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A Friend Virus Mutant That Overcomes *Fv-2*^{rr} Host Resistance Encodes a Small Glycoprotein That Dimerizes, Is Processed to Cell Surfaces, and Specifically Activates Erythropoietin Receptors

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The *env* gene of Friend spleen focus-forming virus (SFFV) encodes a membrane glycoprotein (gp55) that is inefficiently (3 to 5%) processed from the rough endoplasmic reticulum to form a larger dimeric plasma membrane derivative (gp55^{P}). Moreover, the SFFV *env* glycoprotein associates with erythropoietin receptors (EpoR) to cause proliferation of infected erythroblasts [J.-P. Li, A. D. D'Andrea, H. F. Lodish, and D. Baltimore, Nature (London) 343:762–764, 1990]. Interestingly, the mitogenic effect of SFFV is blocked in mice homozygous for the *Fv-2*^{r} resistance gene, but mutant SFFVs can overcome this resistance. Recent evidence suggested that these mutants contain partial *env* deletions that truncate the membrane-proximal extracellular domain of the encoded glycoproteins (M. H. Majumdar, C.-L. Cho, M. T. Fox, K. L. Eckner, S. Kozak, D. Kabat, and R. W. Geib, J. Virol. 66:3652–3660, 1992). Mutant BB6, which encodes a gp42 glycoprotein that has a large deletion in this domain, causes erythroblastosis in DBA/2 (*Fv-2*^{s}) as well as in congenic D2.R (*Fv-2*^{r}) mice. Analogous to gp55, gp42 is processed inefficiently as a disulfide-bonded dimer to form cell surface gp42^{P}. Retroviral vectors with SFFV and BB6 *env* genes have no effect on interleukin 3-dependent BaF3 hematopoietic cells, but they cause growth factor independency of BaF3/EpoR cells, a derivative that contains recombinant EpoR. After binding ^{125}I-Epo to surface EpoR on these factor-independent cells and adding the covalent cross-linking reagent disuccinimidyl suberate, complexes that had immunological properties and sizes demonstrating that they consisted of ^{125}I-Epo–gp55^{P} and ^{125}I-Epo–gp42^{P} were isolated from cell lysates. Contrary to a previous report, SFFV or BB6 *env* glycoproteins did not promiscuously activate other members of the EpoR superfamily. Although the related *env* glycoproteins encoded by dualtropic murine leukemia viruses formed detectable complexes with EpoR, strong mitogenic signalling did not ensue. Our results indicate that the SFFV and BB6 *env* glycoproteins specifically activate EpoR; they help to define the glycoprotein properties important for its functions; and they strongly suggest that the *Fv-2* leukemia control gene encodes an EpoR-associated regulatory factor.

Friend viral erythroleukemia provides an excellent model for analyzing multistep leukemogenesis and the role of host genes in controlling susceptibility to an oncogenic protein (for reviews, see references 4 and 28). The *env* gene of the replication-defective virus component, the spleen focus-forming virus (SFFV), encodes a glycoprotein (gp55) that causes proliferation of late burst-forming and colony-forming erythroblasts (BFU-E and CFU-E, respectively) in susceptible strains of mice (22). gp55 is inefficiently (3 to 5%) processed from the rough endoplasmic reticulum to form a larger dimeric plasma membrane derivative (gp55^{P}) (20, 21). Recent evidence suggested that the SFFV *env* glycoprotein forms a mitogenic complex with erythropoietin receptors (EpoR), perhaps in the rough endoplasmic reticulum (34, 57) or on cell surfaces (7, 18). Another report suggested that gp55 promiscuously activates interleukin 2 receptors (IL-2R) (a member of the EpoR superfamily [2]) and that the structurally related *env* glycoproteins of dualtropic murine retroviruses (MCFs) can also activate EpoR and IL-2R (32). MCF *env* glycoproteins have been implicated in many slowly developing leukemias and lymphomas of mice (9, 11, 15, 17, 44, 53).

Friend and closely related viruses are unique in several respects. First, they are the only known retroviruses that cause rapid neoplasms but lack oncogenes derived from the normal host genome; SFFV contains only retrovirus-specific nucleic acid sequences. Second, their ability to cause erythroblastosis is specific for mice and is controlled by many mouse genes. Although some of these mouse genes restrict replication or transmission of the helper virus (e.g., *Fv-1* and *Fv-4*), the sizes of BFU-E and CFU-E cell pools (e.g., W and S1), or immunological reactions of the host (e.g., *H-2*-linked genes such as *Rfv-1* and *Rfv-2*), others appear to act specifically on SFFV (e.g., *Fv-2* and *Fv-5*), and these might possibly control interaction of gp55 with EpoR or the consequent signal transduction pathway (8, 26, 30, 47). These genes provide a unique resource for analyzing viral and cellular interactions in a pathway of oncogenesis.

The mouse *Fv-2* gene, which maps on the end of chromosome 9 opposite from the EpoR gene (6, 24), is especially interesting because it may directly modulate the EpoR-dependent pathway that is activated by Friend virus. Mice homozygous for the *Fv-2*^{r} allele are resistant to Friend virus-induced erythroblastosis, whereas heterozygotes or *Fv-2*^{s} homozygotes are susceptible. *Fv-2*^{r} homozygotes are also resistant to erythroleukemias induced by a retrovirus that encodes Epo (24), but they are susceptible to all other

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retroviral diseases, including erythroleukemias induced in newborns by the replication-competent Friend murine leukemia virus (MuLV) in the absence of SFFV (44, 49). Resistance of *Fv-2*^{r} homozygotes is not due to a block in infection or in SFFV gene expression (14, 16, 24, 54). In *Fv-2*^{ss}/*Fv-2*^{rr} chimeric mice, infection with Friend virus causes selective proliferation of the *Fv-2*^{ss} erythroblasts (3, 48).

Forced passage of Friend virus in *Fv-2*^{r} homozygotes has yielded novel viruses that cause slowly developing erythroleukemias in these mice as well as in *Fv-2*^{s} strains (19, 36). We recently found that these viruses are SFFV mutants with substantial deletions in the 3′ region of the *env* gene, which encodes the membrane-proximal extracellular domain of the glycoprotein (36). The BB6 mutant has a large 159-base *env* deletion that severely truncates this domain (36). Previously it was not known whether these novel viruses activate EpoR by a process that overcomes *Fv-2*^{r} restriction or whether they activate an alternative receptor or a mitogenic pathway that does not involve *Fv-2* (3, 12–14, 16, 19, 24, 30, 36, 41, 48, 52, 54). Recent evidence that the SFFV-MCF family of *env* glycoproteins can promiscuously activate different receptors (32, 53) is compatible with the latter possibility. In this article we present evidence that the BB6 *env* gene specifically activates EpoR. This has important implications for understanding *Fv-2* control of leukemogenesis.

MATERIALS AND METHODS

Cells. BaF3 (38) and DA-3 (25) IL-3-dependent cells and their derivatives were grown in a solution containing RPMI 1640 medium, 10% fetal bovine serum, 5×10^{-5} M 2-mercaptoethanol, and growth factors (IL-3 provided as 10% WEHI-3–conditioned medium [25], 0.5 U of recombinant mouse Epo [Boehringer-Mannheim, Indianapolis, Ind.], or human recombinant IL-2 [generous gift of Mary Collins, Institute of Cancer Research, London, England]). BaF3 and BaF3/EpoR (34) were kindly provided by Alan D'Andrea (Dana-Farber Cancer Institute, Boston, Mass.). DA-3, a gift from James Ihle (St. Jude Children's Hospital, Memphis, Tenn.), and DA-3/EpoR were described previously (24). BaF3/IL-2Rβ was made as described below. All other cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. These cells include ψ2 ecotropic packaging cells (37), PA12 amphotropic packaging cells (40), NIH 3T3 mouse fibroblasts, and IP/IR erythroleukemia cells that contain helper-free SFFV (50).

Viruses. Viruses maintained in chronically infected NIH 3T3 fibroblasts include Rauscher ecotropic MuLV (46), MCF-IE (9), MCF-13 (11), F-MCF (1) generously donated by Jing-Po Li (New York University Medical School), and the amphotropic MuLV 4070A (11).

Wild-type SFFV (Lilly-Steeves polycythemia strain) was encoded by the molecularly cloned colinear plasmid pL26K (33). A retroviral vector that expresses the BB6 mutant SFFV *env* gene was made by ligating the *Bam*HI-*Kpn*I *env* fragment into the corresponding sites of the pSFF vector (5) to produce pSBB6 (36). A retroviral vector that expresses IL-2Rβ chains was made with cDNA plasmid pIL-2Rβ30 (23). The 2.0-kbp *Pst*I fragment containing the entire coding sequence and a portion of the 3′ noncoding region was subcloned into the *Pst*I site in the symmetrical polylinker of pUC1318 (42). The resulting construct, which was a generous gift of Mary Collins (Institute of Cancer Research), was cut with *Bam*HI to release the 2.0-kbp IL-2Rβ sequence that was then ligated into the *Bam*HI site of the pSFF retroviral vector (5) to obtain pSFF-IL-2Rβ. Helper-free virions were obtained by transfecting these vectors into 1:1 cocultures of ψ2 and PA12 retroviral packaging cells as described previously for ping-pong amplifications (5, 31). A ψ2 cell clone, SBB6i21, was used to produce helper-free ecotropic virions that encode BB6 *env* gp42. BB6 cl 13 cells (36) are a line of NIH 3T3 fibroblasts transfected with pSBB6 and infected with the Friend strain of ecotropic MuLV. Viruses were obtained as culture medium harvests (31). Virus was filtered (0.45 μm pore size) and used immediately or stored in aliquots at −80°C. Cell monolayers (1×10^5 to 2×10^5 cells) in a 25-cm^2 flask or cell suspensions (1×10^6 to 2×10^6 cells) were infected with 2 or 4 ml of virus, respectively, in the presence of 8 μg of Polybrene (Sigma, St. Louis, Mo.) per ml at 37°C for 4 h.

Factor-independent growth assay. Cells were mock infected or infected with viruses and then grown for 48 h in medium containing growth factors. The cells were then washed extensively (at least three times by centrifugation and resuspension with 15 ml of phosphate-buffered saline [GIBCO-BRL, Gaithersburg, Md.]) before being replated at 2×10^5 cells per ml without growth factors. Cultures were then observed daily for 2 weeks for viability and rapid growth. Factor-independent cells were unambiguously detected within 2 to 5 days.

For many experiments, cells resuspended at 10^6 cells per ml were serially diluted (1:10) in medium without growth factors and 0.1 ml of each dilution per well was then plated into 24 wells of a 96-well culture dish. Several days later, the wells were scored for factor-independent growth. These results did not change with prolonged culturing (2 to 3 weeks). Determining the fraction of positive and negative wells for each dilution allowed us to estimate the average number of factor-independent cells plated into the wells. This was done assuming a binomial distribution and calculating the average number of factor-independent cells per well as equal to −log Po/0.44, where Po is the fraction of negative wells in the set of 24 wells (31). Knowing the average number of factor-independent cells per well and the number of cells plated at that dilution enabled us to calculate the percentage of cells that had been converted to factor independency.

Pathogenic assays. DBA/2 (*Fv-2*^{ss}, *H-2*^{d}) mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Breeder pairs of the congenic strain D2.R (*Fv-2*^{rr} *H-2*^{d}) (13) were a generous gift of Frank Lilly (Albert Einstein College of Medicine, Bronx, N.Y.). D2.R mice contain the *Fv-2*^{r} gene of C57BL/6 in the highly permissive DBA/2 genetic background. All mice were bred in the animal facility at the Terre Haute Center for Medical Education. Virus from the medium of BB6 cl 13 cells, containing Friend ecotropic MuLV plus the rescued BB6 retrovirus, was concentrated twofold and then injected in 0.5-ml amounts either diluted or undiluted into tail veins of 10- to 18-week-old mice. Spleens harvested 10 days later were fixed in Bouin's solution for 24 h, transferred to 70% ethanol, and examined for the presence of surface foci. The foci caused by BB6 virus in both DBA/2 and D2.R mice were characteristically smaller and more mottled in their margins than foci caused by wild-type SFFV in DBA/2 mice. Hematocrits and spleen weights were taken at the time of sacrifice.

Protein analyses. Protein immunoprecipitations employed a goat antiserum (made to ecotropic Friend MuLV gp70) that cross-reacts with *env* glycoproteins encoded by SFFV and MCFs (20, 45, 46). After immunoprecipitation, proteins were electrophoresed in polyacrylamide gels in the presence of

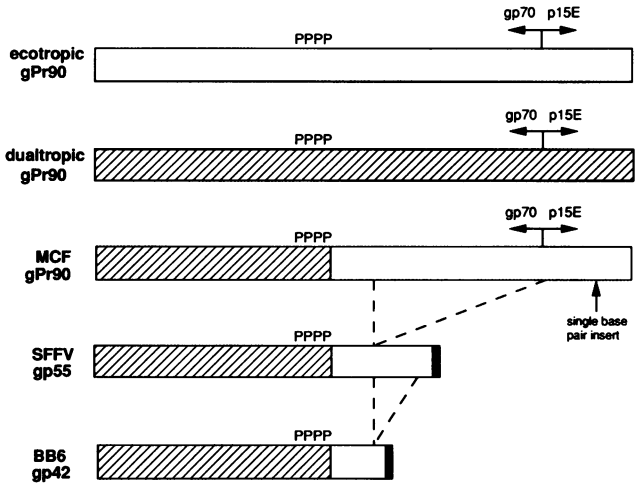

FIG. 1. Diagram of the structures of SFFV-related *env* glycoproteins. The *env* glycoproteins of replication-competent murine retroviruses are synthesized as gPr90 precursors that are cleaved by partial proteolysis to form gp70 and p15E products (for a review, see reference 28). PPPP refers to a proline-rich hypervariable sequence that is approximately 50 amino acids long (29, 35). Dualtropic *env* genes, which are endogenously inherited in mice, recombine with replicating ecotropic MuLVs in vivo to form MCFs (15, 17, 29, 44). Typically, MCFs contain a dualtropic-specific domain that extends from the amino terminus into the proline-rich region. Formation of SFFV is believed to have involved a 585-base deletion and a single base insertion in an MCF *env* gene (28). The single base insert causes a frameshift, and the resulting novel amino acid sequence is indicated by solid rectangles. This novel sequence is highly hydrophobic and serves as the site for membrane attachment of gp55 (20). The frameshift also causes early termination of translation, with loss of 33 amino acids for the carboxyl terminus. The BB6 *env* gene contains an additional 159-base deletion that removes 53 more amino acids from the ecotropic domain (36). The processed sizes of gp55 and gp42 are 409 and 356 amino acids, respectively.

0.1% sodium dodecyl sulfate, transferred onto nitrocellulose membranes, reacted with the same antiserum, and detected by autoradiography after binding ^{125}I-protein A, as previously described (20, 33).

^{125}I-Epo binding studies. ^{125}I-Epo binding to cell surfaces was measured with 3×10^6 cells washed thoroughly by centrifugation and resuspension and then incubated with gentle agitation at 20°C for 3 h in 0.1 ml of Dulbecco's modified Eagle's medium that contained 10% fetal bovine serum, 0.20% sodium azide, and 0.04 μCi of ^{125}I-Epo (300 to 900 Ci/mmol) (Amersham, Arlington Heights, Ill.). Cells were then sedimented through dibutyl phthalate oil (0.15 ml). The bottom of the tube containing the cell pellet was cut off and assayed in a gamma counter. The cell pellet was then analyzed for protein by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, Calif.). ^{125}I-Epo labeling of cells for cross-linking with disuccinimidyl suberate (Pierce, Rockford, Ill.) was done by the method of Casadevall et al. (7) with slight modifications (18).

RESULTS

Pathogenesis caused by the BB6 *env* gene. Figure 1 illustrates the structural relationships among different members of the MCF and SFFV families of recombinant *env* glycoproteins, including the glycoprotein of the BB6 virus that causes a slowly developing erythroleukemia in *Fv-2*^{r} homozygotes or in *Fv-2*^{s} mice 4 to 6 months after infection (36).

TABLE 1. Formation of spleen foci in both DBA/2 (*Fv-2*^{s}) and D2.R (*Fv-2*^{r}) congenic mice by 10 days postinfection with the BB6 variant of Friend virus[a]

BB6 virus dilution[b]	Mouse strain	Spleen wt (g)[c]	Foci[d]
Full-strength	DBA/2	0.154, 0.175	Con, Con
	D2.R	0.165, 0.192	Con, Con
10^{-1}	DBA/2	0.093, 0.125	Con, Con
	D2.R	0.102, 0.221, 0.160	Con, Con, Con
10^{-2}	DBA/2	0.182, 0.143	Con, Con
	D2.R	0.129, 0.105	Con, Con
None (no virus)	DBA/2	0.081, 0.098	0, 0
	D2.R	0.07, 0.085	0, 0

[a] Nearly identical results were obtained in an independent repeat experiment. In addition, the BB6 virus gave similar results 10 days postinfection in BALB/c mice.

[b] The BB6 *env* gene in a retroviral vector was transfected into fibroblasts that contained Friend MuLV helper virus as described in Materials and Methods. Samples (0.5 ml) of virus-containing culture medium were injected intravenously into a tail vein.

[c] Spleen weights for all mice are listed. Normal DBA/2 and D2.R mice are matched controls. Spleen weights for these normal mice rarely exceed 0.10 g. Moreover, helper virus alone causes no change in spleen weights 10 days postinfection.

[d] Foci were small and mottled and were almost completely confluent (Con) even at a dilution of 10^{-4} (data not shown). No foci occur with mice injected with helper virus alone. Wild-type SFFV also causes no foci in D2.R mice (13, 24).

To study the BB6 *env* gene, we ligated it into the pSFF retroviral vector and used rescued virions to infect DBA/2 (*Fv-2*^{s}) and congenic D2.R (*Fv-2*^{r}) mice. This specific congenic study is important because naturally occurring *Fv-2*^{r} strains contain several genes that cause resistance to Friend virus and because the DBA/2 genetic background is completely susceptible at all of these different loci (8). As shown in Table 1, the recombinant retrovirus with the BB6 *env* gene caused abundant spleen foci and mild splenomegaly within 10 days of injection at high dilutions into both DBA/2 and D2.R mice. Similar studies with wild-type SFFV indicated a complete absence of splenomegaly or spleen foci in D2.R mice (e.g., see references 13 and 24).

Activation of EpoR by *env* glycoproteins encoded by wild-type SFFV and by BB6. DA3 and BaF3 are hematopoietic cell lines that require IL-3 for survival and growth (25, 38). However, cellular derivatives that contain recombinant EpoR or IL-2Rβ survive and grow without IL-3 in the presence of Epo or IL-2, respectively (24, 32, 34). As summarized in Table 2, infection with helper-free SFFV and BB6 viruses converted DA3/EpoR and BaF3/EpoR cells to growth factor independency but had no effect on growth factor requirements of any cells that lacked EpoR. Other viruses, including MCFs, ecotropic Rauscher MuLV, and amphotropic MuLV 4070A, also did not abrogate growth factor dependencies of any of these cell lines.

Because DA3 and BaF3 cells spontaneously form growth factor-independent variants at very low frequencies (27; also our results), it was essential to determine whether the growth factor independencies indicated in Table 2 were caused by infection with SFFV and BB6 virus rather than by spontaneous conversions. Moreover, in the cases of viruses that had no effect on growth factor requirements, it was necessary to show that the virus titers were sufficient to infect a substantial number of the cells (see legend to Table 2). For the experiments with SFFV and BB6 virus, two lines

TABLE 2. Effects of retroviral *env* glycoproteins on growth factor requirements of DA-3 and BaF3 hematopoietic cells and on derivatives that contain EpoR or IL-2R

Cells type[a]	Factor(s) required[b]
DA-3	IL-3
DA3/EpoR	IL-3 or Epo
DA3/EpoR/SFFV	None
DA3/EpoR/BB6	None
DA3/SFFV	IL-3
DA3/BB6	IL-3
BaF3	IL-3
BaF3/EpoR	IL-3 or Epo
BaF3/EpoR/SFFV	None
BaF3/EpoR/BB6	None
BaF3/SFFV	IL-3
BaF3/BB6	IL-3
BaF3/IL-2Rβ	IL-3 or IL-2
BaF3/IL-2Rβ/SFFV	IL-3 or IL-2
BaF3/IL-2Rβ/BB6	IL-3 or IL-2
DA3/EpoR/MCF-IE	IL-3 or Epo
DA3/EpoR/MCF-13	IL-3 or Epo
BaF3/EpoR/MCF-1E	IL-3 or Epo
BaF3/EpoR/MCF-13	IL-3 or Epo
BaF3/EpoR/F-MCF	IL-3 or Epo
BaF3/IL-2Rβ/F-MCF	IL-3 or IL-2
BaF3/EpoR/R-MuLV	IL-3 or Epo
BaF3/EpoR/4070A	IL-3 or Epo

[a] The DA-3 and BaF3 cell derivatives that express hemopoietin receptors and/or SFFV or BB6 virus are indicated. These cell derivatives were prepared as described in Materials and Methods. Success with infections with SFFV and BB6 virus was established as shown in Table 3. The infections with MCFs, Rauscher ecotropic MuLV (R-MuLV), and amphotropic MuLV 4070A were shown to be efficient by immunofluorescent detection of cell surface gp70s and, in the case of MCF, by immunoblotting (see Materials and Methods).

[b] Cells that are growth factor dependent die within 1 to 2 days when removed from the factor. Factor-independent cells survive and proliferate in culture medium without any supplements.

TABLE 3. Factor-independent growth assay of BaF3, BaF3/EpoR, and BaF3/IL-2Rβ cells infected with SFFV and BB6 and F-MCF viruses

Expt	Cell type[a,b]	No. of wells with factor-independent cells[a]				% of factor-independent cells[c]
		10,000	1,000	100	10	
1	BaF3/SFFV	0	0	0	0	0
	BaF3/EpoR/SFFV	24	24	21	2	2
	BaF3/BB6	0	0	0	0	0
	BaF3/EpoR/BB6	24	24	24	19	15
	BaF3/F-MCF	0	0	0	0	0
	BaF3/EpoR/F-MCF	0	0	0	0	0
2	BaF3/EpoR	0	0	0	0	0
	BaF3/EpoR/SFFV	24	16	3	0	0.1
	BaF3/EpoR/BB6	24	24	23	9	4.5
	BaF3/EpoR/F-MCF	0	0	0	0	0
	BaF3/IL-2Rβ	0	0	0	0	0
	BaF3/IL-2Rβ/SFFV	0	0	0	0	0
	BaF3/IL-2Rβ/BB6	0	0	0	0	0
	BaF3/IL-2Rβ/F-MCF	0	0	0	0	0

[a] Cells were infected with viruses and maintained in medium with growth factors for 48 h (see Materials and Methods). Cells were pelleted by centrifugation, washed extensively, and resuspended (1×10^6 cells per ml) in medium without growth factors. The cell suspension was serially diluted 1:10, and 0.1-ml aliquots of each dilution were placed into 24 wells of a 96-well culture plate. The number of cells seeded per well is shown directly above each column of data. The data show the number of these wells that contained factor-independent cells.

[b] In studies with F-MCF virus, the infections were efficient as determined by immunofluorescent detection of gp70.

[c] The percentage of cells that were growth factor-independent was calculated from the number of negative wells by using binomial distribution (see Materials and Methods).

of evidence were obtained. First, as illustrated by two experiments in Table 3, substantial numbers of DA3/EpoR and BaF3/EpoR cells that were exposed to our preparations of SFFV and BB6 virus (i.e., 0.1 to 15% of the cells) became converted to growth factor independency. This was determined by removing growth factors and by plating different numbers of the cells into small cultures that were later assayed for cell proliferation. Each dilution of the cells was plated into 24 separate wells, and the data in Table 3 show the number of these wells that contained factor-independent cells. In contrast to these efficient effects of SFFV and BB6 virus on DA3/EpoR and BaF3/EpoR cells, these same virus preparations clearly had no effect on the cell lines that lacked EpoR. Indeed, no spontaneous conversion to growth factor independency was seen in these experiments, suggesting that the rate of spontaneous conversion was less than 10^{-6}. Thus, the effects of SFFV and BB6 virus were rapid, efficient, specific to cells that contain EpoR, and reproducible. Second, the factor-independent cell clones that formed in the cultures were all infected with SFFV and BB6 virus, as indicated by their synthesis of viral *env* glycoproteins. Representative protein blot analyses of these *env* glycoproteins are shown in Fig. 2. Although our DA3 and BaF3 cells all contained an endogenous *env* glycoprotein with an approximate M_r of 85,000, factor-independent cells from the cultures infected with SFFV (Fig. 2A, lane 3, and Fig. 2C, lanes 6 to 10) all contained gp55 and those from the cultures infected with BB6 (Fig. 2A, lanes 4 to 6, Fig. 2B, lanes 2 to 4, and Fig. 2C, lanes 1 to 5) all synthesized gp42. These results strongly suggest that SFFV and BB6 virus specifically interact with EpoR to generate a mitogenic signal.

gp55 and gp42 glycoproteins are processed to cell surfaces, where they complex with EpoR. Glycoprotein gp55 is inefficiently processed from the rough endoplasmic reticulum, and only 3 to 5% reaches cell surfaces (20, 21, 28, 33). The latter component (called $gp55^P$) has large complex N-linked oligosaccharides that contain sialic acid, fucose, and galactose residues plus an O-linked oligosaccharide that is also absent from intracellular gp55 (20, 45). Consequently, $gp55^P$ has a slower electrophoretic mobility than gp55. As shown in Fig. 2, $gp55^P$ was detected in the factor-independent cell lines that had been infected with SFFV. Similarly, a version of gp42 (called $gp42^P$) occurred in the extracts of cells that had been infected with BB6 virus. This observation is compatible with other evidence (see below) that gp42 is partially processed to cell surfaces.

It was previously shown that gp55 forms heterogeneous disulfide bonds in the rough endoplasmic reticulum and that only one disulfide bonded dimer is competent for processing to cell surfaces (20, 21). To determine whether $gp42^P$ also occurs in disulfide-bonded complexes, we compared its electrophoretic mobilities in the presence and absence of a reducing agent. As shown in Fig. 3A, $gp55^P$ and $gp42^P$ components were both evident when the protein samples were reduced prior to electrophoresis. However, these processed glycoproteins migrated as a higher-M_r aggregate(s) in the absence of a reducing agent (Fig. 3B). On the basis of their apparent M_rs, two-dimensional electrophoretic properties, and cross-linking compositions (7, 20, 21), these higher-M_r components appear to be dimers.

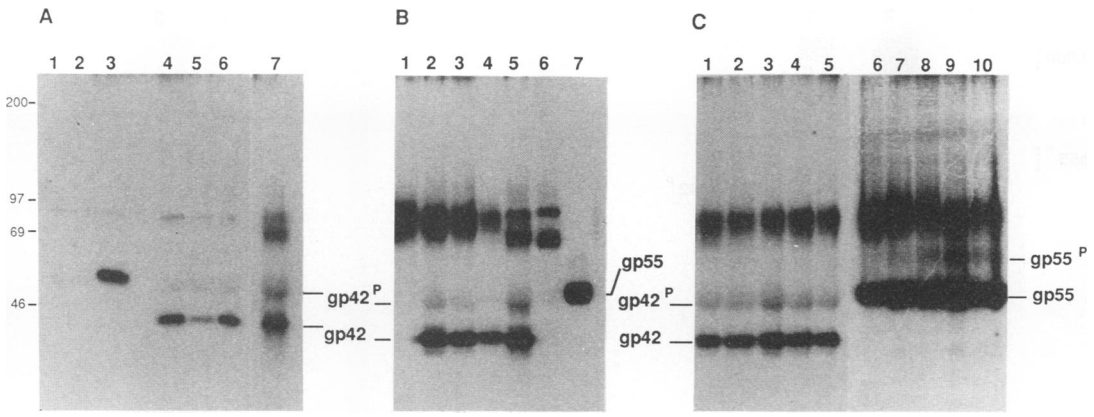

FIG. 2. SFFV and BB6 *env* glycoproteins synthesized in growth factor-independent derivatives of DA3/EpoR and BaF3/EpoR hematopoietic cells. Cell lysates were analyzed by immunoprecipitation followed by electrophoresis and then by blotting and immunodetection with an *env* antiserum (see Materials and Methods). Independently isolated growth factor-independent cultures expressing SFFV gp55 (panel A, lane 3, DA3/EpoR/SFFV cells) or BB6 gp42 (panel A, lanes 4 to 6, DA3/EpoR/BB6 cells, and panel B, lanes 2 to 4, BaF3/EpoR/BB6 cells) are shown. Single cell clones of BaF3/EpoR/BB6 from a factor-independent culture all expressed gp42 and the larger processed form $gp42^P$ (panel C, lanes 1 to 5). Similarly, all single cell clones of BaF3/EpoR/SFFV contained gp55 and $gp55^P$ (panel C, lanes 6 to 10). Other cell lines shown are control cells DA3, DA3/EpoR, and BaF3/EpoR (panel A, lanes 1 and 2, and panel B, lane 1, respectively); SBB6i21, a ψ2 ecotropic packaging clone producing BB6 virus (panel A, lane 7, and panel B, lane 5); ψ2 (panel B, lane 6); and an erythroleukemia cell line (IP/IR) that contains SFFV but no helper virus (panel B, lane 7). Additional proteins detected by our *env* antiserum are an *env* protein in BaF3 cells and their derivatives with an approximate M_r of 85,000 and gPr90 and gp70 in ψ2 and SBBi21 cells. Molecular mass standards were included in all polyacrylamide gel electrophoresis studies. Sizes of molecular mass standards in kilodaltons are shown on the left in panel A.

Recently, Casadevall et al. (7) bound ^{125}I-Epo to EpoR on surfaces of Friend erythroleukemia cells and used covalent cross-linking reagents to study its proximity to other cell surface proteins. Interestingly, their results suggested that ^{125}I-Epo could be cross-linked to $gp55^P$ and to $gp55^P$ dimers, implying that $gp55^P$ might occur in a ternary complex with Epo and EpoR on cell surfaces. To address this issue in a system that includes negative control cells, we first analyzed the binding of ^{125}I-Epo onto our BaF3, BaF3/EpoR, BaF3/EpoR/SFFV, and BaF3/EpoR/BB6 cell lines. Results for two experiments in which an excess amount of ^{125}I-Epo was adsorbed onto surfaces of these cell lines are shown in Table 4. Clearly, ^{125}I-Epo binds extensively and similarly to all of the cells that have EpoR but negligibly to the control BaF3 cells that lack these receptors. The presence of SFFV and BB6 *env* glycoproteins did not significantly reduce adsorption of ^{125}I-Epo onto the cells in the conditions of our studies. We then performed ^{125}I-Epo cross-linking studies with our BaF3/EpoR, BaF3/EpoR/SFFV, and BaF3/EpoR/BB6 cell lines. After cross-linking, the cell extracts were incubated with an antiserum reactive with gp55 and the immunoprecipitated proteins were analyzed by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate. As shown in Fig. 4, ^{125}I-Epo-containing

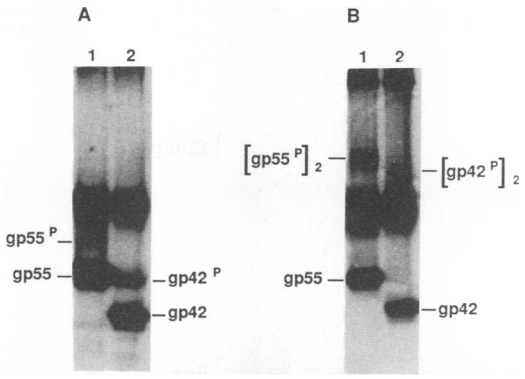

FIG. 3. Effects of reduction on *env* glycoproteins from growth factor-independent BaF3/EpoR/SFFV and BaF3/EpoR/BB6 cell lines. The glycoproteins were immunoprecipitated from cell lysates and were then resolved by electrophoresis followed by transfer to nitrocellulose and detection by immunoblotting. These factor-independent cell lines, which were obtained as described in Results, synthesize gp55 (lanes 1) and gp42 (lanes 2), respectively. When the samples were treated with a reducing agent (A), the more highly processed derivatives ($gp55^P$ in lane 1 and $gp42^P$ in lane 2) were clearly evident. However, when the samples were electrophoresed without reduction, $gp55^P$ and $gp42^P$ selectively electrophoresed as higher-M_r derivatives with apparent M_rs expected for dimers ([$gp55^P$] and [$gp42^P$]; panel B, lanes 1 and 2, respectively).

TABLE 4. Binding of ^{125}I-Epo to hematopoietic cells that contain EpoR plus SFFV or BB6 virus

Expt	Cell type	^{125}I-Epo bound[a] (cpm/mg of protein)
1	BaF3	740, 750, 630
	BaF3/EpoR	61,000, 50,000, 51,000
	BaF3/EpoR/SFFV	56,000, 58,000, 56,000
	BaF3/EpoR/BB6	40,000, 45,000, 41,000
2	BaF3	590, 495, 670
	BaF3/EpoR	48,000, 38,000, 35,000
	BaF3/EpoR/SFFV	28,000, 30,000, 28,500
	BaF3/EpoR/BB6	50,000, 51,000, 43,000

[a] ^{125}I-Epo (0.04 μCi) (300 to 900 Ci/mmol) was incubated at 20°C with 3×10^6 cells in a 0.1-ml volume of Dulbecco's modified Eagle's medium that contained 10% fetal bovine serum and 0.2% sodium azide. After 3 h, the cells were removed from unadsorbed Epo by centrifugation through dibutyl phthalate oil. The pelleted cells were then assayed for radioactivity and for protein. Each cell was analyzed in triplicate, and all results are included.

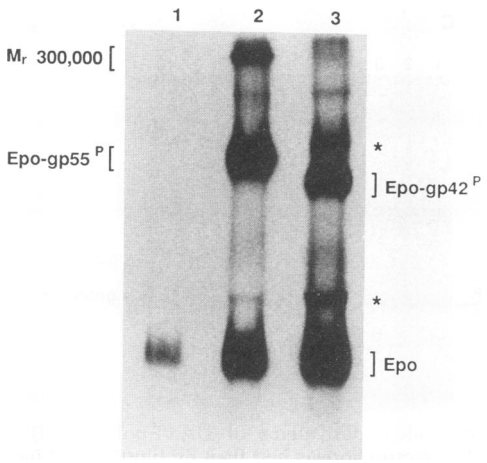

FIG. 4. Cross-linking of ^{125}I-Epo to *env* glycoproteins on the surfaces of hematopoietic cells. ^{125}I-Epo, which adsorbed equally onto BaF3/EpoR, BaF3/EpoR/SFFV, and BaF3/EpoR/BB6 cells (Table 4), was cross-linked to cell surface proteins by incubation with the cross-linking reagent disuccinimidyl suberate in conditions that prevent endocytosis of ^{125}I-Epo (7). Cell extracts were then prepared and used for immunoprecipitations with antiserum that reacts with *env* glycoproteins. The immunoprecipitates were then dissolved by boiling in a buffer that contains 0.1% sodium dodecyl sulfate, and the proteins were analyzed by electrophoresis in polyacrylamide gels. The radioactive ^{125}I-Epo-containing components were then detected by autoradiography. Shown is a film exposed for 1 week. Lanes 1 to 3 are BaF3/EpoR, BaF3/EpoR/SFFV, and BaF3/EpoR/BB6 cells, respectively. The asterisks identify components selectively enriched in the cross-linked product of BaF3/EpoR/BB6 cells (lane 3). The component with an M_r of 300,000 is reproducibly and selectively decreased in this same sample.

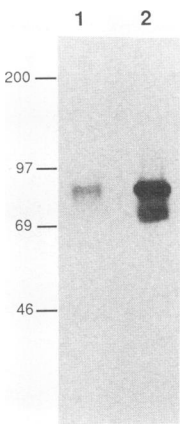

FIG. 5. Analysis of *env* glycoproteins synthesized in BaF3/EpoR cells (lane 1) and in BaF3/EpoR/F-MCF cells (lane 2). The BaF3 cell line contains an endogenous *env*-related glycoprotein that has an apparent M_r of approximately 85,000 (see lane 1 and other figures). After superinfection with F-MCF, these cells synthesized the MCF *env* gPr90 precursor and gp70 product glycoproteins (see lane 2). The BaF3/EpoR/MCF cells also contained MCF gp70-p15E complexes on their surfaces as detected by immunofluorescence, and they released virus with the expected MCF-specific host range. Sizes of molecular mass standards in kilodaltons are on the left of the panel.

complexes were detected in the extracts of the factor-independent cells. The major complexes from the BaF3/EpoR/SFFV and BaF3/EpoR/BB6 cells (lanes 2 and 3) had apparent M_rs of 105,000 and 90,000, respectively, which are consistent with the M_rs of gp55^P (65,000 to 70,000) and gp42^P (50,000) plus ^{125}I-Epo (37,000). We conclude that both gp55 and gp42 are processed to cell surfaces, where they form complexes with EpoR.

Other features of these cross-linking results are noteworthy. First, non-cross-linked ^{125}I-Epo reproducibly occurs in our anti-envelope glycoprotein immunoprecipitates from BaF3/EpoR/SFFV and BaF3/EpoR/BB6 cells in much larger amounts than in the precipitates from BaF3/EpoR negative control cells (Fig. 4; compare lane 1 with lanes 2 and 3). This implies that some non-cross-linked ^{125}I-Epo–EpoR–gp55^P (or gp42^P) ternary complexes occur in the cell extracts in a form that is sufficiently stable for immunoprecipitation. Second, we reproducibly detect other cross-linked components (indicated by asterisks in Fig. 4) in substantially larger amounts in the complexes from the BB6-infected cells compared with the SFFV-infected cells (compare lanes 2 and 3). Conversely, a cross-linked component with an apparent M_r of 300,000 occurs in smaller amounts in the complexes from the BB6-infected cells.

Interaction of MCF *env* glycoproteins with EpoR. We were able to efficiently infect BaF3/EpoR and BaF3/IL-2Rβ cells with MCFs as determined by immunofluorescent detection of the MCF *env* glycoproteins on cell surfaces (i.e., approximately 90% of the cells contained gp70). Protein immunoblotting of MCF-infected BaF3/EpoR cells also clearly showed the presence of the expected MCF *env* glycoproteins in the cells (Fig. 5, lane 2). However, these infected cells all died when deprived of growth factors (Tables 2 and 3).

Nevertheless, MCF gp70 seems to form a relatively small amount of a complex with ^{125}I-Epo and EpoR on the surfaces of cells (Fig. 6, lane 2). The cross-linked component that was immunoprecipitated by antiserum to *env* glycoproteins has an apparent M_r of 110,000, which is compatible

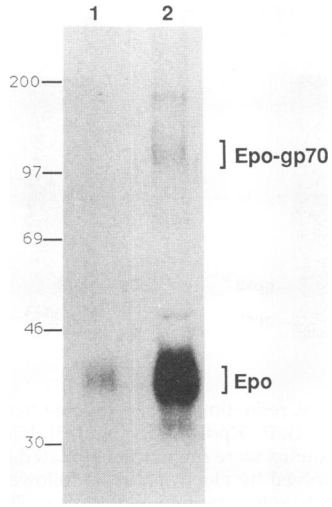

FIG. 6. Cross-linking of ^{125}I-Epo to *env* glycoproteins on the surfaces of cells that contain EpoR plus MCF-specific *env* glycoproteins. The cells used for this study were BaF3/EpoR and BaF3/EpoR/F-MCF (lanes 1 and 2, respectively). The cells were cross-linked with disuccinimidyl suberate and analyzed as described in the legend to Fig. 4. This autoradiograph resulted from a 2-week exposure of the film. Sizes of molecular mass standards in kilodaltons are on the left of the panel.

with the expected size of ^{125}I-Epo–gp70 (Epo electrophoreses with an apparent M_r of 37,000 in our gels). Moreover, the presence of a relatively large amount of ^{125}I-Epo in this lane suggests that non-cross-linked ternary complexes of ^{125}I-Epo–EpoR–gp70 were also present in the cell extracts and were precipitated by the antiserum. Thus, the failure of MCFs to cause survival and proliferation of BaF3/EpoR cells is apparently not due to an inability of MCF gp70 to interact with EpoR. However, the quantity of the cross-linked ^{125}I-Epo–gp70 complex is much lower (ca. 10- to 20-fold less) than the amounts of the complexes reproducibly observed when we use cells that contain gp55^P or gp42^P.

DISCUSSION

SFFV and BB6 *env* glycoproteins activate EpoR. The SFFV and BB6 *env* glycoproteins behave very similarly by all of the criteria we have employed. In particular, they both form dimers that are processed to cell surfaces, where they complex with EpoR (Fig. 3 and 4), and they both specifically activate EpoR to cause factor-independent survival and proliferation of BaF3/EpoR (but not of BaF3 or BaF3/IL-2R) cells (Tables 2 and 3) and erythroblastosis in vivo (36) (Table 1). Because of the structural differences between these glycoproteins (Fig. 1), we infer that these properties do not require the membrane-proximal ecotropic region that is deleted from gp42. However, this ecotropic region must contain a site(s) for negative control by the mouse *Fv-2* locus. These results are compatible with site-directed mutagenesis data, which implied that the dualtropic-specific amino-terminal domain of gp55 is critical for leukemogenesis and that the membrane-proximal ecotropic region is expendable (33). The transmembrane region of gp55, which is retained in gp42, also is important for erythroblastosis (10, 28, 55, 59).

Although gp55-EpoR complexes occur in the rough endoplasmic reticulum (34, 57) as well as on cell surfaces (7), previous genetic and biochemical evidence has implied that the processed plasma membrane gp55^P component may be necessary for pathogenesis (28, 33, 35). SFFV *env* mutants that cause erythroblastosis all encode gp55^P-related components that form cell surface complexes with EpoR (18). The severely truncated but pathogenic BB6 *env* glycoprotein similarly forms a cell surface complex with EpoR (Fig. 4).

Specificity of viral activation of EpoR. It was recently reported that both SFFV and MCFs promiscuously cause factor-independent conversion of both BaF3/EpoR and BaF3/IL-2R cells (32). Moreover, a weak mitogenic effect of an MCF virus was reported for an IL-2-dependent line of rat lymphoma cells (53). In the latter case, MCF infection and withdrawal of IL-2 were followed by a long period of survival with some cell death and with only slight cell growth, during which selection of factor-independent cell clones gradually occurred. In apparent contrast, we find a strong mitogenic effect of SFFV and BB6 that is specific to BaF3/EpoR cells. Although our cross-linking studies suggest a weak interaction of EpoR with MCF *env* glycoproteins on surfaces of BaF3/EpoR/MCF cells (Fig. 6), MCFs do not have a detectable mitogenic effect on BaF3/EpoR or BaF3/IL-2R cells in the conditions of our experiments (Tables 2 and 3).

We believe that the apparent differences between our results and the previous reports must be due to differences in the thresholds of mitogenic signalling that are scored as positive in our respective assays and to the selection conditions that were used. Thus, we infer from our results that activation of EpoR or IL-2R by MCFs or of IL-2R by SFFVs must be much weaker than the activation of EpoR by SFFVs (Tables 2 and 3). Nevertheless, weak receptor activations in the earlier studies could have become amplified by acquisition of viral and/or cellular mutations during the relatively long selection processes that were employed (32, 53). Indeed, there is evidence in both of those reports (32, 53) that changes in the surviving cells did occur during their selections of factor-independent cells. For example, in their work with MCFs, Li and Baltimore (32) found that their factor-independent cell lines contained small *env* glycoproteins similar in size to gp55, whereas our MCF-infected BaF3/EpoR cells contain only the expected gPr90 *env* precursor and gp70 product glycoproteins (Fig. 5). They proposed that MCF *env* mutations might have occurred in their cells. Tsichlis and Bear (53) also clearly observed extensive clonal changes throughout their selection process. Second, in the previous studies with MCFs (and with SFFV activation of IL-2R) (32, 53), it was not shown that the infected cells were efficiently or rapidly converted to factor independency. In contrast, we infected the BaF3/EpoR cells with MCFs and established that the cells were efficiently infected (e.g., see Tables 2 and 3 and Fig. 5) before removing the growth factor. The resulting massive cell death indicated unambiguously that the MCF-infected cells were not all factor independent. Similarly, our titers of SFFV and BB6 were sufficient to infect substantial fractions of the BaF3/IL-2R cells, yet none of these cells survived without growth factors (Table 3). Third, after infecting BaF3/EpoR cells with viruses, we maintained them in the presence of Epo for 48 h before testing them for growth factor independency, whereas Li and Baltimore (32) maintained their cells in IL-3 during the interim period. In our experience, Epo removal results in rapid death of factor-dependent cells. On the contrary, cells removed from IL-3 endure for much longer times and can even grow slowly for several days. When we use their procedure, we occasionally find small numbers of factor-independent cells after 10 days. We believe that formation and selection of these cells occur during the selection process in low concentrations of IL-3.

On the bases of these considerations and the pathogenic effects of these viruses, we suggest that MCF *env* glycoproteins can only weakly activate hemopoietin receptors and that SFFVs strongly activate only EpoR. Thus, in agreement with our results, SFFVs specifically cause proliferation of late BFU-E and CFU-E in vivo and in bone marrow cultures, without rapid effects on other lineages (22, 43); this specifically cannot be ascribed to the SFFV enhancer (14, 51, 56). Similarly, MCFs do not cause erythroblast growth in bone marrow cultures or rapid diseases of multiple hematopoietic lineages in vivo (1, 9, 11, 15, 17, 25, 29, 44, 55). As described above, a weak activation of hemopoietin receptors by MCFs could lead to cell survival and to selection of cellular and viral mutants that favor neoplastic progression. The earlier results of Li and Baltimore (32) and Tsichlis and Bear (53) appear to be compatible with this interpretation.

Mechanism of *Fv-2*^{r} restriction of Friend erythroleukemia. Our results indicate that the BB6 mutant, which causes erythroblastosis in *Fv-2*^{r} homozygotes, acts by the same mechanism as wild-type SFFV. In both cases, the *env* glycoproteins associate with EpoR to induce constitutive mitogenesis. Our evidence that this mitogenic activation is specific to EpoR excludes the involvement of other receptors or pathways in BB6 escape from *Fv-2*^{r} restriction.

We emphasize that our analysis of BB6 activation of EpoR was done with cells (BaF3 and DA3) from *Fv-2*^{s} homozygous

mice (24, 38). This should not cause any problems because SFFV mutants that overcome *Fv-2*^{r} restriction cause indistinguishable diseases in *Fv-2*^{s} or *Fv-2*^{r} mice, almost certainly by the same mechanism (3, 13, 16, 19, 36, 48). Furthermore, stable IL-3-dependent cell lines have not yet been made from *Fv-2*^{r} homozygous mice (34a).

Our data enable us to exclude several explanations for *Fv-2*^{r} restriction. For example, it previously seemed possible that *Fv-2*^{r} homozygotes might have a deficiency or dampening in a step in the mitogenic response pathway that is physically removed and subsequent (i.e., downstream) to the EpoR activation step. Similarly, it seemed possible that *Fv-2*^{r} homozygotes might be resistant to viral pathogenesis because of a reduction in the quantity of EpoR (e.g., due to a decrease in EpoR synthesis or an increase in its degradation). However, both of these ideas would lead to the prediction that SFFV mutants able to overcome *Fv-2*^{r} restriction would have to be superactivators of EpoR. On the contrary, the BB6 mutant is much less rather than more pathogenic than SFFV in *Fv-2*^{s} mice (36). The disease caused by SFFV in *Fv-2*^{s} mice involves massive splenomegaly and polycythemia by 14 to 21 days postinfection, whereas the BB6 virus causes a relatively indolent and slowly developing disease in *Fv-2*^{s} or *Fv-2*^{r} strains, with massive splenomegaly occurring only after 4 to 6 months.

Consequently, we infer that *Fv-2*^{r} restriction directly affects the mitogenic activation of EpoR by SFFV-related *env* glycoproteins. The following two models seem plausible. (i) The *Fv-2*^{s} allele causes a posttranslational modification of EpoR that amplifies the signal resulting from its interaction with Epo and that is required for its activation by SFFV *env*, whereas the truncated BB6 *env* glycoprotein can activate the unmodified EpoR. This model is compatible with the genetic dominance of *Fv-2*^{s} and with other evidence regarding *Fv-2*^{r} restriction (e.g., see references 3, 24, and 52). However, it is difficult to reconcile with conflicting evidence that the bone marrow microenvironment of *Fv-2*^{r} homozygotes may inhibit erythroblastosis (12, 14, 41, 54). (ii) The *Fv-2*-encoded proteins could associate with EpoR to control its activation by various ligands including gp55^P and Epo (24). According to a simple version of this model, the restriction of SFFV pathogenesis might be due to steric interference between the "ecotropic bulge" in gp55^P and the *Fv-2*^{r} protein; gp42^P lacks this bulge and consequently would activate EpoR (Fig. 1). Presumably, the *Fv-2*^{s} protein would differ from the *Fv-2*^{r} protein at the site of interference, thereby allowing both gp55^P and gp42^P to activate EpoR. However, the MCF *env* glycoprotein, which has an even larger ecotropic domain (Fig. 1), would be inhibited by both *Fv-2*^{s} and *Fv-2*^{r} proteins (55). This model is compatible with *Fv-2*^{s} dominance in heterozygotes and with evidence that *Fv-2*^{r} proteins might actively inhibit mitogenesis (52). Moreover, it is known that EpoR is associated with many accessory proteins (7, 39, 58) and that some of these proteins, such as p85 and p100 subunits, can be displaced by gp55^P (7). Some of these proteins might occur in the bone marrow microenvironment as extracellular factors that adsorb onto the EpoR (12, 14, 41). Our results suggest that several accessory proteins in the EpoR complex are differentially displaced by gp55^P and by gp42^P (Fig. 4), which is consistent with this model for *Fv-2*^{r} restriction.

Although additional studies will be required to identify *Fv-2* proteins and to understand their mechanism of action, both of the plausible models described in the previous paragraph suggest that these proteins interact closely with EpoR to control its activation by the SFFV family of *env* glycoproteins. Consequently, this system will very likely provide a mouse genetic model for analyzing the role of accessory proteins in hemopoietin receptor function. Moreover, it may enable us to understand at the molecular level how a host gene controls activity of an oncogenic protein.

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