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Fusion of the Erythropoietin Receptor and the Friend Spleen Focus-Forming Virus gp55 Glycoprotein Transforms a Factor-Dependent Hematopoietic Cell Line

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The Friend spleen focus-forming virus (SFFV) gp55 glycoprotein binds to the erythropoietin receptor (EPO-R), causing constitutive receptor signaling and the first stage of Friend erythroleukemia. We have used three independent strategies to further define this transforming molecular interaction. First, using a retroviral selection strategy, we have isolated the cDNAs encoding three fusion polypeptides containing regions of both EPO-R and gp55. These fusion proteins, like full-length gp55, transformed the Ba/F3 factor-dependent hematopoietic cell line and localized the transforming activity of gp55 to its transmembrane domain. Second, we have isolated a mutant of gp55 (F-gp55-M1) which binds, but fails to activate, EPO-R. We have compared the transforming activity of this gp55 mutant with the EPO-R–gp55 fusion proteins and with other variants of gp55, including wild-type polycythemia Friend gp55 and Rauscher gp55. All of the fusion polypeptides and mutant gp55 polypeptides were expressed at comparable levels, and all coimmunoprecipitated with wild-type EPO-R, but only the Friend gp55 and the EPO-R–gp55 fusion proteins constitutively activated wild-type EPO-R. Third, we have examined the specificity of the EPO-R–gp55 interaction by comparing the differential activation of murine and human EPO-R by gp55. Wild-type gp55 had a highly specific interaction with murine EPO-R; gp55 bound, but did not activate, human EPO-R.

The Friend and Rauscher murine leukemia virus complexes induce a rapid and fatal erythroleukemia in adult mice (4, 30). It is the replication-defective spleen focus-forming virus (SFFV) contained in these complexes that initiates the disease. Both Friend and Rauscher SFFV encode a 55-kDa glycoprotein, gp55, which is necessary and sufficient for the initiation of leukemia (1). Friend SFFV gp55 (F-gp55), encoded by the polycythemia-inducing strain of SFFV, has recently been shown to bind to the normal erythropoietin receptor (EPO-R) expressed in infected erythroblasts, resulting in constitutive activation of EPO-R, EPO-independent growth of the infected cells, and the first stage of Friend erythroleukemia (4, 17, 22).

Several features of the F-gp55 polypeptide affect its leukemogenic activity. F-gp55 is transport defective, with the majority of its expression remaining intracellular (16, 19, 35, 40). Its cell surface form appears to be a prerequisite for its leukemogenicity (21). Also, it is the transmembrane region of F-gp55 that is required for its ability to generate EPO-independent erythroblasts in vivo (8, 37, 38).

The precise molecular interaction between F-gp55 and EPO-R is poorly understood. Most of the EPO-R–gp55 complex is formed in the endoplasmic reticulum of infected cells (41), although cross-linked complexes of EPO-R and F-gp55 have also been detected at the cell surface (7). In addition, the transmembrane region of EPO-R is critical for its interaction with gp55, and at least one binding site for gp55 is present in the extracytoplasmic region of EPO-R (44). Furthermore, the molecular interactions between EPO-R and other naturally occurring gp55 variants, such as the anemia variant [F-gp55 (FVA)] (8) or the Rauscher variant (R-gp55) (6), have not been previously described.

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In this current study, we have taken three independent approaches to further define the molecular interaction between gp55 and EPO-R that causes transformation. First, we have used a retroviral selection strategy to generate fusion proteins, containing regions of both EPO-R and gp55, which have transforming activity. The fusion proteins, like full-length gp55, activate a coexpressed wild-type EPO-R, resulting in constitutive growth of Ba/F3 cells. The fusion proteins, expressed in the absence of wild-type EPO-R, have no transforming activity. In addition, the fusion proteins localized the EPO-R binding and activation domains of gp55 to amino acids 329 to 409, including the transmembrane domain. Surprisingly, the dualtropic *env*-specific sequences of gp55 were completely deleted from the fusion proteins, without loss of transforming activity.

Second, we have isolated a mutant of gp55 which binds EPO-R but fails to transform cells. We have compared this mutant (F-gp55-M1) with wild-type F-gp55, R-gp55, and EPO-R–gp55 fusion proteins. F-gp55 binding to EPO-R is therefore necessary but not sufficient for EPO-R activation.

Third, we have analyzed the species specificity of the interaction between gp55 and EPO-R. The functional interaction between F-gp55 and murine EPO-R (mEPO-R) was highly specific. F-gp55 and the fusion proteins bound, but failed to activate, human EPO-R (hEPO-R), even though the murine and human EPO-R polypeptides share 82% amino acid identity (18, 39).

MATERIALS AND METHODS

Cell culture and transfection. Culture conditions of interleukin-3 (IL-3)-dependent, EPO-dependent, and factor-independent murine Ba/F3 cells have been previously described (14, 43). Ba/F3 is a murine pro-B-lymphocyte line that has an absolute dependence on IL-3 for growth (22). Expression of

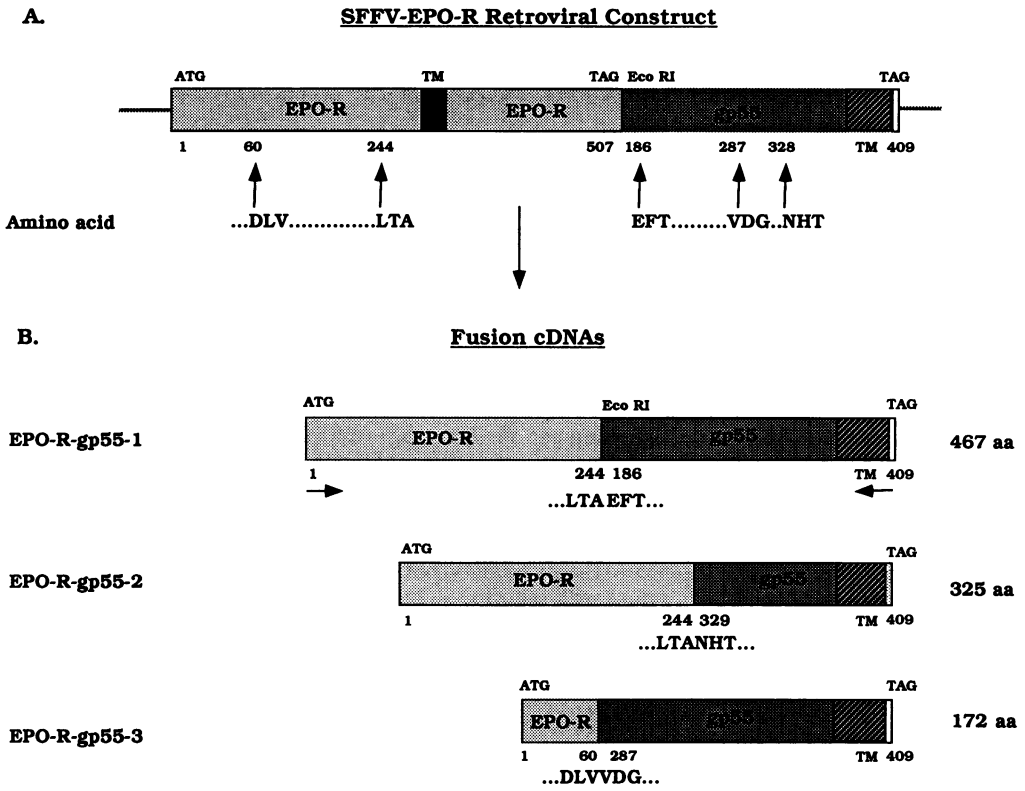

FIG. 1. EPO-R-gp55 fusion proteins result from internal deletions of the EPO-R and gp55 coding regions. (A) Schematic of the original retroviral vector (SFFV-EPO-R) containing the full-length wild-type EPO-R cDNA and the 3′ region of the F-gp55 cDNA. EPO-R amino acids (light shading), gp55 sequences (darker shading), the EPO-R transmembrane domain (black shading), and the gp55 transmembrane region (hatched area) are indicated. Amino acid positions are indicated by numbers below the boxes. The capital letters below the boxes represent amino acid sequences. (B) Schematic of three fusion cDNAs, isolated by PCR of genomic DNA by using a 5′ primer for the EPO-R and a 3′ primer for the F-gp55. The shadings are as in panel A. Primer positions are indicated by arrows at the bottom of the first fusion protein. The capital letters below the boxes represent amino acid (aa) sequences at the sites of fusion.

wild-type EPO-R alone in Ba/F3 cells confers EPO-dependent growth. Expression of EPO-R and gp55 in Ba/F3 cells confers factor-independent growth. Cultures of murine Psi-2, PA12, and PA317 packaging cells were maintained in Dulbecco modified Eagle medium with 10% fetal calf serum (FCS). Cultures of murine E86 ecotropic producer cells (27) were maintained in Dulbecco modified Eagle medium with 6% fetal calf serum and 4% calf serum.

Retroviral infection strategy and isolation of factor-independent Ba/F3 cells. The SFFV-EPO-R construct (Fig. 1) was stably cotransfected into Psi-2 ecotropic packaging cells with pSV2*neo* and selected in G418 (1.5 mg/ml) for 10 to 14 days. Individual G418-resistant colonies were subcloned and screened for expression of EPO-R (14, 44). Psi-2 subclones were mixed with either PA12 or PA317 amphotropic packaging cells to amplify retroviral titers (5).

After 3 to 7 days, viral supernatants from the packaging cell mixture were used to infect Ba/F3 cells (5×10^5) growing in RPMI 1640 medium supplemented with WEHI-conditioned medium (mIL-3 medium) (14). Forty-eight hours after infection, the cells were washed twice with Hanks balanced salt solution–20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.4) and resuspended in RPMI 1640 with 10% FCS without supplemental EPO or IL-3. Factor-independent subclones were isolated by limiting dilution, and individual subclones were expanded and analyzed for the expression of EPO-R- or gp55-related polypeptides. No factor-independent Ba/F3 cells were isolated from mock-infected Ba/F3 cultures.

pLXSN (28, 36) constructs expressing gp55 variant polypeptides (see below) were transfected into E86 packaging cells (27) by the calcium phosphate precipitation method (32). Following 14 days of selection in G418 (1.5 mg/ml), G418-resistant E86 clones were isolated (14). After the E86 subclones had grown to confluence (5 days), helper-free viral supernatants were used to infect Ba/F3 cells (5×10^5). Infected Ba/F3 subclones were selected in G418-supplemented medium (14) and assayed for factor-dependent growth.

Plasmid construction of rescued fusion cDNAs and gp55 variant cDNAs. The EPO-R–gp55 fusion cDNAs were isolated by polymerase chain reaction (PCR) of genomic DNA from the factor-independent Ba/F3 subclones. A 5′ EPO-R primer contained the first 10 codons of EPO-R, including the initiation codon, and a 3′ gp55 primer contained the last 10 codons of gp55, including the termination codon. The PCR-isolated fusion cDNAs were subcloned into the *Xho*I-*Bam*HI sites of pLXSN, generously supplied by D. Miller (28). The nucleotide sequence of the fusion cDNAs was confirmed by dideoxy sequence analysis.

The F-gp55-M1 (mutant gp55) cDNA was isolated by PCR of genomic DNA from the subcloned Ba/F3-F-gp55-M1 cells. The 5′ primer contained the first 10 codons of gp55, including the initiation codon, and the 3′ primer contained

the last 10 codons of gp55, including the termination codon. The PCR-isolated cDNA was cloned into pBluescript (26), and the nucleotide sequence was determined. The F-gp55-M1 cDNA was subcloned into pLXSN as described above, and the restriction enzyme pattern was verified.

The cDNAs encoding the wild-type R-gp55 and F-gp55 cDNAs were subcloned into the *Xho*I site of pLXSN, and their orientation was confirmed by restriction enzyme digestion. The sequence of these cloned cDNAs was compared with the published sequences for F-gp55 (2, 9, 40) and R-gp55 (6).

Growth characteristics of Ba/F3 subclones. Individual subclones expressing EPO-R–gp55 fusion polypeptides or gp55 variants were assayed for EPO-dependent or IL-3-dependent growth by the (MTT) reduction assay (14). Subclones were screened for growth in RPMI 1640 medium plus 10% FCS (with no supplemental growth factor) or in the same medium supplemented with either recombinant hEPO or murine IL-3 (14). Three Ba/F3 subclones for each cDNA construct were examined for factor-independent, EPO-dependent, or IL-3-dependent growth.

Metabolic labeling and immunoprecipitation. Ba/F3 subclones were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine (^{35}S-Express; NEN) as previously described (14). Cell lysates were prepared, and labeled proteins were immunoprecipitated with antipeptide antiserum raised against the N terminus of EPO-R or with a goat polyclonal anti-envelope gp70 antiserum, which reacts with all of the gp55 variants. Proteins were eluted in gel sample buffer and run on 10% acrylamide–sodium dodecyl sulfate gels as previously described (14).

Generation of Ba/F3 subclones expressing either wild-type mEPO-R or wild-type hEPO-R. Ba/F3 cells growing in IL-3 were coelectroporated with PXM-mEPO-R (13) plus pSV2*neo* or with PXM-hEPO-R (18) plus pSV2*neo*. G418-resistant cells were selected, three independent subclones were isolated, and expression of either mEPO-R or hEPO-R was confirmed by immunoprecipitation.

EPO surface binding assay. Ba/F3 subclones expressing EPO-R or EPO-R–gp55 fusion polypeptides were used for binding studies. Ba/F3 cells (10^6) were washed and incubated with radiolabeled recombinant human EPO (1 nM) in 200 μl of RPMI 1640 medium with 10% FCS for 2 h at 4°C either in the absence or in the presence of excess (10 nM) unlabeled EPO. The cells were washed with RPMI 1640 plus 10% FCS, and cell-associated radioactivity was measured by using an Auto-Gamma counter (Packard). Radiolabeled recombinant hEPO (1,000 to 1,500 cpm/fmol) was prepared by the iodine monochloride method as previously described and retained full biological activity (13).

RESULTS

Isolation of factor-independent Ba/F3 subclones which express mutant EPO-R and gp55 polypeptides. We initially used a retroviral selection strategy in Ba/F3 cells to isolate the EPO-R–gp55 fusion proteins. Ba/F3 cells growing in IL-3 were infected with an SFFV retroviral construct (SFFV-EPO-R) (Fig. 1). This retroviral construct contained the 3′ region of the SFFV *env* gene (22), encoding amino acids 186 to 409 of gp55. The 5′ region of *env* had been excised and replaced with full-length wild-type EPO-R cDNA.

We predicted that during retroviral replication, random point mutations in the retroviral genome would generate transforming variants of the EPO-R polypeptide, as previously described (42). After 2 days, infected cells were transferred to medium without supplemental IL-3, and seven factor-independent subclones were isolated. The subclones were next assayed for the presence of EPO-R- or gp55-related polypeptides by immunoprecipitation, since SFFV-EPO-R contained the coding regions of both EPO-R and gp55 (Fig. 1A). All of the subclones expressed EPO-R; four Ba/F3 subclones expressed a full-length 64-kDa EPO-R polypeptide (Fig. 2A, lanes 4 to 7). These polypeptides were subsequently shown to contain a single point mutation (R129C) which has been previously shown to induce constitutive activation of EPO-R (data not shown) (23, 42). Three other factor-independent subclones (lanes 1 to 3) expressed

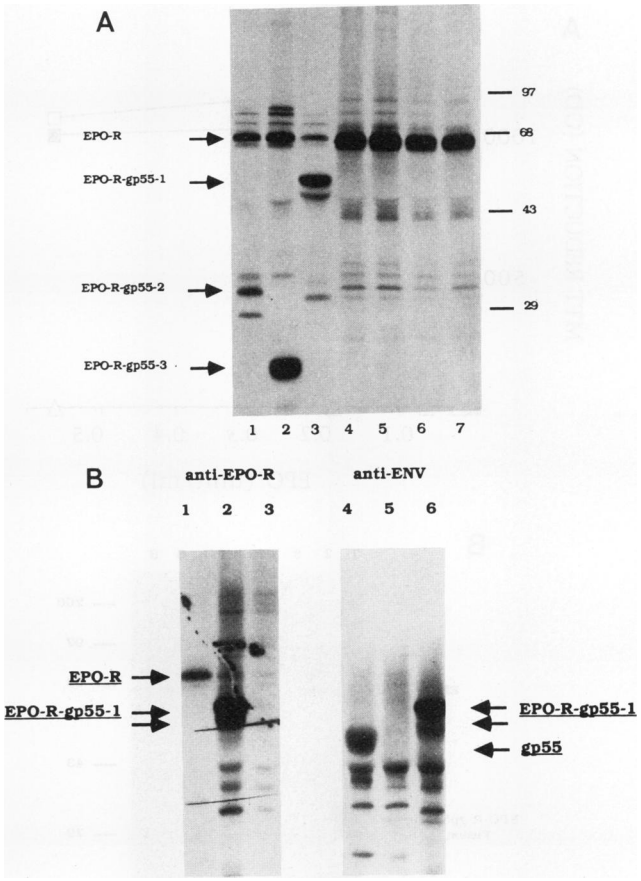

FIG. 2. Expression of constitutive EPO-R polypeptides or EPO-R–gp55 fusion proteins by factor-independent Ba/F3 subclones. Ba/F3 parental cells growing in IL-3 were infected with retroviral supernatants from an SFFV-EPO-R producer cell line as previously described (22). After 3 days, the Ba/F3 cells were transferred to medium without IL-3, and seven factor-independent Ba/F3 subclones were isolated by limiting dilution. (A) Seven subclones were metabolically labeled with [^{35}S]cysteine-methionine, and labeled proteins were extracted and immunoprecipitated with a polyclonal antiserum raised against the N terminus of EPO-R (lanes 1 to 7). Arrows indicate the fusion proteins and the full-length EPO-R. Sizes are indicated in kilodaltons. (B) Radiolabeled proteins from one of these factor-independent subclones (panel A, lane 3) were immunoprecipitated with either an anti-EPO-R antiserum or an antienvelope antiserum. Radiolabeled proteins were immunoprecipitated with an anti-EPO-R antiserum from either Ba/F3-EPO-R (lane 1), factor-independent Ba/F3-EPO-R–gp55-1 (lane 2), or parental Ba/F3 (lane 3) cells. Alternatively, radiolabeled proteins were immunoprecipitated with antienvelope antiserum from either Ba/F3-gp55 (lane 4), parental Ba/F3 (lane 5), or factor-independent Ba/F3-EPO-R–gp55-1 (lane 6) cells.

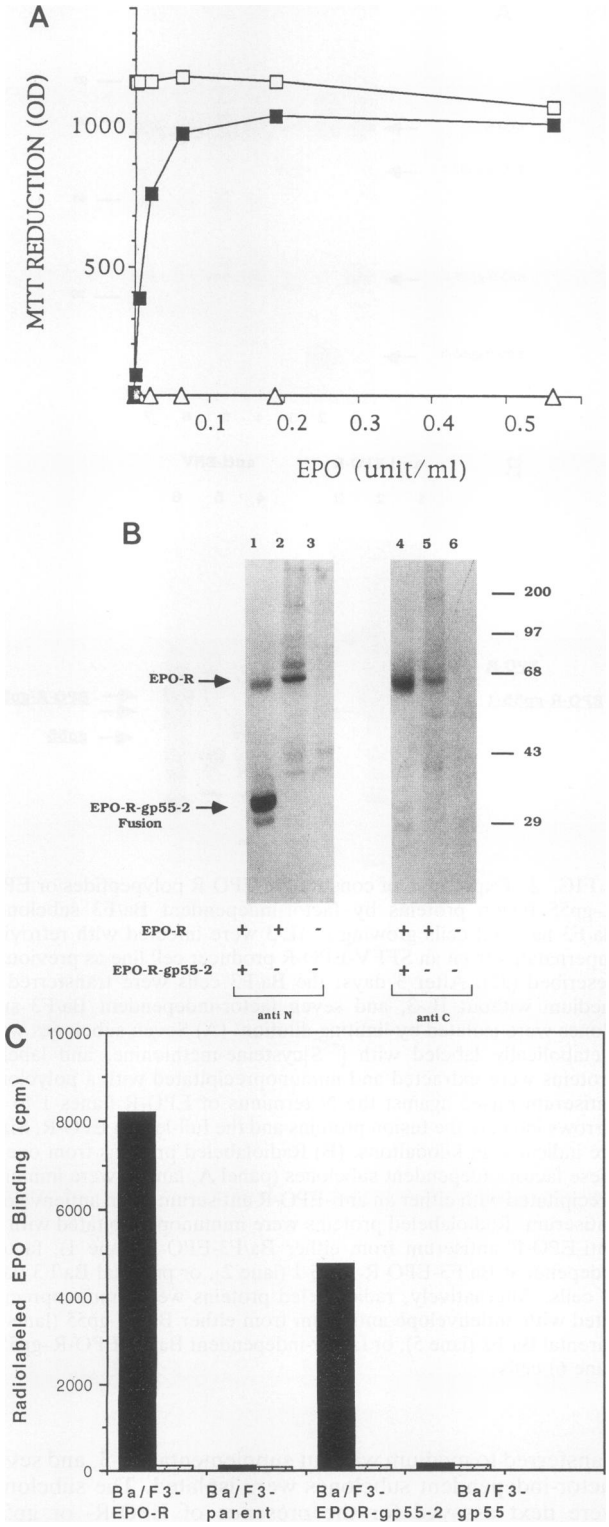

FIG. 3. Evidence that the EPO-R-gp55 fusion proteins form complexes with wild-type EPO-R and activate cell growth. Ba/F3 or Ba/F3-EPO-R cells were infected with a retrovirus (pLXSN-EPO-R-gp55-2) encoding the EPO-R–gp55-2 fusion protein and were subcloned by limiting dilution. (A) EPO-dependent growth characteristics were measured by the MTT assay (14). OD, optical density. Ba/F3 cells expressed either the EPO-R–gp55 fusion protein alone (open triangles), the wild-type EPO-R polypeptide (closed squares), or the EPO-R–gp55 fusion protein plus wild-type EPO-R (open squares). (B) Ba/F3 subclones expressing both EPO-R and the EPO-R–gp55-2 fusion protein (lanes 1 and 4), EPO-R alone (lanes 2 and 5), or neither heterologous protein (lanes 3 and 6) were metabolically labeled, and the labeled proteins were immunoprecipitated with antiserum (22) against the N terminus (lanes 1 to 3) or the C terminus (lanes 4 to 6) of EPO-R. The C-terminal antiserum recognizes epitopes of EPO-R which are absent from EPO-R–gp55-2 (22). Sizes are indicated in kilodaltons. (C) The indicated Ba/F3 subclones (10^6 cells) were incubated with radiolabeled EPO in the absence (black box) or presence (hatched box) of excess unlabeled EPO as described in Materials and Methods. After 2 h at 4°C, the cells were washed and cell-associated radioactivity (counts per minute) was measured. Ba/F3-EPO-R cells (expressing only EPO-R), parental Ba/F3 cells, Ba/F3-EPO-R–gp55-2 cells (expressing only the fusion protein), and Ba/F3-gp55 cells (expressing only F-gp55) were used.

the full-length wild-type EPO-R polypeptide (64 kDa) and, unexpectedly, an additional novel polypeptide (fusion protein) of either 40 kDa (EPO-R–gp55-2), 18 kDa (EPO-R–gp55-3), or 60 kDa (EPO-R–gp55-1). These subclones probably resulted from multiple proviral integrations (5), accounting for the presence of both the full-length EPO-R and the smaller forms in each lane.

One polypeptide (EPO-R–gp55-1; Fig. 2A, lane 3) immunoprecipitated with both an antibody against EPO-R and an antibody against the viral envelope protein (Fig. 2B), suggesting that it was a fusion protein containing epitopes for both antisera. The anti-EPO-R antiserum immunoprecipitated the EPO-R–gp55-1 from this factor-independent subclone (Fig. 2B, lane 2). As a positive control, the antiserum immunoprecipitated the wild-type EPO-R from a Ba/F3-EPO-R subclone (lane 1). The antienvelope antiserum also immunoprecipitated the EPO-R–gp55-1 from the same factor-independent subclone (lane 6). As a positive control, this antiserum immunoprecipitated the wild-type gp55 from a Ba/F3-gp55 subclone expressing only F-gp55 (lane 4). The top two arrows at the right of Fig. 2B indicate alternate forms of EPO-R–gp55-1 which probably result from differential carbohydrate processing (41).

Transforming retroviral variants result from internal deletions causing in-frame fusions of EPO-R and gp55 coding regions. To prove that the novel polypeptides (Fig. 2A, lanes 1 to 3) are fusions of EPO-R and gp55, we next cloned their corresponding cDNAs. Genomic DNA from the clonal factor-independent subclones expressing the putative fusion proteins was amplified by PCR, using a 5′ primer from the EPO-R coding region and a 3′ primer from the gp55 coding region (Fig. 1B, arrows). The PCR-generated cDNAs were subcloned into pBluescript and sequenced. The full-length retroviral cDNA (Fig. 1A) was not detected, but 1.2-, 0.5-, and 1.6-kb specific PCR products were isolated from the cell lines characterized in Fig. 2A, lanes 1, 2, and 3, respectively. All of the novel 1.2-, 0.5-, and 1.6-kb cDNAs were found to be fusion products of EPO-R and gp55 cDNAs, confirming that the novel polypeptides were fusion proteins. The EPO-R cDNA sequence in the fusion cDNAs, as in the original pSFFV-EPO-R construct, was wild type (13). No PCR products were amplified from genomic DNA from parental Ba/F3 cells (data not shown), which confirmed that the new PCR products resulted from novel proviral integrations in the Ba/F3 genome.

The sizes of the PCR-amplified fragments and their nucleotide sequences (Fig. 1B) revealed that the fusion constructs had originated from internal deletions of the original retroviral genome, generating in-frame fusions of the EPO-R and

TABLE 1. Comparison of sequence changes of F-gp55 and the F-gp55-M1 variant[a]

Amino acid position mutated	Wild-type codon (amino acid)	
	Wild type F-gp55	F-gp55-M1
82	ATG (Met)	ATA (Ile)
84	GAG (Asp)	GGA (Gly)
187	TTC (Phe)	TTA (Leu)
253	CCC (Pro)	CTC (Leu)

[a] Nucleotide sequence changes that do not produce an amino acid sequence change are not listed. The Leu-253 is also found in our wild-type F-gp55 sequence and differs from the published sequence (Pro-253) (2, 9, 40).

gp55 coding regions. The fusion cDNAs encode EPO-R–gp55 fusion proteins which contain the amino-terminal hydrophobic leader sequence and extracytoplasmic region of EPO-R and the carboxy-terminal region (transmembrane region) of gp55 (Fig. 1B). These smaller novel polypeptides (40, 18, and 60 kDa) probably arose from splice sites in the parental retroviral genome, producing in-frame fusions of the EPO-R and gp55 coding regions. The sizes of the coding regions of the PCR-generated fusion cDNAs agreed well with the sizes of the corresponding fusion proteins (Fig. 2A). Interestingly, the 5′ fusion site is identical for EPO-R–gp55-1 and EPO-R–gp55-2, but the 3′ fusion site differs for all three variants. The 5′ and 3′ fusion sites are atypical splice consensus sequences (29) (data not shown).

An EPO-R–gp55 fusion protein binds wild-type EPO-R and transforms Ba/F3 cells. We next tested the fusion cDNAs for transforming activity in Ba/F3 cells (Fig. 3). The fusion cDNA encoding the EPO-R–gp55-2 fusion protein, which contains the smallest region of gp55 of all the fusion polypeptides, was subcloned into the Moloney virus-based expression vector pLXSN. Ba/F3 and Ba/F3-EPO-R cells were infected with the prepared virus.

Ba/F3 cells expressing the EPO-R–gp55-2 fusion protein alone failed to grow in EPO, whereas Ba/F3 cells expressing both EPO-R and the EPO-R–gp55-2 fusion protein showed EPO-independent growth. Ba/F3-EPO-R cells showed dose-dependent growth in EPO (Fig. 3A). For these experiments, multiple Ba/F3 subclones expressing fusion proteins and EPO-R were analyzed from four independent infections. Despite a relatively low level of fusion protein expression in some subclones, cell growth was EPO independent (data not shown).

We next confirmed that the Ba/F3 subclones expressed EPO-R and the fusion protein by metabolic labeling and immunoprecipitation (Fig. 3B). An anti-N-terminal EPO-R antiserum immunoprecipitated both the EPO-R polypeptide (64 kDa) and the EPO-R–gp55-2 fusion polypeptide (40-kDa doublet) (Fig. 3B, lane 1). The size of EPO-R–gp55-2 is identical to the size of the fusion protein of the original factor-independent Ba/F3 isolate (Fig. 2A, lane 1). EPO-R, expressed alone, is 66 kDa in Ba/F3-EPO-R cells (Fig. 3B, lane 2). A block in maturation accounts for the smaller molecular mass (64 kDa) of the EPO-R in lane 1. Consistent with this result, we have previously shown that coexpression of EPO-R and full-length F-gp55 blocks the carbohydrate processing of wild-type EPO-R (41). An anti-C-terminal EPO-R antiserum whose epitope is absent from the fusion protein still coimmunoprecipitates EPO-R and a fraction of EPO-R–gp55-2 (Fig. 3B; compare lanes 1 and 4), suggesting that these proteins form a complex in the cell.

Previous studies have suggested a correlation between the

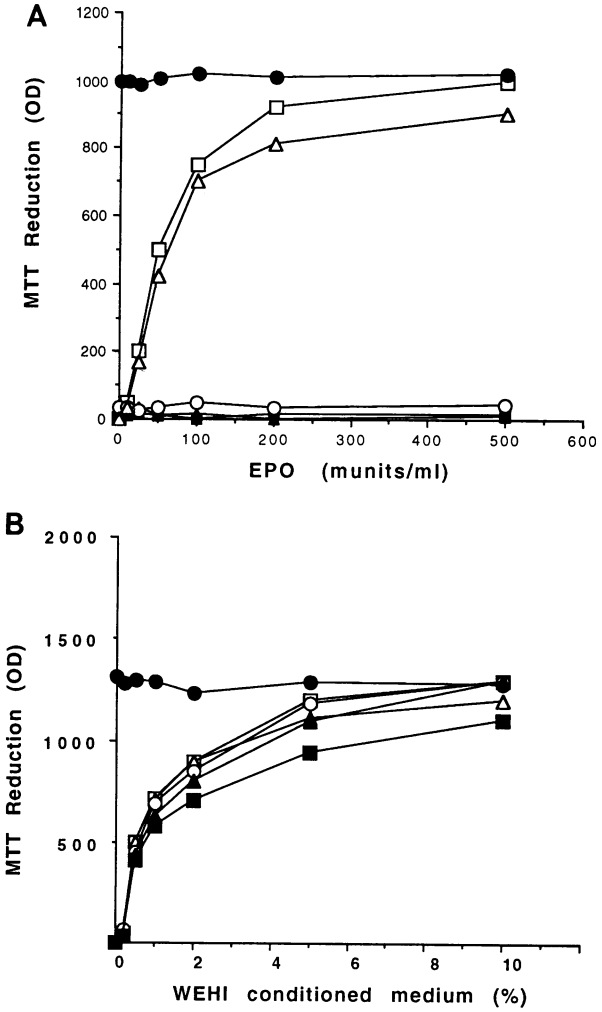

FIG. 4. Failure of the R-gp55 and F-gp55-M1 variants to activate the EPO-R to signal cell growth (A) EPO-dependent growth characteristics were measured by the MTT assay. Ba/F3 subclones expressed either F-gp55-M1 alone (closed triangles), F-gp55 alone (closed squares), R-gp55 alone (open circles), EPO-R plus R-gp55 (open squares), EPO-R plus F-gp55-M1 (open triangles), or EPO-R plus EPO-R–F-gp55 (closed circles). (B) IL-3-dependent growth characteristics of the Ba/F3 subclones. Symbols are the same as in panel A. OD, optical density.

cell surface expression of gp55 and its transforming activity (21). The cell surface expression of EPO-R–gp55-2 was therefore analyzed by radiolabeled EPO binding (Fig. 3C). Ba/F3 subclones were incubated with a saturating (1.0 nM) concentration of radiolabeled EPO, in the presence or absence of excess unlabeled EPO. Ba/F3 cells that expressed either wild-type EPO-R or the EPO-R–gp55-2 fusion protein had specific binding for EPO, demonstrating that these polypeptides were transported to the cell surface. Ba/F3 cells which expressed F-gp55 alone or no heterologous protein had no specific binding.

Isolation of a mutant gp55 (F-gp55-M1) which fails to activate EPO-R. As a second approach to analyzing the gp55–EPO-R interaction, we next isolated a nontransforming mutant of gp55 (F-gp55-M1). A high-titer mixture of retroviruses, one encoding full-length EPO-R and one encoding gp55 (22), was used to infect parental IL-3-dependent Ba/F3 cells. The Ba/F3 cells were subcloned after coinfection; most

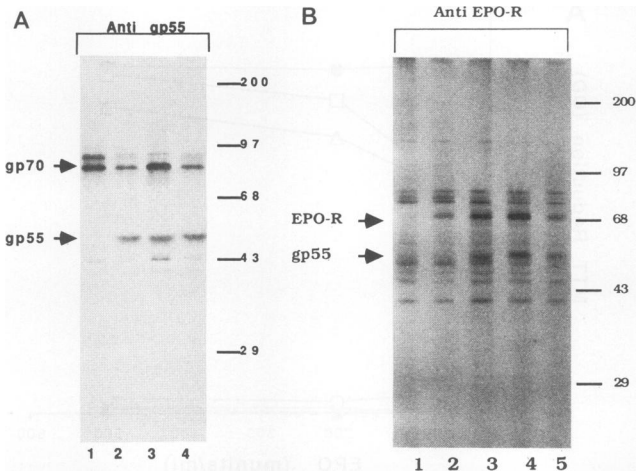

FIG. 5. Evidence that R-gp55 and F-gp55-M1 bind and coimmunoprecipitate with EPO-R. (A) Ba/F3 cells were infected with pLXSN (no cDNA insert) (lane 1), pLXSN-R-gp55 (lane 2), pLXSN-F-gp55-M1 (lane 3), or pLXSN-F-gp55 (lane 4). G418-resistant cells were selected, and labeled proteins were immunoprecipitated with the antienvelope antiserum. (B) Ba/F3-EPO-R cells were infected with pLXSN (no cDNA insert) (lane 2), pLXSN-R-gp55 (lane 3), pLXSN-F-gp55-M1 (lane 4), or pLXSN-F-gp55 (lane 5). G418-resistant cells were selected, and labeled proteins were immunoprecipitated with the anti-N-terminal EPO-R antiserum. Lane 1, Ba/F3 parental cells. Sizes are indicated in kilodaltons.

expressed both EPO-R and gp55, and most were growth factor independent. One Ba/F3 subclone (Ba/F3-F-gp55-M1) coexpressed both EPO-R and gp55 but remained dependent on EPO for growth. Superinfection of Ba/F3-F-gp55-M1 cells with wild-type SFFV gp55 caused factor-independent growth (data not shown) suggesting that this Ba/F3 subclone expressed a mutant gp55 (F-gp55-M1).

The full-length F-gp55-M1 cDNA was isolated by PCR of genomic DNA, using a 5′ primer and 3′ primer specific for the gp55 cDNA. The F-gp55-M1 cDNA was subcloned, sequenced, and found to have three point mutations in the extracytoplasmic region of gp55 and several point mutations which did not change the amino acid sequence (Table 1). There is also a P253L mutation in the extracytoplasmic region of F-gp55-M1 which is present in our wild-type F-gp55 and which differs from the published sequence (2, 9, 40). These point mutations were confirmed from four independent PCR isolates.

gp55 binding is necessary but not sufficient for the constitutive activation of the EPO-R. We next tested the mutant gp55 (F-gp55-M1) and a naturally occurring variant of gp55, R-gp55, for EPO-R binding and activation. The cDNAs encoding wild-type polycythemia F-gp55, F-gp55-M1, or R-gp55 were subcloned into the Moloney retroviral expression vector pLXSN (28). Ba/F3 or Ba/F3-EPO-R cells were infected with helper-free retroviral supernatants encoding these variants and assayed for factor-dependent growth (Fig. 4). In the absence of EPO-R, Ba/F3 cells which expressed F-gp55, R-gp55, or F-gp55-M1, did not grow in EPO (Fig. 4A). These subclones remained dependent on IL-3 for growth (Fig. 4B). Ba/F3-EPO-R cells that were infected with the retrovirus encoding F-gp55 were growth factor independent (Fig. 4). These data confirmed that F-gp55 alone, isolated from the SFFV genome and encoded by a different retrovirus, is sufficient to induce factor-independent growth (1). Ba/F3-EPO-R cells expressing R-gp55 or F-gp55-M1 had identical growth in EPO or IL-3 compared with the uninfected Ba/F3-EPO-R cells (Fig. 4).

To verify expression and coimmunoprecipitation of the gp55 variants, we next performed immunoprecipitations on these Ba/F3 subclones (Fig. 5). Uninfected parental Ba/F3 cells expressed an immunoreactive 70-kDa envelope protein which immunoprecipitated with a polyclonal antienvelope antiserum (Fig. 5A, lane 1). This gp70 does not bind or activate EPO-R (22). Ba/F3 cells that were infected with either pLXSN-R-gp55, pLXSN-F-gp55-M1, or pLXSN-F-gp55 expressed these gp55 variant polypeptides (lane 2, 3, or 4, respectively). Uninfected Ba/F3-EPO-R cells expressed wild-type EPO-R alone (66 to 68 kDa) (Fig. 5B, lane 2). With an anti-EPO-R N-terminal antiserum, EPO-R coimmunopre-

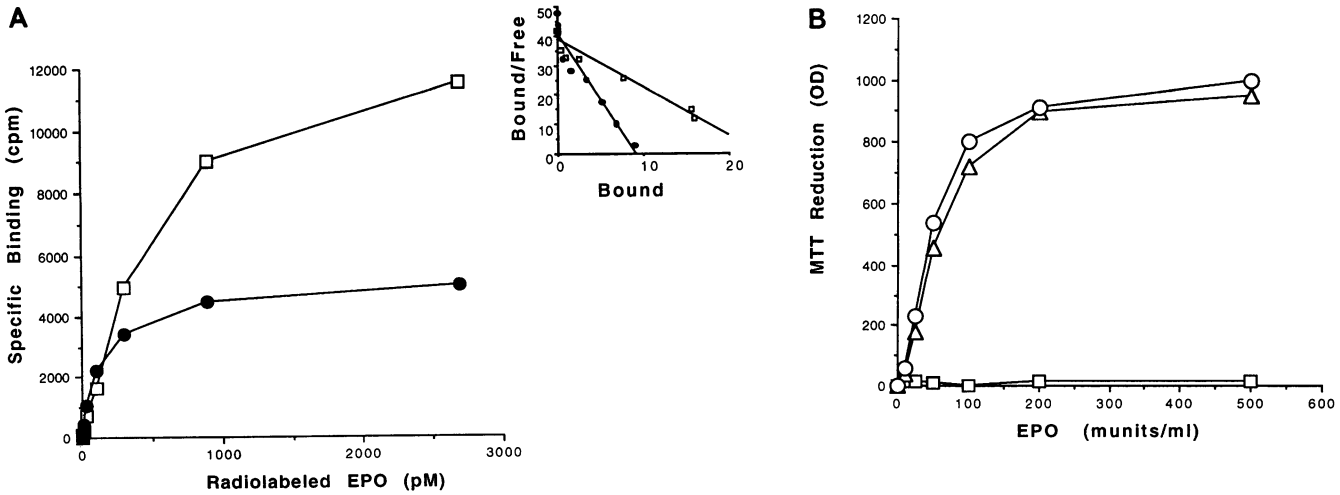

FIG. 6. Characterization of Ba/F3 subclones expressing either mEPO-R or hEPO-R. Ba/F3 cells growing in IL-3 were stably transfected with either the mEPO-R or hEPO-R cDNA, and Ba/F3 subclones were isolated. (A) Equilibrium binding of radiolabeled recombinant hEPO to Ba/F3-mEPO-R (open squares) and Ba/F3-hEPO-R (closed circles) cells and Scatchard analysis (inset). (B) EPO-dependent growth characteristics measured by the MTT assay for Ba/F3-mEPO-R (open triangles), Ba/F3-hEPO-R (open circles), and parental Ba/F3 (open squares) cells. OD, optical density.

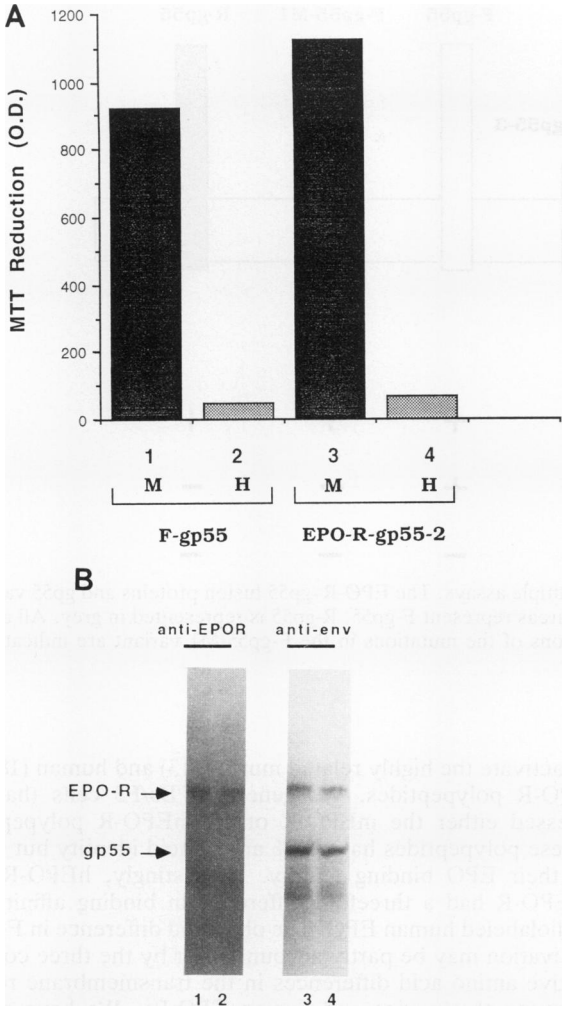

FIG. 7. Activation by gp55 of the mEPO-R and not of the hEPO-R. (A) Ba/F3 subclones expressing mEPO-R (M) or hEPO-R (H) were infected with a retrovirus encoding either F-gp55 (columns 1 and 2) or EPO-R–gp55-2 fusion protein (columns 3 and 4). Infected Ba/F3-mEPO-R (black bars) and Ba/F3-hEPO-R (shaded bars) cells were washed, transferred to medium without supplemental growth factor, and tested by the MTT assay after 48 h. O.D., optical density. (B) Radiolabeled proteins were immunoprecipitated with the indicated antisera from Ba/F3-mEPO-R (lanes 1 and 3) and Ba/F3-hEPO-R (lanes 2 and 4) cells. The cells shown in lane 3 and 4 had been infected with pLXSN-F-gp55.

cipitates with R-gp55 (lane 3), F-gp55-M1 (lane 4), and wild-type F-gp55 (lane 5), demonstrating that all gp55 variants form complexes with the wild-type EPO-R.

To ensure that the expression level of the gp55 variants did not account for the differences in the EPO-dependent growth characteristics, we infected Ba/F3-EPO-R cells multiple times and isolated multiple subclones. One subclone expressed very low levels of wild-type F-gp55 (data not shown). Despite this relatively low level of expression, these cells were transformed to EPO-independent growth. Comparatively high levels of F-gp55-M1 and R-gp55 expression were seen for some of the subclones, yet these subclones remained EPO dependent for growth.

The interaction between gp55 and mEPO-R is species specific. In a third strategy, we compared the interaction of gp55 with the murine and human EPO-R polypeptides, which are 82% identical in primary amino acid sequence. Ba/F3 cells were stably transfected with either mEPO-R or hEPO-R cDNA. Scatchard analysis of Ba/F3 cells expressing mEPO-R demonstrated 3,100 receptors per cell surface with a single affinity of 240 pM (Fig. 6A). Ba/F3-hEPO-R cells had 1,820 receptors per cell surface with an affinity constant of 87 pM. The higher affinity of hEPO-R for EPO, which has not been previously described, may be due to the use of radiolabeled hEPO for these binding studies. Ba/F3 cells expressing either mEPO-R or hEPO-R had similar EPO dose-dependent growth (Fig. 6B), with one-half maximal growth at approximately 50 mU of EPO per ml (5 pM) for Ba/F3-mEPO-R and 20 mU of EPO per ml (2 pM) for Ba/F3-hEPO-R. These Ba/F3 subclones also had similar IL-3-dependent growth (data not shown).

The Ba/F3 subclones were next infected with the virus encoding F-gp55 to compare the ability of F-gp55 to activate mEPO-R or hEPO-R. mEPO-R was constitutively activated by F-gp55 to signal cell growth (Fig. 7A), but hEPO-R was not activated. This differential activation was not due to the level of expression of the heterologous proteins. Four independent Ba/F3 subclones expressing either mEPO-R or hEPO-R were infected, and comparable results were obtained each time. Also, the EPO-R–gp55-2 fusion protein activated the murine but not the human EPO-R (Fig. 7A). To ensure that F-gp55 expression was equivalent, we next immunoprecipitated F-gp55 from both infected cell lines (Fig. 7B). Both mEPO-R and hEPO-R were expressed at equivalent levels, and both bound and coimmunoprecipitated with F-gp55.

DISCUSSION

We have used three independent strategies to define the regions of EPO-R and gp55 required for their transforming molecular interaction. First, using a retroviral selection strategy, we have isolated fusion proteins containing regions of both EPO-R and gp55, which have transforming activity in Ba/F3 cells. These fusion proteins form stable complexes with wild-type EPO-R and thereby activate wild-type EPO-R to signal constitutively. The fusion proteins (Fig. 2 and 8) contain different regions of the amino terminus of EPO-R and of the carboxy terminus of F-gp55. All fusion proteins contain at least the transmembrane domain of F-gp55, confirming earlier studies indicating that this region of gp55 is required for the transforming activity of the protein in vivo (8, 36). The fusion proteins expressed alone in Ba/F3 cells do not abrogate the IL-3 dependence of Ba/F3 cells. They are transforming only when coexpressed with wild-type EPO-R, which has its own critical cytoplasmic signaling domain (14, 15).

The EPO-R–gp55 fusion proteins help localize the transforming region of gp55. The entire dualtropic *env*-specific sequence of gp55 (amino acids 1 to 331) can be substituted with EPO-R sequence without loss of EPO-R binding or activation. Previous studies had suggested that these dualtropic sequences were essential for pathogenic activity in vivo (3, 20, 24). Also, the highly conserved CWLC sequence (amino acids 306 through 309) (34) can be deleted from the fusion protein and is therefore not required for EPO-R binding and activation.

The EPO-R–gp55 fusion proteins also further localize the EPO-R binding domain of gp55. The EPO-R sequence of the fusion proteins may contribute to EPO-R binding by mediating oligomerization; however, EPO-R–gp55-3 contains only the amino-terminal 38 amino acids of EPO-R (after the

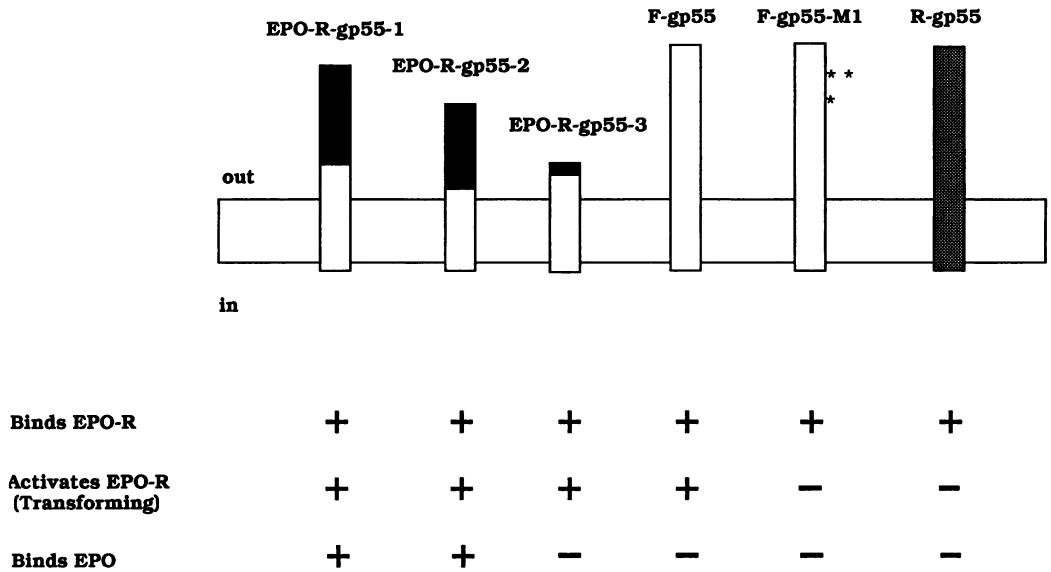

FIG. 8. Analysis of EPO-R–gp55 fusion proteins and gp55 variants by multiple assays. The EPO-R–gp55 fusion proteins and gp55 variants are shown schematically. The black areas represent EPO-R, and the white areas represent F-gp55. R-gp55 is represented in grey. All assays were performed in stable Ba/F3 cell transfectants. The approximate locations of the mutations in the F-gp55-M1 variant are indicated by asterisks.

signal peptide is cleaved). More likely, the EPO-R region of the fusion proteins promotes proper conformation and cell surface localization of the fusion proteins. The minimal site required for EPO-R binding must therefore reside between amino acids 329 and 409 of gp55. Of this region, there are 50 amino acids which are extracytoplasmic and 30 amino acids which are transmembrane (40). Interestingly, most of this critical gp55 sequence (47 of 50 extracytoplasmic residues and 6 of 30 transmembrane residues) can be further deleted without loss of leukemogenic activity in vivo (25). Taken together, these results implicate transmembrane residues 384 to 409 as the critical transforming domain of gp55.

Using a second strategy, we have isolated a transforming mutant of F-gp55 (F-gp55-M1) which binds to EPO-R but fails to activate cell growth. This F-gp55 mutant contains three noncontinuous point mutations in the extracytoplasmic region of F-gp55 (Table 1) but contains a wild-type F-gp55 transmembrane domain. These three mutations fall in a dualtropic extracytoplasmic region which has previously been shown to be required for cell surface translocation of F-gp55 and for leukemogenicity in vivo (21). Importantly, the F-gp55-M1 mutant demonstrates that the transmembrane region of F-gp55 is necessary but not sufficient for EPO-R activation. Even though the F-gp55-M1 polypeptide binds to wild-type EPO-R, it may not assume the proper conformation to translocate to the cell surface or to activate EPO-R signaling.

We have compared the F-gp55-M1 mutant with R-gp55, a naturally occurring variant of gp55. R-gp55, when coexpressed with EPO-R, binds and coimmunoprecipitates with EPO-R; however, these cells remain dependent on EPO for growth (Fig. 8), irrespective of the level of expression of R-gp55. This result is consistent with the in vivo effects of R-gp55, which induces a polyclonal proliferation of erythroblasts that remain EPO dependent for growth (30). Like R-gp55, the anemia strain protein F-gp55 (FVA) does not abrogate EPO-dependent growth of HCD cells (31).

In a third strategy, we have compared the ability of F-gp55 to activate the highly related murine (13) and human (18, 39) EPO-R polypeptides. We generated Ba/F3 cells that expressed either the mEPO-R or the hEPO-R polypeptide. These polypeptides have 82% amino acid identity but differ in their EPO binding affinity. Interestingly, hEPO-R and mEPO-R had a threefold difference in binding affinity for radiolabeled human EPO. The observed difference in F-gp55 activation may be partly accounted for by the three conservative amino acid differences in the transmembrane region between the murine and human EPO-Rs. We have previously shown that the interaction between F-gp55 and EPO-R is also cytokine receptor specific; F-gp55 does not activate the endogenous IL-3 receptor of Ba/F3 cells (22) and does not activate the endogenous IL-2 receptor β chain (p75 subunit) of CTLL-2 cells (33), even though the IL-3 and IL-2 receptors are related proteins in the EPO-R superfamily (10, 12).

The factor-independent Ba/F3 subclones described in this study are leukemogenic in vivo (data not shown). It is therefore likely that the EPO-R–gp55 fusion proteins themselves have leukemogenic activity. Previous studies have demonstrated a direct correlation between the transforming activity of a viral oncoprotein in Ba/F3 cells in vitro and its leukemogenic activity in vivo (11). The Ba/F3 cell system described in this study has provided a rapid selection of new transforming retroviral gp55 variants such as the fusion proteins as well as a rapid assay of preexisting gp55 mutants (such as R-gp55).

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