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A Dominant Negative Erythropoietin (EPO) Receptor Inhibits EPO-Dependent Growth and Blocks F-gp55-Dependent Transformation

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The erythropoietin receptor (EPO-R), a member of the cytokine receptor superfamily, can be activated to signal cell growth by binding either EPO or F-gp55, the Friend spleen focus-forming virus glycoprotein. Activation by F-gp55 results in constitutive EPO-R signalling and the first stage of Friend virus-induced erythroleukemia. We have generated a truncated form of the EPO-R polypeptide [EPO-R(T)] which lacks the critical cytoplasmic signal-transducing domain of the EPO-R required for EPO- or F-gp55-induced cell growth. EPO-R(T) specifically inhibited the EPO-dependent growth of EPO-R-expressing Ba/F3 cells without changing the interleukin-3-dependent growth of these cells. In addition, Ba/F3 cells that coexpressed wild-type EPO-R and EPO-R(T) were resistant to transformation by F-gp55 despite efficient expression of the F-gp55 transforming oncoprotein in infected cells. EPO-R(T) inhibited the EPO-dependent tyrosine phosphorylation of wild-type EPO-R, the tyrosine kinase (JAK2), and the SH2 adaptor protein (Shc). In conclusion, the EPO-R(T) polypeptide is a dominant negative polypeptide which specifically interferes with the early stages of EPO-R-mediated signal transduction and which prevents Friend virus transformation of erythroblasts.

The erythropoietin receptor (EPO-R), a 507-amino-acid (aa) membrane protein (12), exerts its biological activity in erythroid precursors through the binding of its 34-kDa glycoprotein ligand, thereby functioning as the primary regulator of erythroid mitogenesis and differentiation. The Friend spleen focus-forming virus (SFFV) glycoprotein (F-gp55) binds to the EPO-R, causing constitutive receptor signalling and the first stage of Friend virus-induced erythroleukemia (2). There is no amino acid similarity between EPO and F-gp55, and each protein appears to bind to a discrete site on the EPO-R (5). Little is known of the mechanisms by which the EPO-R transduces a growth signal. Activation of the EPO-R by EPO or F-gp55 (44) results in the tyrosine phosphorylation of the EPO-R cytoplasmic region (9, 15, 27), and this phosphorylation correlates with mitogenic activity.

Although the EPO-R does not itself contain a tyrosine kinase catalytic domain (13), it contains a critical regulatory domain which interacts with cytoplasmic tyrosine kinases and other cytoplasmic effector molecules. Recent studies demonstrated that EPO activates the cytoplasmic tyrosine kinase (JAK2) (43). The tyrosine kinase Fes has been shown to be tyrosine phosphorylated in response to EPO in TF-1 cells, a human erythroleukemia cell line (18). Other tyrosine kinases may be recruited to the EPO-R, as observed with a variety of cytokine receptors (31); however, their identification remains elusive. EPO stimulation results in the rapid tyrosine phosphorylation of the EPO-R (9, 15, 27), JAK2 (43), and the SH2 adaptor protein (Shc) (8, 10). A number of other biochemical events have been associated with activation of the EPO-R, including an increase in the activities of phosphatidylinositol 3-kinase (11, 20, 25), $p21^{ras}$ (40), and Raf-1 (4), as well as an increase in the phosphorylation of p120 GAP (40). The relative sequence of these events and their importance to EPO-induced growth and differentiation have not been established.

Homodimerization is likely to play a role in EPO-R signal transduction, as shown by multiple studies. First, the growth hormone receptor, a related member of the cytokine receptor superfamily, forms a homodimeric complex with a single molecule of growth hormone (7, 14), suggesting the possibility of an analogous model for the EPO-R. Second, an Arg-to-Cys substitution (aa 129) in the extracellular domain of the EPO-R results in EPO-independent growth of transfected Ba/F3 cells (45) and in constitutively formed homodimers of the EPO-R complex (42). Third, a recent report (29) demonstrated oligomerization of wild-type EPO-R and a truncated EPO-R (−221) mutant by coimmunoprecipitation.

Functional interactions between receptor subunits have been demonstrated with dominant negative mutants of several growth factor receptors, including the epidermal growth factor receptor (22, 36), the fibroblast growth factor receptor (1), and the tumor necrosis factor receptor (3), although the actual mechanism of wild-type receptor inhibition is not known. Ueno et al. (41) demonstrated that a truncated, dominant negative mutant of the platelet-derived growth factor receptor inhibits the wild-type platelet-derived growth factor receptor by forming heterodimeric, nonproductive complexes which are defective for autophosphorylation. We were therefore interested in testing the ability of a truncated EPO-R to affect EPO-dependent signal transduction and in perturbing the interaction between wild-type EPO-R and F-gp55.

In this study, we examined the biological properties of a truncated EPO-R(−221) mutant (13, 29), hereafter called EPO-R(T). We showed that the overexpression of EPO-R(T) relative to wild-type EPO-R produced a dominant negative phenotype which specifically inhibited the EPO-mediated but not the interleukin-3 (IL-3)-mediated growth of hematopoietic cells. Also, EPO-R(T) efficiently blocked the transformation of hematopoietic cells by the F-gp55 polypeptide. The coexpression of wild-type EPO-R and EPO-R(T) blocked the EPO-

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dependent tyrosine phosphorylation of wild-type EPO-R, JAK2, and Shc, confirming that this event is required for mitogenic activity. The dominant negative activity of EPO-R(T) supports a model of homodimerization for EPO-R signalling.

MATERIALS AND METHODS

Cells and cell culture. Ba/F3 cells (32) were maintained as previously described (13) in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum (FCS) and 10% (vol/vol) conditioned medium from WEHI-3B cells as a source of IL-3 (IL-3 medium).

Plasmid construction. EPO-R(T) was generated by the PCR by introducing a stop codon (TAG) at aa 248 of the EPO-R. The mutant EPO-R(T) cDNA sequence was confirmed by dideoxy DNA sequence analysis and subcloned into mammalian expression vector PXM (13) or retroviral vector PLXSN (26).

DNA transfection of Ba/F3 cells. Ba/F3 cells (10^7 cells) were transfected by coelectroporation with PXM-EPO-R or PXM-EPO-R(T) cDNA (linearized with *Nde*I) and with PSV2neo (linearized with *Acc*I) as previously described (13). Selection with G418 (1.0 mg/ml) in 10% WEHI-3B-conditioned medium was initiated 48 h after electroporation. Selected cells were subcloned by limiting dilution, and individual subclones were expanded in G418-containing IL-3 medium and analyzed for the presence of the wild-type EPO-R or EPO-R(T) polypeptide by metabolic labeling and immunoprecipitation as previously described (38). Alternatively, Ba/F3 cells growing in IL-3 medium were infected with retroviral construct PLXSN-EPO-R(T) (13).

Flow cytometric analysis. Ba/F3 subclones expressing various EPO-R mutants were washed in RPMI 1640 medium–10% FCS without supplemented EPO or murine IL-3 (plain medium) and resuspended in phosphate-buffered saline. The cells (10^6) were incubated with a single saturating concentration of a protein A-purified polyclonal antiserum (10 μg/ml) directed against the extracytoplasmic domain of human EPO-R in a 200-μl reaction mixture. The cells were washed and stained with a fluorescein isothiocyanate (FITC)-conjugated anti-mouse second antibody. The cells were analyzed with FACScan (Becton Dickinson, San Jose, Calif.).

Immunoprecipitation of EPO-R and F-gp55 in Ba/F3 cell extracts. Ba/F3 subclones were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine as previously described (13). Labeled proteins were immunoprecipitated with antisera against the amino terminus of mouse EPO-R, the carboxyl terminus of mouse EPO-R, or F-gp55 (13).

EPO-dependent growth characteristics of Ba/F3 subclones. Individual subclones expressing the EPO-R or other heterologous polypeptides were screened for growth in RPMI 1640 medium supplemented with 10% FCS and various concentrations of recombinant human EPO (without supplemental WEHI-3B-conditioned medium). Cell growth and viability were measured with the MTT (dimethylthiazole diphenyltetrazolium bromide) reduction assay as previously described (13). Electroporations with each mutant were performed three times. When resulting transfectants were isolated and tested for EPO responsiveness, identical results were obtained each time.

F-gp55-dependent growth characteristics of Ba/F3 subclones. Subclones expressing the EPO-R and EPO-R(T) polypeptides were infected with a high-titer (SFFV) retroviral supernatant encoding the F-gp55 polypeptide (38). Approximately 5×10^5 Ba/F3 cells growing in IL-3 medium were infected by cocultivation with E86 producer cells. 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 plain medium. The infected cells were screened for growth and viability in plain medium with the MTT reduction assay. In addition, the SFFV-infected Ba/F3 subclones were grown for 72 h in IL-3 medium. These infected cells were screened for productive SFFV infection by metabolic labeling and immunoprecipitation of the F-gp55 polypeptide with a goat polyclonal anti-envelope antiserum.

Analysis of EPO-R, JAK2, and Shc tyrosine phosphorylation. Ba/F3 subclones were starved in RPMI 1640 medium–10% FCS for 4 h and stimulated for various times. Cell lysates were prepared as described previously (39). For monoclonal antibody 4G10 immunoprecipitations, a cell lysate (500 μg of protein) was incubated with 4G10-protein A-Sepharose (40 μg) overnight. The Sepharose beads were washed three times with 50 mM Tris-HCl (pH 8.0)–150 mM NaCl–0.05% Triton X-100. Samples were then prepared for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and electrophoretic transfer. After the membrane was blocked, Western blotting (immunoblotting) was performed by incubation with 10 μg of protein A-purified murine anti-EPO-R peptide antibody per ml for 1 h. The membrane was washed, incubated with horseradish peroxidase (HRP)-protein A (1:5,000) (Amersham, Arlington Heights, Ill.) for 30 min, and washed extensively. Immune complexes for all Western immunoblotting experiments were detected by use of ECL (Amersham).

An immunoprecipitation was conducted with 2×10^7 cell equivalents and with an anti-JAK2 antibody as described previously (43). The membrane was blocked, incubated with 1 μg of antiphosphotyrosine monoclonal antibody 4G10 per ml for 1 h, washed, and incubated with HRP-sheep anti-mouse immunoglobulin G (IgG) (1:5,000) (Amersham) for 30 min. Following ECL immunodetection, the membrane was incubated in a buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% (wt/vol) SDS, and 100 mM β-mercaptoethanol at 50°C for 1 h. The membrane was blocked, reprobed with the anti-JAK2 antibody (1:1,000), and washed. HRP-protein A (1:5,000) was added for 30 min, and the membrane was washed before ECL detection.

An immunoprecipitation was performed with 2×10^7 cell equivalents and with 8 μg of an anti-Shc polyclonal antibody (Transduction Laboratories, Lexington, Ky.) (33). The membrane was blocked before the addition of an HRP-conjugated monoclonal antibody against phosphotyrosine (HRP-RC20) (1:2,500) (Transduction Laboratories). After 1 h of incubation, the membrane was washed before ECL detection. The same nitrocellulose membrane was washed overnight, blocked, and then cut into two pieces. The upper membrane was incubated with the anti-Shc polyclonal antibody (1:1,000) for 1 h, washed, incubated with HRP-protein A (1:5,000) for 30 min, and washed. To the lower nitrocellulose membrane, anti-Grb2 monoclonal antibody (1:500) (Transduction Laboratories) was added for 1 h and the membrane was washed and incubated with HRP-sheep anti-mouse IgG (1:5,000) for 30 min.

RESULTS

Expression of EPO-R and EPO-R(T) in a hematopoietic cell line. We initially synthesized cDNA encoding a truncated form of murine EPO-R [EPO-R(T)], which lacks the critical signal-transducing domain previously described (13, 17, 30) and contains only the Box 1 sequence (17). This cDNA was subcloned into retroviral vector PLXSN (26). The construct

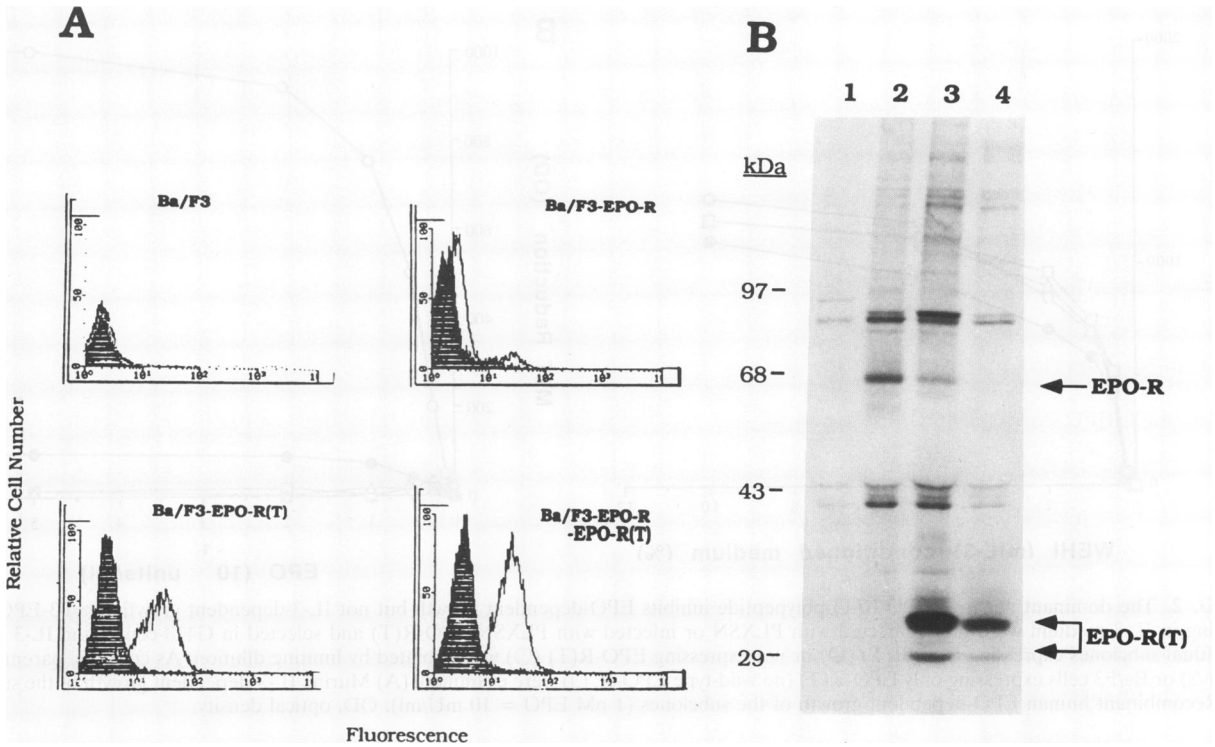

FIG. 1. Cell surface expression of wild-type EPO-R and EPO-R(T) polypeptides in Ba/F3 cells. (A) Ba/F3 cells expressing the indicated polypeptides were incubated with 5 nM anti-human EPO-R polyclonal antibody and stained with FITC-conjugated anti-rabbit IgG antibody. Fluorescence was analyzed by FACScan analysis. The dark histogram shows staining with FITC-conjugated anti-rabbit IgG alone, and the light histogram shows staining with anti-human EPO-R polyclonal antibody, as detected with FITC-conjugated anti-rabbit IgG. (B) Cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine. Immunoprecipitation with an anti-EPO-R N terminus antiserum was performed as previously described (13). Lanes: 1, Ba/F3; 2, Ba/F3-EPO-R; 3, Ba/F3-EPO-R-EPO-R(T) subclone 10; 4, Ba/F3-EPO-R(T) subclone 16. Nonspecific bands are observed at 90 and 40 kDa.

was transfected into the ecotropic packaging cell line, E86, and a helper-free stock of PLSXN-EPO-R(T) was generated (38). The expression of full-length EPO-R and EPO-R(T) in infected Ba/F3 cells was confirmed by FACScan analysis (Fig. 1A). Parental Ba/F3 cells had no detectable cell surface EPO-R. Ba/F3 cells transfected with PXM-EPO-R (Ba/F3-EPO-R cells) expressed low levels of EPO-R. Ba/F3 cells infected with PLXSN-EPO-R(T) [Ba/F3-EPO-R(T) cells] expressed high levels of cell surface EPO-R(T). The relative levels of expression of the EPO-R and EPO-R(T) polypeptides were dependent on the expression vector used.

To further confirm that the various Ba/F3 subclones expressed the heterologous proteins, we performed immunoprecipitation with an anti-EPO-R N terminus antiserum (Fig. 1B). Parental Ba/F3 cells did not express the EPO-R polypeptide (lane 1). Ba/F3-EPO-R cells expressed only the full-length 66-kDa EPO-R polypeptide (lane 2). Ba/F3 cells transfected with both PXM-EPO-R and PLXSN-EPO-R(T) [Ba/F3-EPO-R-EPO-R(T) cells] expressed both the full-length EPO-R polypeptide and EPO-R(T) polypeptides which migrated as a 27- and 31-kDa doublet (lane 3). These two polypeptides are differentially glycosylated forms of EPO-R(T) (44). Ba/F3-EPO-R(T) cells expressed only the EPO-R(T) polypeptide (lane 4).

EPO-R(T) inhibits EPO-induced growth of Ba/F3-EPO-R cells. We next tested the effect of EPO-R(T) expression on EPO-dependent cell growth (Fig. 2). For each of the four cell types shown in Fig. 1B, we isolated individual Ba/F3 subclones by limiting dilution and tested IL-3- or EPO-dependent growth. Parental Ba/F3 cells were dependent on murine IL-3 for growth and did not grow in response to EPO. Ba/F3-EPO-R cells demonstrated dose-dependent growth in response to EPO (Fig. 2B), consistent with previous reports (23, 44). Ba/F3-EPO-R(T) cells did not grow in response to EPO but bound radiolabeled EPO at the cell surface (13). Ba/F3-EPO-R cells infected with PLXSN-EPO-R(T) showed minimal EPO-dependent growth (Fig. 2B). The inhibition of EPO-dependent growth was confirmed with multiple, independently isolated Ba/F3-EPO-R-EPO-R(T) subclones (see below). Even at EPO concentrations as high as 5 nM, Ba/F3-EPO-R-EPO-R(T) cells failed to grow in response to EPO. The expression of the EPO-R(T) polypeptide did not affect the growth of Ba/F3-EPO-R cells in response to IL-3, suggesting that EPO-R(T) specifically inhibited EPO-R-mediated but not IL-3-R-mediated proliferation (Fig. 2A).

The dominant negative EPO-R(T) polypeptide blocks F-gp55-induced transformation of Ba/F3 cells. Previous studies showed that the envelope glycoprotein (F-gp55) of SFFV binds to EPO-R and activates receptor signalling (21, 23). While the bulk of the interaction between F-gp55 and EPO-R occurs in an intracellular compartment (44), the complex of F-gp55 and EPO-R has also been detected at the cell surface (5, 16). We next tested EPO-R(T) for its ability to block F-gp55-mediated activation of wild-type EPO-R. Parental Ba/F3 cells, Ba/F3-EPO-R cells, or Ba/F3-EPO-R-EPO-R(T) cells were infected with a retrovirus encoding F-gp55 (38). Infected subclones were isolated by limiting dilution and analyzed for IL-3-dependent or EPO-dependent growth (Fig.

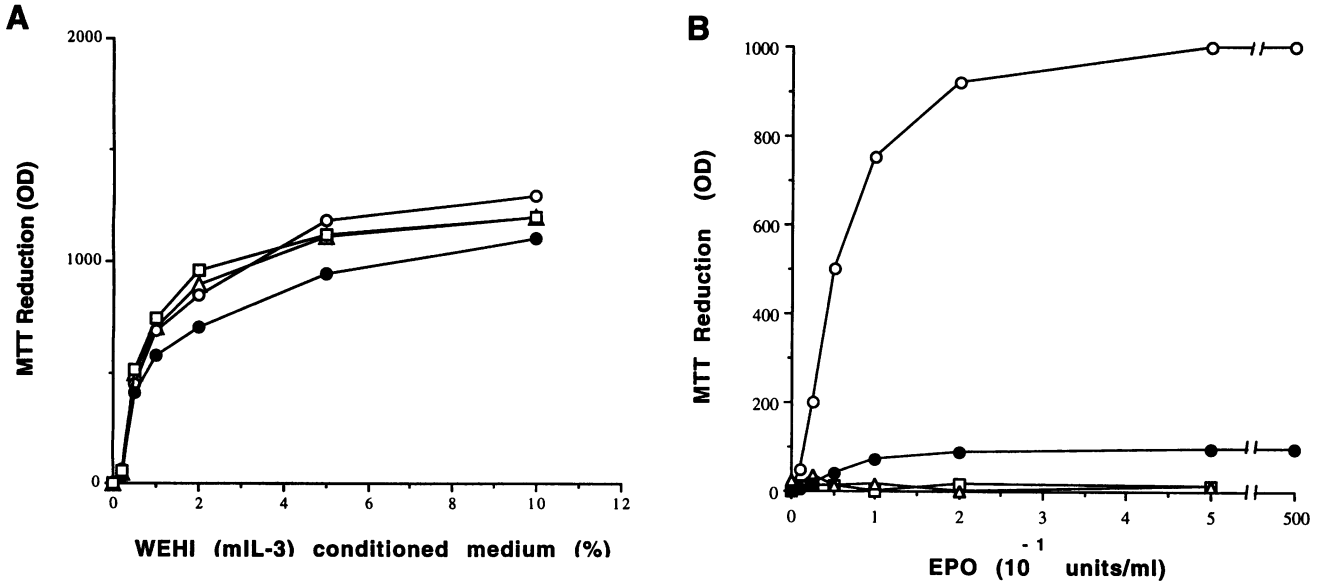

FIG. 2. The dominant negative EPO-R(T) polypeptide inhibits EPO-dependent growth but not IL-3-dependent growth. Ba/F3-EPO-R cells growing in IL-3 medium were mock infected with PLXSN or infected with PLXSN-EPO-R(T) and selected in G418-containing IL-3 medium. Individual subclones expressing EPO-R(T) (●) or not expressing EPO-R(T) (○) were isolated by limiting dilution. As controls, parental Ba/F3 cells (△) or Ba/F3 cells expressing only EPO-R(T) (no wild-type EPO-R; □) were examined. (A) Murine IL-3-dependent growth of the subclones. (B) Recombinant human EPO-dependent growth of the subclones (1 pM EPO = 10 mU/ml). OD, optical density.

3). Ba/F3-EPO-R cells expressing F-gp55 were transformed to growth factor independence (Fig. 3). Ba/F3-EPO-R-EPO-R(T) cells expressing F-gp55 remained dependent on IL-3 for growth (Fig. 3A) and failed to grow in response to EPO (Fig. 3B). No growth factor-independent subclones of Ba/F3-EPO-R-EPO-R(T) were isolated following a number of independent infections with the F-gp55-encoding virus.

We next analyzed four Ba/F3 subclones for the presence of wild-type EPO-R, EPO-R(T), and F-gp55 polypeptide (Fig. 4). Two Ba/F3-EPO-R subclones which grew in response to EPO (Ba/F3-EPO-R subclones 1 and 2) expressed 66-kDa wild-type EPO-R (Fig. 4, lanes 2 and 4). Two Ba/F3-EPO-R-EPO-R(T) subclones which failed to grow in response to EPO [Ba/F3-EPO-R-EPO-R(T) subclones 8 and 10] expressed both wild-type EPO-R and EPO-R(T) (Fig. 4, lanes 1 and 3). All four subclones analyzed by immunoprecipitation expressed similar levels of wild-type EPO-R (Fig. 4, lanes 1 to 4). The Ba/F3-EPO-R-EPO-R(T) subclone infected with F-gp55 was strictly

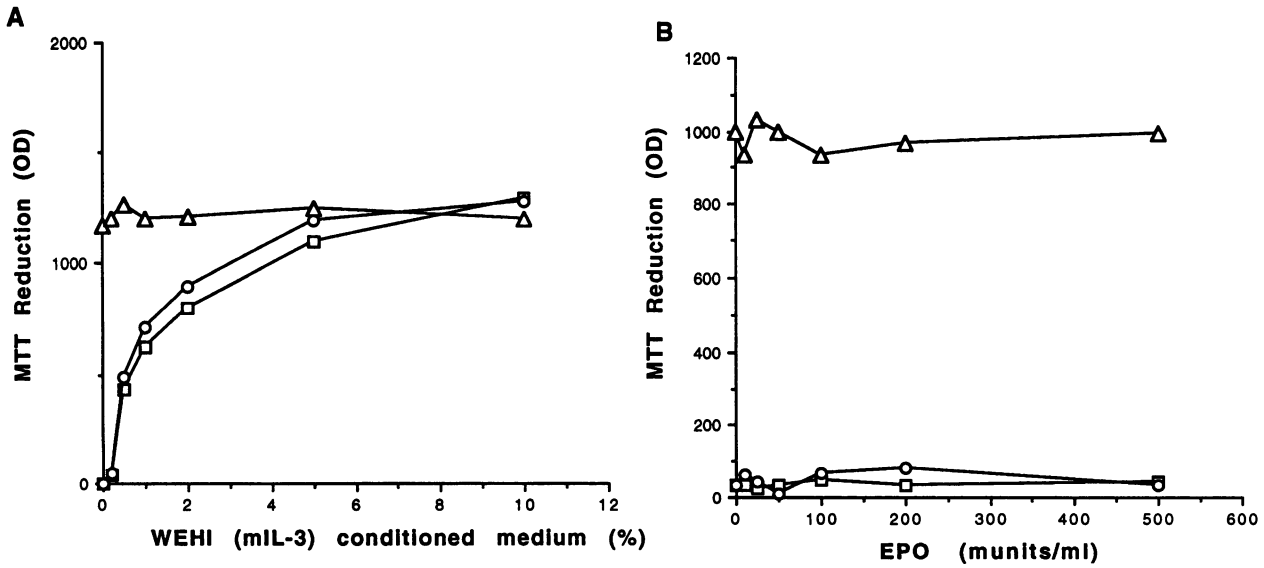

FIG. 3. The dominant negative EPO-R(T) polypeptide blocks F-gp55-induced transformation of Ba/F3-EPO-R cells. Ba/F3 subclones expressing either wild-type EPO-R (△), wild-type EPO-R plus EPO-R(T) (○), or no heterologous protein (□) were infected with a retroviral supernatant encoding F-gp55 (38). F-gp55-expressing cells were isolated. (A) Murine IL-3-dependent growth of F-gp55-expressing cells. (B) Recombinant human EPO-dependent growth of the same subclones. OD, optical density.

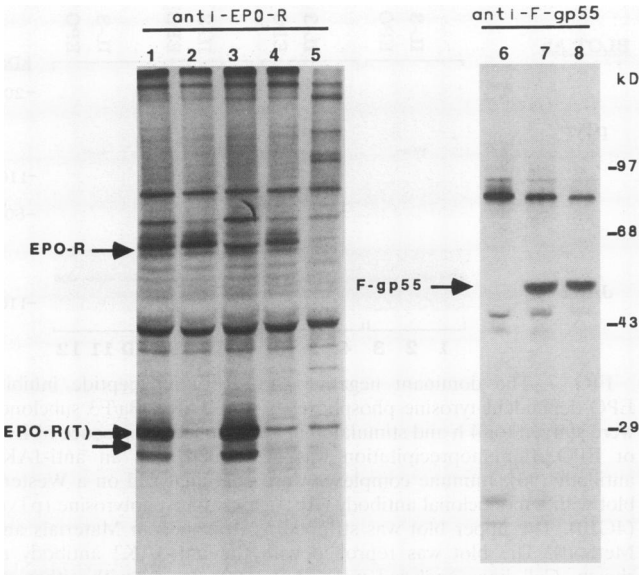

FIG. 4. Expression of wild-type EPO-R, EPO-R(T), and F-gp55 in infected Ba/F3 subclones. Ba/F3 cells expressing the indicated heterologous polypeptides were metabolically labeled and lysed. ^{35}S-labeled polypeptides were immunoprecipitated with either an anti-EPO-R N terminus antiserum (lanes 1 to 5) or an anti-F-gp55 antiserum (lanes 6 to 8) as previously described (38). Lanes: 2 and 8, Ba/F3-EPO-R subclone 1; 4, Ba/F3-EPO-R subclone 2; 1 and 7, Ba/F3-EPO-R-EPO-R(T) subclone 8; 3, Ba/F3-EPO-R-EPO-R(T) subclone 10; 5 and 6, parental Ba/F3 cells.

dependent on IL-3 (Fig. 3A). However, F-gp55 was expressed in Ba/F3-EPO-R subclone 1 (Fig. 4, lane 8) and Ba/F3-EPO-R-EPO-R(T) subclone 8 (Fig. 4, lane 7). Therefore, the lack of conversion of Ba/F3-EPO-R-EPO-R(T) subclone 3 to growth factor independence is not due to a lack of F-gp55 protein expression.

Overexpression of EPO-R(T) is required for the inhibition of EPO-dependent growth but not for the inhibition of F-gp55-dependent transformation. To evaluate the importance of the EPO-R(T) expression level to the dominant inhibitory effect, we isolated several independent Ba/F3-EPO-R-EPO-R(T) subclones (Fig. 5). Ba/F3-EPO-R cells were infected with PLXSN-EPO-R(T), and 10 individual infected subclones [Ba/F3-EPO-R-PLXSN-EPO-R(T) subclones] were isolated by limiting dilution in G418-containing IL-3 medium (Fig. 5A). These subclones expressed variable levels of EPO-R(T) in an immunoprecipitation analysis with an anti-EPO-R N terminus antiserum. Two subclones which expressed the lowest levels of EPO-R(T) (Fig. 5A, lanes 4 and 6) grew in response to EPO, while all other subclones were inhibited for EPO-dependent growth. Increased expression of EPO-R(T) in all other subclones probably resulted from the stronger promoter activity of the PLXSN-EPO-R(T) vector than of the PXM-EPO-R vector in Ba/F3 cells. These results suggested that the overexpression of EPO-R(T) was required for the dominant inhibitory effect.

To confirm these results, parental Ba/F3 cells were transfected by electroporation with PXM-EPO-R and/or PXM-EPO-R(T) (Fig. 5B). After selection in G418-containing IL-3 medium, these cells were capable of growth in response to IL-3 or EPO. Since the same PXM promoter was utilized to express both wild-type EPO-R and EPO-R(T), we expected similar levels of expression. These transfected cells were metabolically labeled, and the labeled proteins were immunoprecipitated with an anti-EPO-R antiserum. Figure 5B, lane 3, verifies that the full-length EPO-R polypeptide (66 kDa) and EPO-R(T) (29 kDa) were expressed in approximately equal amounts.

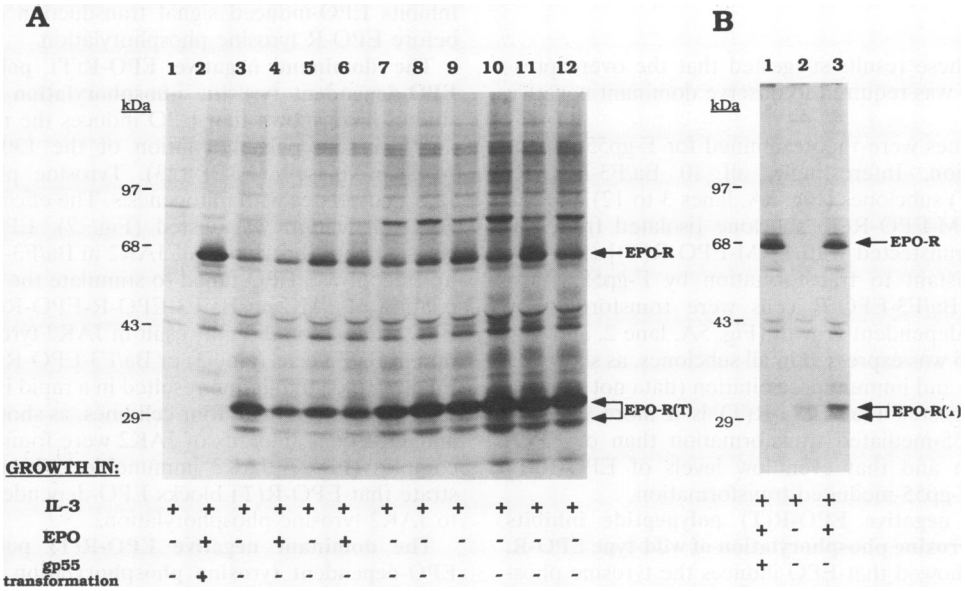

FIG. 5. Overexpression of EPO-R(T) is required for dominant negative inhibition of EPO-dependent growth. Individual Ba/F3-EPO-R-EPO-R(T) subclones were isolated by limiting dilution. Cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine, and immunoprecipitation was performed with anti-EPO-R N terminus antiserum as previously described (13). Growth in response to IL-3 or EPO and transformation by F-gp55 is shown by a plus sign. (A) Cell lines were as follows (lanes): Ba/F3 (1), Ba/F3-EPO-R (2), and Ba/F3-EPO-R-PLXSN-EPO-R(T) subclones 1 to 10 (3 to 12). (B) Cell lines were as follows (lanes): Ba/F3-EPO-R (1), Ba/F3 (2), and Ba/F3-EPO-R-PXM-EPO-R(T) (3). The migration of wild-type EPO-R and EPO-R(T) is shown by arrows.

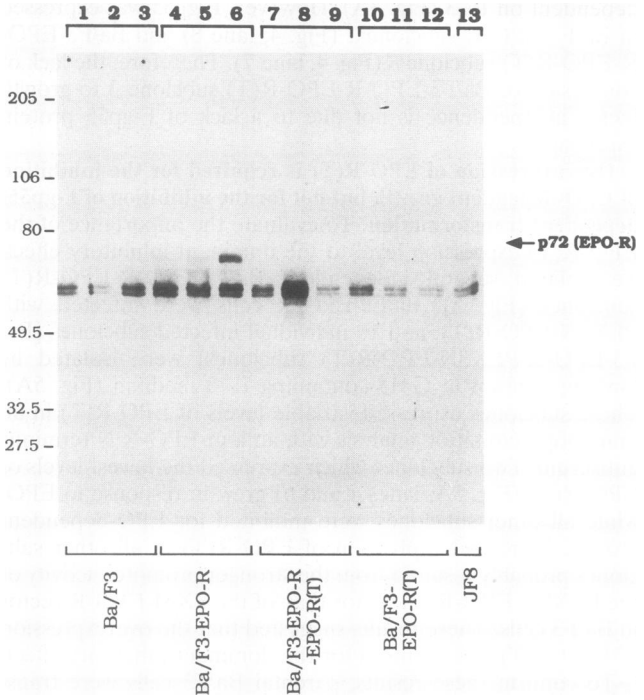

FIG. 6. The dominant negative EPO-R(T) polypeptide inhibits EPO-dependent tyrosine phosphorylation of wild-type EPO-R. Ba/F3 subclones were starved for 4 h and then stimulated in the presence or absence of IL-3 or EPO. Immunoprecipitation was performed with an antiphosphotyrosine monoclonal antibody (4G10) (39). Immune complexes were next analyzed on a Western blot with an antiserum against the carboxy terminus of EPO-R. BaF3-EPOR-EPO(JF8) is an autocrine cell line and was used as a positive control. Stimulation was done with no growth factor (lanes 1, 4, 7, and 10), IL-3 (lanes 2, 5, 8, and 11), or EPO (lanes 3, 6, 9, and 12). Tyrosine-phosphorylated EPO-R is at 72 kDa (arrow). The doublet seen at 60 kDa in all lanes is nonspecific. Molecular mass standards (in kilodaltons) are indicated.

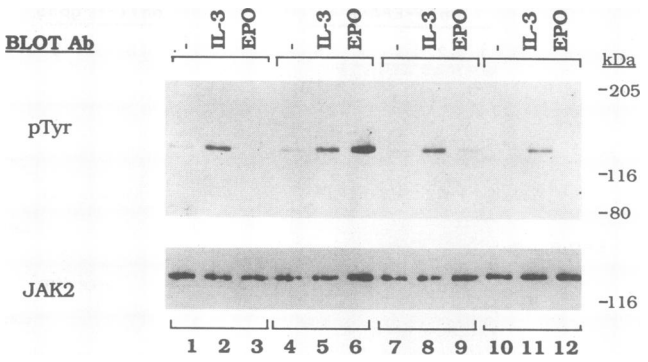

FIG. 7. The dominant negative EPO-R(T) polypeptide inhibits EPO-dependent tyrosine phosphorylation of JAK2. Ba/F3 subclones were starved for 4 h and stimulated in the absence or presence of IL-3 or EPO. Immunoprecipitation was performed with an anti-JAK2 antibody (43). Immune complexes were next analyzed on a Western blot with a monoclonal antibody (Ab) against phosphotyrosine (pTyr) (4G10). The upper blot was stripped as described in Materials and Methods. The blot was reprobed with the anti-JAK2 antibody as shown. Cell lines analyzed were Ba/F3 transfected with either no heterologous protein (lanes 1 to 3), Ba/F3-EPO-R (lanes 4 to 6), Ba/F3-EPO-R-EPO-R(T) (lanes 7 to 9), and Ba/F3-EPO-R(T) (lanes 10 to 12). Stimulation was done with no growth factor (lanes 1, 4, 7, and 10), IL-3 (lanes 2, 5, 8, and 11), or EPO (lanes 3, 6, 9, and 12). Molecular mass standards are indicated. Ab, antibody.

Taken together, these results suggested that the overexpression of EPO-R(T) was required to observe dominant negative activity.

All of these clones were then examined for F-gp55-dependent transformation. Interestingly, all 10 Ba/F3-EPO-R-PLXSN-EPO-R(T) subclones (Fig. 5A, lanes 3 to 12) and the Ba/F3-EPO-R-PXM-EPO-R(T) subclone [isolated from Ba/F3-EPO-R cells transfected with PXM-EPO-R(T)] (Fig. 5B, lane 3) were resistant to transformation by F-gp55. As a positive control, Ba/F3-EPO-R cells were transformed by F-gp55 to EPO-independent growth (Fig. 5A, lane 2, and Fig. 5B, lane 1). F-gp55 was expressed in all subclones, as shown by metabolic labeling and immunoprecipitation (data not shown). These results suggested that EPO-R(T) is a more effective inhibitor of F-gp55-mediated transformation than of EPO-dependent growth and that even low levels of EPO-R(T) effectively block F-gp55-mediated transformation.

The dominant negative EPO-R(T) polypeptide inhibits EPO-dependent tyrosine phosphorylation of wild-type EPO-R. Previous studies showed that EPO induces the tyrosine phosphorylation of the cytoplasmic tail of EPO-R (9, 15, 27). Tyrosine phosphorylation of EPO-R correlates with its mitogenic signal. To identify the cellular level at which EPO-R(T) exerts its inhibitory effect, we next assayed EPO-R tyrosine phosphorylation in transfected cells (Fig. 6). Various Ba/F3 subclones were depleted of growth factor and stimulated with either IL-3 or EPO. EPO, but not IL-3, stimulated the tyrosine phosphorylation of EPO-R in Ba/F3-EPO-R cells (lane 6), consistent with previous reports (9, 15, 27). In contrast, EPO did not stimulate the tyrosine phosphorylation of EPO-R in Ba/F3-EPO-R-EPO-R(T) cells (lane 9). As a positive control, an autocrine cell line (39) expressing both EPO-R and EPO (lane 13) demonstrated low levels of constitutive phosphorylation of the EPO-R polypeptide in the absence of exogenous growth factor. These results demonstrate that EPO-R(T) inhibits EPO-induced signal transduction at an early stage, before EPO-R tyrosine phosphorylation.

The dominant negative EPO-R(T) polypeptide inhibits EPO-dependent tyrosine phosphorylation of JAK2. Recent studies have shown that EPO induces the rapid, dose-dependent tyrosine phosphorylation of the 130-kDa cytoplasmic tyrosine kinase (JAK2) (43). Tyrosine phosphorylation of JAK2 correlates with mitogenesis. The effect of EPO-R(T) on JAK2 activation was tested (Fig. 7). EPO stimulated the tyrosine phosphorylation of JAK2 in Ba/F3-EPO-R cells (lane 6). In contrast, EPO failed to stimulate the tyrosine phosphorylation of JAK2 in Ba/F3-EPO-R-EPO-R(T) cells (lane 9). EPO stimulation did not result in JAK2 tyrosine phosphorylation in Ba/F3 cells (lane 3) or Ba/F3-EPO-R(T) cells (lane 12). Finally, IL-3 stimulation resulted in a rapid increase in tyrosine phosphorylation in all four cell lines, as shown in lanes 2, 5, 8, and 11. Equal amounts of JAK2 were found in each immune complex (Fig. 7, JAK2 immunoblot). These results demonstrate that EPO-R(T) blocks EPO-dependent signalling prior to JAK2 tyrosine phosphorylation.

The dominant negative EPO-R(T) polypeptide inhibits EPO-dependent tyrosine phosphorylation of Shc. The SH2 adaptor protein Shc (33) is tyrosine phosphorylated in response to EPO-R activation (8, 10). The phosphorylation of Shc leads to the binding of Grb2 (37), a 23-kDa adaptor protein consisting of an SH2 domain sandwiched between SH3 domains (24). Grb2, in turn, binds the mammalian SOS protein (6), which acts as a nucleotide exchanger, converting $p21^{ras}$ to

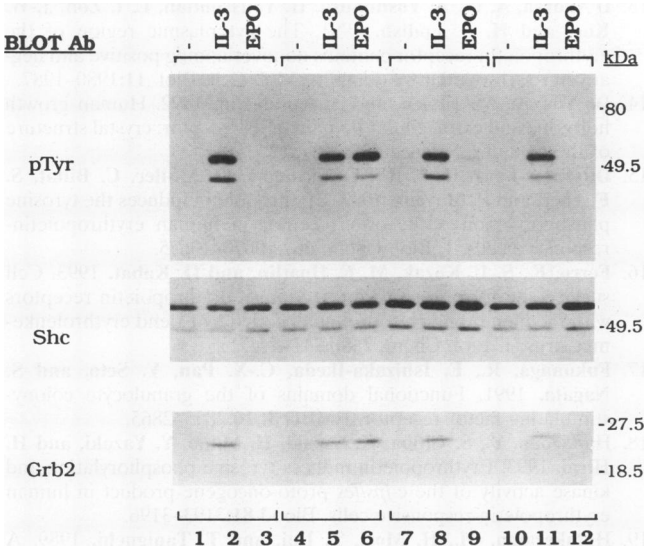

FIG. 8. The dominant negative EPO-R(T) polypeptide inhibits EPO-dependent tyrosine phosphorylation of Shc and the Grb2-Shc association. Ba/F3 subclones were starved for 4 h and stimulated in the absence or presence of IL-3 or EPO. Immunoprecipitation was performed with an anti-Shc polyclonal antibody. Immune complexes were directly analyzed by Western blotting with HRP-RC20 (pTyr). Western blotting was then performed with the anti-Shc polyclonal antibody. Finally, the membrane was incubated with an anti-Grb2 monoclonal antibody, and Western blotting was performed. Cell lines analyzed were Ba/F3 transfected with no heterologous protein (lanes 1 to 3), Ba/F3-EPO-R (lanes 4 to 6), Ba/F3-EPO-R-EPO-R(T) (lanes 7 to 9), and Ba/F3-EPO-R(T) (lanes 10 to 12). Stimulation was done with no growth factor (lanes 1, 4, 7, and 10), IL-3 (lanes 2, 5, 8, and 11), or EPO (lanes 3, 6, 9, and 12). Molecular mass standards are indicated. Ab, antibody.

the active GTP-bound state. In hematopoietic cells, Shc migrates at 52 and 46 kDa, representing products of alternative translational start sites (33). In Ba/F3-EPO-R cells, Shc was phosphorylated in response to EPO (Fig. 8, lane 6). The tyrosine phosphorylation of Shc was decreased in Ba/F3-EPO-R-EPO-R(T) cells stimulated with EPO (lane 9). Ba/F3 and Ba/F3-EPO-R(T) cells did not demonstrate the tyrosine phosphorylation of Shc in response to EPO (lanes 3 and 12, respectively). IL-3 resulted in the rapid tyrosine phosphorylation of Shc in all subclones (lanes 2, 5, 8, and 11). Equivalent amounts of Shc were immunoprecipitated from each cell lysate (Fig. 8, Shc immunoblot). In each case, the activation of Shc tyrosine phosphorylation resulted in the coimmunoprecipitation of Grb2 (Fig. 8, Grb2 immunoblot).

DISCUSSION

We have studied the biological properties of EPO-R(T), which contains only the Box 1 sequence (17) and thereby lacks the critical positive regulatory domain previously described (13, 19, 34). The overexpression of EPO-R(T) inhibits the EPO-dependent growth induced by wild-type EPO-R and blocks the transformation induced by F-gp55. The coexpression of the dominant negative form of EPO-R blocks the EPO-dependent tyrosine phosphorylation of full-length EPO-R and the EPO-dependent tyrosine phosphorylation of the cytoplasmic effector proteins JAK2 and Shc. The tyrosine phosphorylation of EPO-R and JAK2 has been shown to correlate with mitogenic activity and sustained cell growth (43).

Several possible mechanisms could account for the inhibitory effect of EPO-R(T). First, EPO-R(T) may compete with wild-type EPO-R for available EPO at the cell surface. This mechanism seems unlikely, since inhibition by EPO-R(T) is observed even at high (5 nM) concentrations of EPO. Second, EPO-R(T), when overexpressed, may interfere with the normal biosynthesis and cell surface translocation of wild-type EPO-R. This mechanism is unlikely, since wild-type EPO-R and EPO-R(T) each demonstrate efficient cell surface transport in singly transfected cells. Also, the expression of EPO-R(T) has no effect on the level of expression of wild-type EPO-R in infected cells, as judged by immunoprecipitation (Fig. 4). Third, EPO-R(T) may compete with wild-type EPO-R for binding to some unidentified cytoplasmic signalling protein. Although EPO-R(T) contains the Box 1 sequence, earlier studies showed that sequences carboxy terminal to Box 1 are required for JAK2 activation (43). Recent studies demonstrated that EPO-R and a truncated EPO-R identical in structure to EPO-R(T), described here, could be efficiently coimmunoprecipitated with an anti-EPO-R C terminus antibody (29). When these three possible mechanisms are taken together, the dominant negative effect of EPO-R(T) is consistent with a model of homodimerization or homooligomerization of wild-type EPO-R.

Interestingly, the coexpression of the EPO-R(T) polypeptide and the full-length EPO-R polypeptide also blocks the F-gp55 transformation of Ba/F3 cells to growth factor independence. There are multiple mechanisms which could account for this inhibition of F-gp55 transformation. First, the overexpression of EPO-R(T) on the cell surface could interfere with retroviral infection and the subsequent expression of transduced F-gp55. This was not the case, however, since all Ba/F3-EPO-R-EPO-R(T) subclones expressed F-gp55 following infection. Second, F-gp55 could preferentially bind to EPO-R(T); however, our results did not demonstrate the coimmunoprecipitation of F-gp55 and EPO-R(T). Third, EPO-R(T) could block the productive interaction between EPO-R and F-gp55. This latter mechanism is supported by our data (Fig. 3B). Our analysis of 10 independent subclones of Ba/F3-EPO-R-EPO-R(T) cells (Fig. 5) demonstrated that EPO-R(T) is a more effective inhibitor of F-gp55-mediated growth than of EPO-mediated growth. Eight of the clones failed to grow in response to EPO, but all 10 clones were resistant to F-gp55 transformation. The presence of even a low level of EPO-R(T) in the cells prevented the coimmunoprecipitation of F-gp55 and wild-type EPO-R. These results suggest that EPO is a more potent activator of EPO-R than is F-gp55, although other explanations are possible. Finally, the demonstration of a dominant negative EPO-R(T) polypeptide which blocks F-gp55-induced transformation further supports the specific role that F-gp55 plays in Friend virus-induced erythroleukemia.

EPO-R(T) specifically inhibits growth mediated by wild-type EPO-R and not by the IL-3R complex. This fact suggests that EPO-R(T) forms nonproductive oligomeric complexes with wild-type EPO-R and not with components of the IL-3R complex. Consistent with this model, the β_{IL-3} or β_c polypeptides do not coimmunoprecipitate with EPO-R from Ba/F3 cell lysates. In addition, EPO-R(T) does not appear to be inhibitory by its interaction with some other signal-transducing polypeptide which is common to the EPO-R and IL-3R signalling pathways. Accordingly, the IL-3-induced tyrosine phosphorylation of JAK2 and Shc in Ba/F3-EPO-R-EPO-R(T) cells is normal.

Finally, the activation of EPO-R results in the rapid, dose-dependent tyrosine phosphorylation of EPO-R (9, 15, 27) and of several cellular substrates (18, 35), including JAK2 (43) and Shc (8, 10). Our data show that the overexpression of EPO-R(T) specifically blocks early tyrosine phosphorylation events, further supporting the critical importance of these proteins in EPO-R-mediated signal transduction. Further studies will be required to determine whether the dominant negative EPO-R(T) polypeptide actually blocks the direct association of JAK2 or Shc with EPO-R.

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