cell surfaces (20). In transit, this component is modified by addition of an O-linked oligosaccharide and complex N-linked oligosaccharides that have fucose, galactose, and sialic acids (20, 25, 51). Evidence that the cell surface dimeric form of gp55 is required for pathogenesis (18, 35) is consistent with other indications that dimerization may be involved in activating EpoR and other members of the cytokine and growth hormone superfamily of receptors (2, 8, 47, 58). Strong support for this dimerization model has been derived from X-ray crystallographic studies of human growth hormone complexed with its receptor (8, 15). Additional evidence, including results of chemical cross-linking and gentle isolation studies, have indicated that EpoR is a large complex protein assemblage with many components, including JAK2 protein tyrosine kinase (5, 12, 18, 24, 28, 39, 40, 59), the SH2 adapter protein (Shc), Grb2 (9, 10), and phosphatidylinositol 3-kinase (11, 21, 40, 43). Moreover, gp55 displaces several proteins from this assemblage (5, 18). It has been proposed that EpoR dimerization triggers cross-phosphorylation and activation of adjacent JAK2 molecules, with resulting phosphorylation of tyrosines on the 66,000-M_r EpoR component and on other proteins (59).

A fascinating property of gp55 that distinguishes it from other known oncoproteins is the severe host range limitation of its activity. For example, gp55 has no pathogenic activity in rats (14) or even in some inbred strains of mice (16, 34). On the basis of these host range limitations, many polymorphic mouse genes have been identified that control susceptibility to Friend virus (6, 27, 49, 50, 54, 56), and these provide a unique resource for analyzing virus-host interactions in a pathway of leukemogenesis. Mice homozygous for the *Fv-2*^{r} allele are resistant to SFFV-induced erythroblastosis, although they synthesize gp55 and are susceptible to all other retroviral diseases (16, 19, 23, 28, 34, 36, 57). Recently, we found that mutant SFFVs selected for the ability to overcome this resistance encode gp55-related glycoproteins with scattered point mutations plus partial deletions in their ecotropic domains (36). The fact that polymorphism at the *Fv-2* locus controls the activity of gp55 and that this control can be overcome by alterations of gp55 implies that *Fv-2*-encoded proteins interact directly or indirectly with gp55 (28, 36).

As an initial step in analyzing the molecular basis for the

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† This article is dedicated to the memory of Carl Glen Ross.

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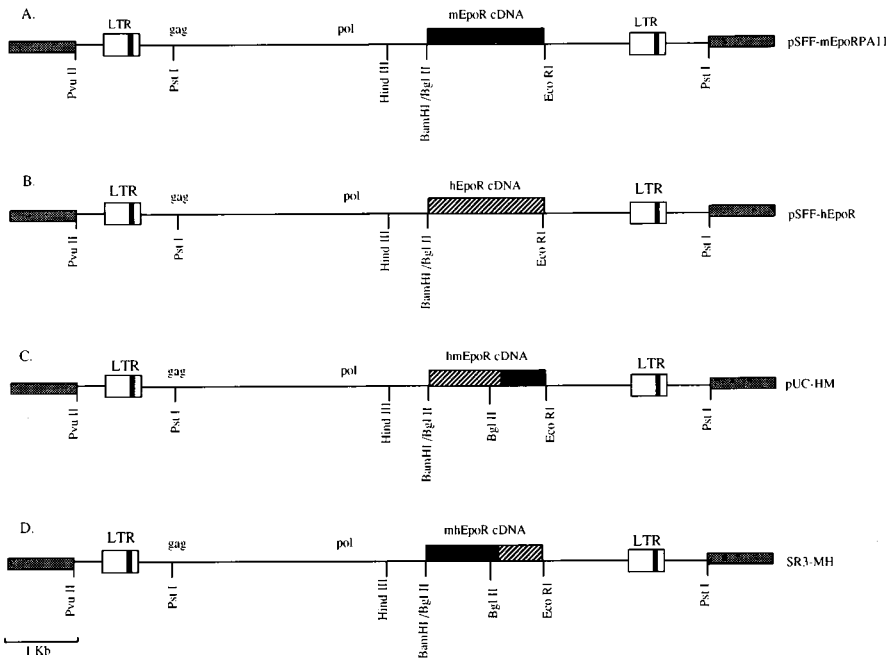

FIG. 1. Maps of retroviral constructs. The relative sizes and restriction sites used to produce the retroviral constructs encoding mEpoR, hEpoR, and chimeric EpoR are shown. The pSFF retroviral vector has been described (4). Murine and human sequences are shown as black and striped boxes, respectively. (A) pSFF-mEpoRPA11. (B) pSFF-hEpoR. The mEpoR and hEpoR restriction fragments used to construct pSFF-EpoR reciprocal chimeric receptors are shown: pUC-HM (C) encodes the hEpoR up to 36 amino acids beyond the end of the transmembrane region; the remainder of the C-terminal protein is murine. SR3-MH encodes the reciprocal receptor (D). The plasmids encoding EpoR were transfected into cocultures of packaging cells to produce helper-free EpoR-encoding virions (see Materials and Methods). LTR, long terminal repeat.

resistance of nonmurine species to gp55-induced erythroblastosis, we expressed human EpoR (hEpoR) and mouse EpoR (mEpoR) in interleukin 3 (IL-3)-dependent hematopoietic cells and studied their activation by Epo and by SFFV-encoded glycoproteins. mEpoR and hEpoR contain 507 and 508 amino acids, respectively, and they are 76% identical in sequence (12, 24). Mouse BaF3 cells were used for this analysis because they have been employed for previous studies of SFFV and EpoR (13, 18, 26, 28, 30, 33, 55, 62, 64), they are from a susceptible strain of mice, and they spontaneously convert to growth factor independence at a negligibly low rate (18, 28). Comparable cells are not available from *Fv-2*^{r} homozygous mice. However, we have initiated parallel studies with human cells and have obtained preliminary results compatible with those described here (21a). Our data indicate that hEpoR is resistant to gp55-mediated activation but that it is efficiently activated by mutant forms of gp55 that contain deletions in their ecotropic domains.

MATERIALS AND METHODS

Cells. Retroviral packaging cell line ψ-2 (37) and PA12 (41) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. IL-3-dependent hematopoietic BaF3 cells (38) were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 5×10^{-5} M β-mercaptoethanol with 10% WEHI-3 conditioned medium as an IL-3 source. ψ-2 and PA12 cells were transfected with retroviral vectors encoding EpoR and *env* glycoproteins to produce helper-free virions by ping-pong amplification as previously described (4, 29). The retroviral vectors encoding gp55 of wild-type SFFV (Lilly-Steeves polycythemic strain) (32) and the mutant SFFV *env* genes BB6 (28) and PvuΔ (18) have been described. BaF3 cells that were infected with the EpoR-encoding virions and selected for growth in Epo were maintained in medium containing recombinant mouse Epo at 0.5 U/ml (Boehringer Mannheim, Indianapolis, Ind.).

Plasmid constructions. The retroviral vector encoding mEpoR (pSFF-EpoRPA11) was constructed from the ligation of an *Eco*RI-*Bam*HI fragment of pSFF (3), an 800-bp *Bgl*II-*Nhe*I fragment from pXM190 (a generous gift of A. D'Andrea, Dana-Farber Cancer Institute, Boston, Mass.), and a 790-bp *Nhe*I-*Eco*RI fragment of pXM190 containing the 3′ end of the EpoR gene without its polyadenylation signal. The *Eco*RI site of the latter fragment was constructed by treating a *Sty*I site (EpoR nucleotide position 1547 [12]) with Klenow polymerase, which fortuitously introduced a stop codon at the natural stop site in the EpoR sequence and also created an *Eco*RI restriction site in the proper orientation for insertion into the *Bam*HI-*Eco*RI cloning site of pSFF. pSFF-hEpoR was constructed from a three-way ligation of the following fragments: a *Bam*HI-*Xho*I pSFF vector fragment, a *Bgl*II-*Bss*HII fragment, and a *Bss*HII-*Xho*I fragment from the hEpoR-encoding plasmid p18 (a gift from G. Wong, Genetics

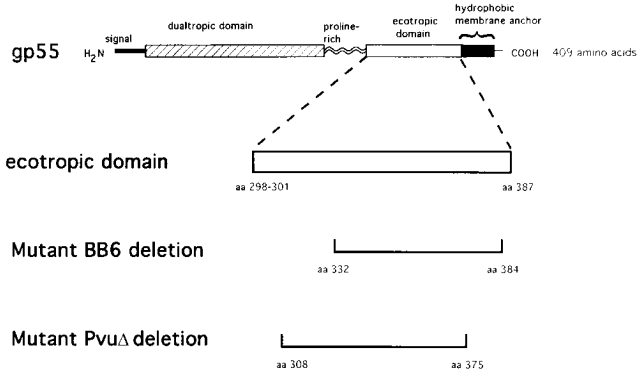

FIG. 2. Structures of gp55 and of deletions that occur in SFFV mutants that overcome resistance of *Fv-2*^{r} homozygotes. Mutant BB6 was isolated by forced passage of wild-type SFFV in *Fv-2*^{r} mice (57). In contrast, mutant PvuΔ was isolated in cell culture on the basis that it activates EpoR and encodes a small glycoprotein (18), and it was later shown that it causes pathogenesis in NIH/Swiss mice (*Fv-2*^{ss}). Studies in this report suggest that it overcomes *Fv-2*^{r} resistance. The structure of gp55, which has been reviewed (25), includes an amino-terminal polytropic (also called dual-tropic) domain that has a proline-rich sequence at its carboxyl terminus, an ecotropic domain (shown in white), and a hydrophobic membrane anchor. gp55 lacks any cytosolic extension.

TABLE 1. Pathogenesis by the PvuΔ mutant of SFFV in DBA/2 (*Fv-2*^{ss}) and in D2.R16 (*Fv-2*^{rr}) congenic mice[a]

Virus[b]	Mouse strain	% Hematocrit (spleen weight [g]) on postinfection day[c]:		
		22	28	39
PvuΔ	DBA/2	72 (1.1)	85 (1.3)	75 (1.1)
		73 (0.78)	85 (1.3)	82 (1.9)
		80 (0.90)		83 (1.4)
				84 (1.9)
PvuΔ	D2R.16	72 (0.63)	86 (0.80)	83 (0.8)
		74 (0.71)	83 (1.0)	84 (1.1)
		75 (0.58)		84 (1.1)
				85 (1.2)
				85 (1.4)
None[d]	DBA/2	41–45 (0.09–0.13)[d]		

[a] The D2.R16 mouse colony was maintained in standard housing separate from other mice used in this study. The founder pairs were generously provided by Frank Lilly (Albert Einstein College of Medicine, Bronx, N.Y.). DBA/2 mice were obtained commercially and were maintained under normal housing conditions.

[b] The virus was a mixture of the PvuΔ SFFV mutant and the nonpathogenic biologically cloned B4 strain of Friend murine leukemia virus helper (22, 23, 44). The virus was a passaged virus stock as previously described (22, 23).

[c] Injections consisted of 0.5 ml given intravenously (36). At the indicated postinfection day, the mice were sacrificed and their hematocrits and spleen weights were measured.

[d] Hematocrits and spleen weights for control DBA/2 and D2.R16 mice are ranges observed typically for uninfected disease-free mice of the ages and sexes used for this study, as previously reported (23). The helper virus used for this work, the B4 isolate of Friend murine leukemia virus, does not cause significant hematopoietic abnormalities (44).

Institute, Cambridge, Mass.). A *Bgl*II site common to both receptors (nucleotide position 866, mEpoR numbering [12]) was used to make the in-frame connection between the hEpoR and mEpoR for the construction of the chimeras (Fig. 1). The chimeric EpoR constructions were verified by DNA sequencing.

^{125}I-Epo binding assays. Cells growing in Epo (BaF3-hEpoR and BaF3-mEpoR) or IL-3 (BaF3) were pelleted, washed, and resuspended in medium without growth factors for 1 h at 37°C. The cells (1×10^6 to 8×10^6) were then pelleted and incubated with 0.03 mCi of ^{125}I-Epo (300 to 900 Ci/mmol) (Amersham Corp., Arlington Heights, Ill.) in 0.1 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 0.2% sodium azide for 3 h at 20°C. Cells with bound ^{125}I-Epo were separated from excess unbound ^{125}I-Epo by pelleting through 0.15 ml of dibutyl phthalate oil. The cell pellets were clipped from the bottoms of the tubes and assayed for radioactivity in a gamma counter.

Factor-independent growth assays. For factor-independent growth assays, BaF3 cells or BaF3-EpoR cells were infected with SFFV, PvuΔ, or BB6 virus for 2 h at 37°C in the presence of 8 μg of Polybrene per ml. The cells were pelleted by centrifugation and resuspended for 48 h in medium containing growth factor. The cells were then sedimented by centrifugation, washed twice with phosphate-buffered saline (Gibco BRL, Gaithersburg, Md.), and resuspended in complete medium without growth factors and seeded into microtiter wells or flasks to allow for selection of factor-independent cells.

Analysis of proteins. For detection of SFFV, BB6, and PvuΔ *env* glycoproteins by Western blotting (immunoblotting), cell lysates were immunoprecipitated with an anti-Friend leukemia virus gp70 antiserum that cross-reacts with *envs* encoded by SFFV and mink cell focus-forming virus (20, 51, 52) and were electrophoresed on polyacrylamide gels under reducing or nonreducing conditions in the presence of 1% sodium dodecyl sulfate. The proteins were then transferred to nitrocellulose membranes, incubated with the same antibody, and detected with ^{125}I-labeled protein A as described previously (20, 32). EpoR protein was detected similarly with an antibody made by injecting rabbits with a synthetic peptide (APSPSLPDPKFESKC) matching the amino terminus of the mature mEpoR sequence (12).

RESULTS

Mutant PvuΔ is highly pathogenic in DBA/2 (*Fv-2*^{ss}) and in congenic D2.R16 (*Fv-2*^{rr}) mice. Structures of glycoproteins coded for by wild-type and mutant SFFVs relevant to this study are diagrammed in Fig. 2. Whereas mutant BB6 was isolated by repetitive forced passages in homozygous (*Fv-2*^{r}) mice (57), mutant PvuΔ formed spontaneously in culture and was isolated on the basis of its small size and ability to activate EpoR (18). Recently, we described its *env* gene sequence and its ability to cause advanced erythroleukemia in NIH/Swiss (*Fv-2*^{s}) mice (22). Because its deletion overlaps that of mutant BB6, we tested the possibility that PvuΔ might also overcome *Fv-2*^{rr} resistance. In contrast to uninfected disease-free DBA/2 (*Fv-2*^{ss}) or D2.R16 (*Fv-2*^{rr}) congenic mice, which have hematocrits of 45 to 50% and spleen weights of 0.09 to 0.13 g (16, 19), mice injected with PvuΔ plus a nonpathogenic helper virus (44) uniformly developed very high hematocrits (72 to 85%) and spleen weights (0.58 to 1.9 g) by 22 to 39 days postinfection (Table 1), indicative of splenic erythropoiesis with resulting erythrocytosis and polycythemia. Wild-type Friend virus complex or helper virus alone does not cause significant disease in D2.R16 mice (16, 23).

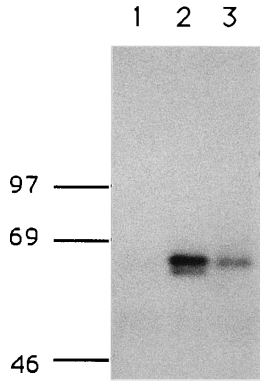

FIG. 3. mEpoR and hEpoR expressed in murine retroviral packaging cells. Cell lysates from ψ-2 and PA12 cocultures transfected with pSFF-mEpoRPA11 and pSFF-hEpoR were immunoprecipitated with an anti-mEpoR antibody (see Materials and Methods). The lysates were run on electrophoresis gels under reducing conditions and transferred to nitrocellulose membranes. The membranes were incubated with anti-EpoR antibody followed by ^{125}I-protein A. Lane 1, untransfected coculture; lane 2, ψ-2 and PA12 cells expressing mEpoR; lane 3, ψ-2 and PA12 cells expressing hEpoR. Sizes of molecular mass standards are indicated in kilodaltons on the left.

Production of BaF3 cells expressing hEpoR or mEpoR. By 13 days after transfecting pSFF-mEpoR and pSFF-hEpoR vectors (Fig. 1) into cocultures of ψ-2 (37) and PA12 (41) retroviral packaging cells, efficient vector amplifications had occurred and the cultures expressed EpoRs with the expected M_r of 66,000 (Fig. 3, lanes 2 and 3). Helper-free virions harvested from the latter cocultures and used to infect IL-3-dependent mouse BaF3 cells enabled these cells to grow with Epo as the only growth factor. However, independently made BaF3-hEpoR cells grew much more slowly after initial transfer into Epo than BaF3-mEpoR cells, and it required approximately 2 weeks of selection in Epo before the cells with hEpoR were proliferating rapidly. As shown in Table 2, the resulting cell lines bound ^{125}I-Epo.

TABLE 2. ^{125}I-Epo binding of BaF3 and BaF3-EpoR derivatives

Cell	Bound ^{125}I-Epo (cpm)[a]	Growth factor required
BaF3	521	IL-3
BaF3-mEpoR	12,300	IL-3 or Epo
BaF3-hEpoR	26,200	IL-3 or Epo

[a] Binding was done on triplicate samples of 5×10^6 cells with a saturating amount of ^{125}I-Epo as described previously (28).

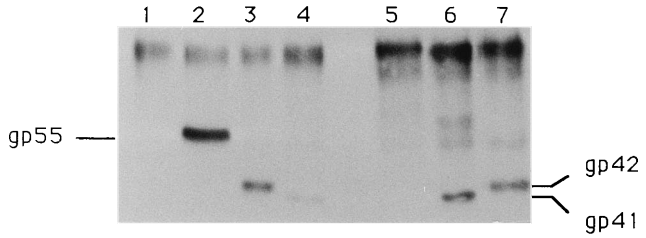

FIG. 4. SFFV, BB6, and PvuΔ *env* glycoproteins expressed by factor-independent BaF3-EpoR. The proteins were immunoprecipitated, electrophoresed, and transferred to nitrocellulose membranes for detection by immunoblotting with an anti-envelope glycoprotein antiserum (see Materials and Methods). Lane 1, BaF3-mEpoR; lane 2, BaF3-mEpoR-SFFV; lane 3, BaF3-mEpoR-BB6; lane 4, BaF3-mEpoR-PvuΔ; lane 5, BaF3-hEpoR; lane 6, BaF3-hEpoR-PvuΔ; lane 7, BaF3-hEpoR-BB6. BaF3 cells express an endogenous 85,000-M_r protein (18, 28).

Interactions of mEpoR and hEpoR with gp55 and with smaller glycoproteins coded for by SFFV mutants BB6 and PvuΔ. Previous studies have indicated that BaF3-mEpoR cells but not BaF3 cells become growth factor independent after expression of the SFFV glycoprotein gp55 (13, 18, 28, 30, 33, 62, 64). Similarly, the growth factor independence of BaF3-mEpoR cells specifically occurs after infection with the BB6 mutant that overcomes resistance of *Fv-2*^{r} homozygotes (28, 36) and after infection with the PvuΔ mutant (18).

Surprisingly, BaF3 cells with hEpoR were converted to growth factor independence by the BB6 and PvuΔ SFFV mutants but not by the wild-type SFFV (Table 3). To establish that these conversions were efficiently caused by the viruses rather than by rare spontaneous loss of growth factor dependence, the cells were washed away from growth factors 48 h after infection and were then diluted to low concentrations. Each dilution of the cells was plated into 24 wells of a microtiter plate, and the number of wells with factor-independent cells was later determined by detection of sustained cell growth. As illustrated in Table 3, the virus-induced frequencies of conversion to growth factor independence were many orders of magnitude larger than spontaneous conversion frequencies. Indeed, in positive assays, 0.1 to 5% of the infected cells became growth factor independent and vigorous proliferation was observed in the culture wells within 2 to 3 days, whereas in negative cases conversions were not observed, indicating that substantially less than 10^{-4}% of the cells were growth factor independent. Identical results were obtained in nine independent assays using these methods.

To verify that the factor-independent cells that formed in these assays contained SFFV-related viruses, extracts of the cell cultures were analyzed for viral-encoded glycoproteins. As illustrated in Fig. 4, the factor-independent BaF3-mEpoR cells

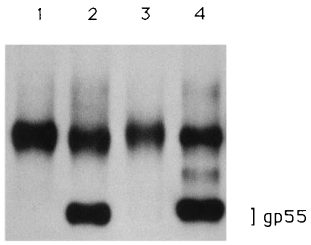

FIG. 5. Immunoblot of adapted BaF3-hEpoR cells that became factor independent after SFFV infection. Cell lysates were analyzed with an anti-envelope glycoprotein antiserum as described in the legend to Fig. 4. Lanes 1 and 3, an endogenous 85,000-M_r protein expressed by BaF3 cells; lane 2, BaF3-mEpoR-SFFV; lane 4, adapted BaF3-hEpoR-SFFV.

TABLE 3. Factor-independent growth assays of BaF3 and BaF3-EpoR derivatives after infection with SFFV, BB6, and PvuΔ viruses[a]

Expt	Cell	Virus	No. of wells in a 24-well plate with factor-independent cells at indicated no. of cells plated/well				Approximate conversion (%)[b]
			10,000	1,000	100	10	
1	BaF3-mEpoR	SFFV	24	15			0.1
	BaF3-mEpoR	BB6	24	24			>0.3
	BaF3-hEpoR	SFFV	0	0			$<10^{-4}$
	BaF3-hEpoR	BB6	24	22			0.2
2	BaF3	SFFV	0	0	0	0	$<10^{-4}$
	BaF3-mEpoR	SFFV	24	24	14	1	0.9
	BaF3-hEpoR	SFFV	0	0	0	0	$<10^{-4}$
3	BaF3-mEpoR	SFFV	24	24	4	1	0.18
	BaF3-mEpoR	BB6	24	24	24	5	3.0
	BaF3-mEpoR	PvuΔ	24	22	9	2	0.46
	BaF3-hEpoR	SFFV	0	0	0	0	$<10^{-4}$
	BaF3-hEpoR	BB6	24	15	7	2	0.34
	BaF3-hEpoR	PvuΔ	24	24	21	7	2.6

[a] Fresh or frozen viral harvests were used to infect cells for 3 h at 37°C in the presence of 8 μg of Polybrene per ml. After growth for 48 h in medium with growth factors, cells were suspended in medium without growth factors at a concentration of 10^5 cells per ml and at additional 10-fold dilutions. Each cell dilution (0.1 ml) was then plated into 24 wells of a test plate. The wells were observed for proliferating factor-independent cells.

[b] Approximate conversion (%) is conversion events per 100 cells. To calculate the number of conversion events (n) per 100 wells, we used $n = \log P_O/-0.0044$, where P_O is the fraction of wells negative for cell growth (28). The average number of conversions per well is $n/100$. This is divided by the number of cells per well in the assay sample, and the resulting number is multiplied by 100 to give the approximate percent conversion.

that had been infected with SFFV, BB6, and PvuΔ viruses contained the expected gp55, gp42, and gp41 glycoproteins (Fig. 4, lanes 2 to 4, respectively). Similarly, the factor-independent BaF3-hEpoR cells that had been infected with BB6 and PvuΔ contained gp42 (Fig. 4, lane 7) or gp41 (Fig. 4, lane 6), respectively. BaF3 cells also contain an endogenous, 85,000-M_r, *env*-related protein that has been previously observed (18, 28) and that is present irrespective of SFFV infections. In some cases, small amounts of this endogenous component are seen also in the form of smaller degradation products (see below). Factor-independent cells that grow in these assays contain virus-encoded *env* glycoproteins even when the virus infections occur at low multiplicities (28).

Although the positive results described above were unambiguous, we observed several notable complexities in the negative assays. Specifically, in several assays, the BaF3-hEpoR cells infected with SFFV seemed to be marginally stimulated. This was seen as a slower cell death compared with that of uninfected BaF3-hEpoR cells following removal of growth factors, with occasional slight cell growth for several days. However, these infected cells ultimately failed to survive even when Epo was added at several time points in efforts to rescue the partially stimulated cells. Furthermore, one derivative line of BaF3-hEpoR cells that had been selected by continuous growth in Epo for longer than 3 months appeared to be slightly susceptible to SFFV. This derivative line also bound substantially more ^{125}I-Epo than freshly isolated BaF3-hEpoR cells (results not shown). In this case, the SFFV-infected cells did not die after the removal of growth factors, and a few of the survivors gradually began to grow after 3 weeks. The survivors contained gp55 (Fig. 5). We emphasize these results because

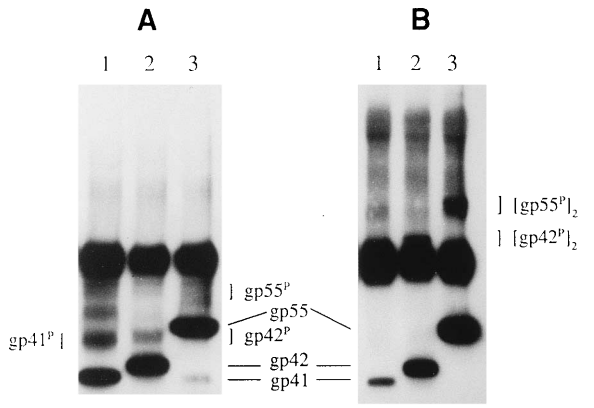

FIG. 6. Immunoblot showing the effects of reduction on electrophoretic mobilities of processed and unprocessed components of the gp41, gp42, and gp55 glycoproteins. Cell lysates were immunoprecipitated with anti-envelope glycoprotein antiserum, electrophoresed under reducing or nonreducing conditions, and transferred to nitrocellulose membranes. The anti-envelope antiserum was used for immunoblot detection as described in Materials and Methods. In panel A, the proteins were analyzed after treatment with a reducing agent. Lane 1, BaF3-mEpoR-PvuΔ; lane 2, BaF3-mEpoR-BB6; lane 3, BaF3-mEpoR-SFFV. In addition to the endogenous protein component with an M_r of approximately 80,000 to 90,000, these lysates contain large amounts of the unprocessed gp41, gp42, or gp55 components and smaller amounts of the processed components, indicated with the superscript p. The p components selectively disappear in the absence of a reducing agent (panel B), and aggregated components with higher M_rs are observed in these conditions. The lanes in panel B are as described for panel A.

they indicate that gp55 may marginally stimulate hEpoR and because they establish that the results can depend on the cells employed (i.e., on their prior selection and adaptation to growth in Epo) and on the experimental outcomes scored as positive.

Dimerization of SFFV-related *env* glycoproteins that activate EpoR. The SFFV and BB6 *env* glycoproteins are processed inefficiently from the rough endoplasmic reticulum as disulfide-bonded dimers to form $gp55^p$ and $gp42^p$, respectively (18, 28). To determine if processed forms of gp41 (i.e., $gp41^p$) also occur in disulfide-bonded complexes, the electrophoretic mobilities of the *env* glycoproteins were compared in the absence or presence of a reducing agent. As shown by the immunoblot analysis in lane 1 of Fig. 6A, a $gp41^p$ component is present at an M_r of approximately 50,000 in the lysates that were treated with a reducing agent. Similarly, $gp42^p$ and $gp55^p$ components are also present under these conditions (Fig. 6A, lanes 2 and 3). In contrast, when these lysates were not treated with a reducing agent, the $gp41^p$, $gp42^p$, and $gp55^p$ components were reduced in relative quantities and components with higher M_rs were observed. As shown in lanes 2 and 3 of Fig. 6B, $gp42^p$ and $gp55^p$ selectively appear as derivatives with higher M_rs, although $gp42^p$ is barely visible above the large endogenous *env*-related glycoprotein that occurs in all BaF3 cell lysates (18, 28). Compared with those of $gp42^p$, the smaller $gp41^p$ dimers are presumably masked by this endogenous component (Fig. 6B, lane 1). These results are compatible with other evidence that gp55 is processed from the endoplasmic reticulum to cell surfaces as a disulfide-bonded dimer (20). Evidently, $gp41^p$ and $gp42^p$ are also processed as disulfide-bonded complexes.

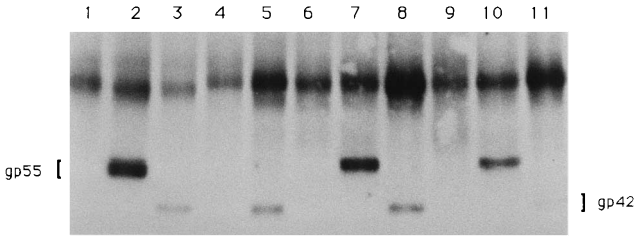

FIG. 7. Immunoblot of factor-independent BaF3-EpoR derivatives expressing BB6- and SFFV-encoded *envs*. Cell lysates were analyzed with *env* antiserum as described in the legend to Fig. 4. Lane 1, BaF3-mEpoR; lane 2, BaF3-mEpoR-SFFV; lane 3, BaF3-mEpoR-BB6; lane 4, BaF3-hEpoR; lane 5, BaF3-hEpoR-BB6; lane 6, BaF3–SR3-MH; lane 7, BaF3–SR3-MH–SFFV; lane 8, BaF3–SR3-MH–BB6; lane 9, BaF3–pUC-HM; lane 10, BaF3–pUC-HM–SFFV; lane 11, BaF3–pUC-HM–BB6 (the light gp42 band in lane 11 was clearly seen in a longer exposure of this blot).

TABLE 4. Factor-independent growth assays of BaF3 cells and BaF3-EpoR derivatives after infection with SFFV and BB6 viruses

Cell	Virus	No. of wells in a 24-well plate with factor-independent cells at indicated no. of cells plated/well[a]				Approximate conversion (%)
		10,000	1,000	100	10	
BaF3-mEpoR	SFFV	24	24	24	10	5.3
BaF3-hEpoR	SFFV	0	0	0	0	$<10^{-4}$
BaF3–pUC-HM	SFFV	24	20	5	0	0.23
BaF3–SR3-MH	SFFV	24	21	3	0	0.15
BaF3-mEpoR	BB6	24	24	24	17	12.1
BaF3-hEpoR	BB6	24	15	0	0	0.1
BaF3–pUC-HM	BB6	24	24	9	2	0.6
BaF3–SR3-MH	BB6	24	24	3	0	0.13

[a] BaF3 cells expressing chimeric receptors and infected with SFFV were not convincingly proliferating until days 6 to 8 after the removal of growth factors. This judgment is based on the number, refractility, and shape of the cells and the appearance of doublets. BaF3-mEpoR cells infected with SFFV were vigorously proliferating by days 3 to 4 after the removal of growth factors. The cells infected with BB6 virus were also all vigorously proliferating by days 2 to 3 after the removal of growth factors. The percent conversion was calculated as described in footnote b of Table 2.

Analysis of interspecies EpoR chimeras. Cytosolic domains of hEpoR and mEpoR were switched to produce reciprocal chimeric receptors (Fig. 1). As expected, the BaF3-chimeric receptor cells grew in Epo and bound ^{125}I-Epo (results not shown). However, as with the BaF3-hEpoR cells, the cells with both chimeric EpoR required an initial adaptation period prior to rapid proliferation in Epo. When superinfected with SFFV and later removed from growth factors, cells with both chimeric EpoR slowly became factor independent for survival and proliferation after lag periods that were not observed for BaF3-mEpoR cells (Table 4). Three independent assays confirmed these results (data not shown). The cell dilution analysis implied that conversions of the BaF3-chimeric EpoR cells to growth factor independence were unambiguously caused by SFFV infection. However, the efficiencies of conversions for these cells were somewhat lower than those for BaF3-mEpoR cells (Table 4) infected with the same preparation of SFFV. Moreover, as anticipated, the factor-independent cells contained the expected SFFV-encoded glycoproteins (Fig. 7). These results suggest that gp55 was capable of activating both chimeras in the conditions of this analysis but to a lesser degree than intact mEpoR.

DISCUSSION

The PvuΔ mutant of SFFV overcomes *Fv-2^{rr}* resistance of mice. In the presence of a nonpathogenic helper virus, the PvuΔ mutant strongly stimulates erythroblastosis and polycy-

themia in both DBA/2 (*Fv-2*^{ss}) and in congenic D2.R16 (*Fv-2*^{rr}) mice (Table 1). Unlike the other SFFV mutants that overcome *Fv-2*^{rr} resistance and that were selected by repetitive forced passages in resistant mice, PvuΔ was isolated in cell culture on the basis of its small size and ability to activate EpoR (18) and it lacks the multiple scattered point mutations present in these other isolates (36). Our results strongly imply that deletions in the ecotropic domain are responsible for this host range expansion, that mutants with deletions in this domain will predictably have this extended host range, and that the entire ecotropic domain is not essential for pathogenesis. Nevertheless, the mutant SFFVs that overcome *Fv-2*^{rr} resistance are less pathogenic than wild-type SFFV in *Fv-2*^{ss} mice (19, 22, 28). Consequently, ecotropic sequences of gp55 have positive or negative effects on pathogenesis depending on the *Fv-2* alleles of the host mice.

The BaF3 cell culture system for EpoR analysis. We used the IL-3-dependent BaF3 cell culture system (38) to express hEpoR and mEpoR and to analyze SFFV *env* glycoproteins. Although this system provides a powerful means to detect activation of EpoR by viral-encoded glycoproteins (13, 18, 26, 28, 30, 33, 55, 62, 64), our results suggest that it has several limitations that must be considered, especially in interpreting negative data. For example, in carefully controlled studies, it is clear that hEpoR is reproducibly highly resistant to activation by gp55 (Table 3). However, this resistance is not absolute because the BaF3-hEpoR cells infected with SFFV survive much longer in the absence of growth factors than uninfected control BaF3-hEpoR cells or SFFV-infected BaF3 cells. Furthermore, a derivative line of BaF3-hEpoR cells that had been selected by continuous growth in Epo for longer than 3 months contained an increased content of hEpoR and was marginally competent for activation by gp55 (Fig. 5). Similar adaptive changes were reported for BaF3 cells that express receptors for granulocyte-macrophage colony-stimulating factor or that express a truncated form of EpoR (45, 53). Thus, assay results may depend to some extent on the passage and selection history of the cells and on the experimental outcomes that are scored as positive. Previous investigators concluded that BaF3-hEpoR cells did (26) or did not (55) become factor independent after SFFV infections.

We have employed this assay in controlled studies and have based our conclusions on stringent positive criteria rather than on negative results. Specifically, we conclude that a retroviral *env* glycoprotein activates an EpoR if viral infection of the newly prepared BaF3-EpoR cells converts the cells to growth factor independence as indicated by a cell dilution analysis (18, 28) (Tables 3 and 4) and if the resulting factor-independent cells express the viral-encoded glycoprotein (Table 3 and Fig. 4 and Table 4 and Fig. 7). Generally, in our positive assays, 0.1 to 5% of the BaF3-EpoR cells were converted to factor independence by infection with the SFFV-related viruses and the resulting factor-independent cells all expressed the expected glycoproteins. Notably, however, in some assays the SFFV-related viruses converted different cells with different efficiencies (Tables 3 and 4), suggesting that every infected cell may not grow as a clone in our dilution assays. This could possibly indicate that high-level SFFV glycoprotein expression may be necessary for EpoR activation and clonal growth in these cases. In contrast, in the negative assays, conversions were never observed, indicating that they occurred at frequencies substantially lower than 10^{-4}% (Tables 3 and 4). Our conclusion that hEpoR is highly resistant to SFFV-mediated activation but is susceptible to BB6 and PvuΔ viruses is based on these criteria. By these same standards, both reciprocal hEpoR-mEpoR chimeras were activated by SFFV (Table 4 and Fig. 6). Nevertheless, we emphasize that hEpoR may be weakly activated by gp55 and that our conclusions may not apply to other cell cultures or experimental conditions. The need for caution in interpreting results obtained with these cell culture assays has also been emphasized by others (46).

General implications. Despite these experimental limitations, we believe that our results have important implications concerning the mechanisms of EpoR activation and of SFFV host range restriction. Our observation that hEpoR is resistant to activation by gp55 but is efficiently activated by BB6 and PvuΔ mutants demonstrates that hEpoR contains sites for interaction with SFFV-related *env* glycoproteins. The ecotropic sequences that are eliminated by deletions in BB6 gp42 and PvuΔ gp41 (Fig. 2) must interfere with activation of hEpoR in BaF3 cells.

It is intriguing that the same ecotropic domain deletions that overcome *Fv-2*^{rr} host range restriction in mice also overcome the block in activation of hEpoR that occurs in BaF3 cells. This raises the possibility that different SFFV host range restrictions are related and that deleting ecotropic sequences from gp55 might expand the host range to include not only *Fv-2*^{rr} mice but also other species.

The overlapping PvuΔ and BB6 mutants both encode glycoproteins that form disulfide-bonded oligomers (Fig. 6) that are processed to cell surfaces (22, 28). In contrast, many SFFV mutants that are nonpathogenic encode glycoproteins that remain as monomers in the rough endoplasmic reticulum (18, 31, 32). Our results support the hypothesis that one or more of the eight cysteines in the dual-tropic domain of gp55 may be involved in the dimerization that is critical for pathogenesis.

We were surprised that both reciprocal mEpoR-hEpoR chimeras can be activated by gp55 (Table 4 and Fig. 7) because gp55 is situated exclusively extracellularly (1, 3, 7, 60, 61) (Fig. 2), whereas one of the chimeras (pUC-HM) has mEpoR sequences only in the cytosol. This strongly suggests that a factor(s) in the BaF3 cytosol that interacts differently with hEpoR and mEpoR can critically affect activation by gp55. Compatible evidence was recently derived from studies of mEpoR–IL-3 receptor and mEpoR–IL-2 receptor chimeras (26).

Our interpretation of these results relies on evidence that EpoR consists of a transmembrane assemblage of protein subunits and associated factors including JAK2 protein tyrosine kinase (5, 12, 18, 24, 28, 39, 59) and perhaps *Fv-2*-encoded proteins (34) and that activation by Epo or gp55 probably involves dimerization, thereby changing multiple subunit interactions and activating JAK2 by transphosphorylation (2, 8, 15, 18, 58, 59). This results in tyrosine phosphorylation of the 66,000-M_r EpoR component (10, 17, 42) and in additional changes in its interactions with cytosolic factors such as Shc and Grb2 (9, 10). According to this model, the ability of gp55 or truncated derivatives to activate EpoR would depend on the dimer structure of the gp55 cell surface component (20), on the strength of its association with EpoR, on steric constraints imposed by other proteins of the assemblage, and on the energetics involved in changing multiple protein-protein interactions on both sides of the membrane. Because hEpoR and mEpoR presumably differ in their interactions with viral glycoproteins and with other proteins of the mouse BaF3 cells, the steric and energetic requirements for activation by SFFV glycoproteins or by Epo would differ for these receptors and for their reciprocal chimeras. This model of EpoR could explain how multiple factors on both sides of the membrane can influence SFFV activity. Our results are compatible with the hypothesis that one of these associated proteins is encoded by or controlled by the polymorphic *Fv-2* gene.

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