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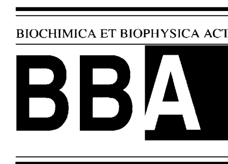

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Erythropoietin hypersensitivity in primary familial and congenital polycythemia: Role of tyrosines Y^{285} and Y^{344} in erythropoietin receptor cytoplasmic domain

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Abstract

Erythropoietin receptor (EPOR) gene mutations leading to truncations of the cytoplasmic, carboxy-terminal region of EPOR have been described in some patients with primary familial and congenital polycythemia (PFCP), a disorder characterized by isolated erythrocytosis and increased sensitivity of erythroid progenitors to Epo. We studied the role of EPOR in the pathogenesis of PFCP and the requirement for intracytoplasmic tyrosine residues Y^{285} and Y^{344} in generation of Epo hypersensitivity phenotype. Interleukin-3-dependent hematopoietic cells were engineered to express variant human EPORs using retrovirus-mediated gene transfer. We introduced tyrosine to phenylalanine substitutions in EPOR-ME, a naturally occurring, mutant human EPOR (G5881T), truncated by 110 carboxy-terminal amino acids and associated with autosomal dominantly inherited PFCP. Cells expressing EPOR-ME exhibited increased Epo sensitivity compared to cells expressing wild type EPOR. Mutation of Y^{285} alone had a relatively minor effect on Epo hypersensitivity whereas mutation of Y^{344} resulted in loss of increased Epo sensitivity. Expression of a tyrosine-null truncated EPOR conferred further decrease of Epo-mediated proliferation suggesting that both Y^{285} and Y^{344} may contribute to proliferation signals. In the context of EPOR-ME, Y^{344} was required for Epo-induced Stat5 tyrosine phosphorylation. The positive effect of either Y^{285} or Y^{344} on cellular proliferation was associated with Epo-induced tyrosine phosphorylation of Stat1. These findings suggest that both tyrosine residues Y^{285} and Y^{344} in the cytoplasmic domain of EPOR-ME may contribute to increased Epo sensitivity that is characteristic of PFCP phenotype.
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1. Introduction

The production of blood cells is regulated by various extracellular stimuli including a network of hematopoietic cytokines that exert their biologic effects through specific interactions with their cognate cell surface receptors. Erythropoietin (Epo) is a glycoprotein hormone that is the principal hematopoietic cytokine required for the regulation of mammalian erythropoiesis [1–3]. The biologic effects of Epo in erythroid progenitor cells are mediated through its binding to EPOR, its specific cell surface receptor [4–6]. The EPOR is a member of the Type I cytokine receptor family that includes cellular transmembrane receptors for factors such as granulocyte-colony stimulating factor (G-CSF), many of the interleukins, prolactin and growth hormone [7,8]. Typical of other members of the cytokine receptor family, the EPOR exhibits no intrinsic tyrosine kinase enzymatic activity and Epo–EPOR signaling is associated with activation of the cytoplasmic Janus protein tyrosine kinase Jak2 [9] that is required for EPOR function [10,11]. The binding of Epo to the extracellular region of EPOR leads to a conformational change of receptor homodimers resulting in the rapid tyrosine phosphorylation of Jak2 upon receptor engagement [12,13] and activation of Jak2 in turn is associated with the phosphorylation of EPOR

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on its cytoplasmic tyrosine residues that are recognized by various Src homology 2 (SH2)-domain containing signaling molecules including Stat5, a cryptic cytoplasmic transcription factor that is converted into its active form following its tyrosine phosphorylation by Jak2 [14,15].

The cytoplasmic domain of EPOR contains conserved subdomains including box 1 and box 2 motifs within the membrane-proximal region that are important for Jak2 activation [16,17] and Epo-induced mitogenic signaling [18,19]. The membrane-distal cytoplasmic domain of EPOR, a region that is dispensible for mitogenic signaling in cell lines and for in vivo erythropoiesis [20], is required for the activation of a number of intracellular signaling molecules such as mitogen activated protein kinases [21], the protein tyrosine phosphatase SHP-2 [22], phosphatidylinositol 3-kinase [23,24] as well as negative regulators of EPOR signaling such as SHP-1 [25–27] and CIS [28–30] that normally downregulate EPOR signaling activity. Previous experiments in hematopoietic cell lines expressing carboxy-terminal truncations of murine EPORs have suggested a negative role for the membrane-distal domain on receptor function [31,32].

Primary familial and congenital polycythemia (PFCP), also known as familial erythrocytosis, is a proliferative disorder affecting bone marrow progenitor cells of the erythroid lineage leading to isolated erythrocytosis in the peripheral blood [33,34]. The disorder is typically associated with an autosomal dominant mode of inheritance although it may also occur sporadically. The clinical features of PFCP include marked increase of the red blood cell mass associated with normal leukocyte and thrombocyte counts, low or low-normal serum Epo levels, absence of splenomegaly and lack of progression to clonal bone marrow disorders and leukemia. Erythroid progenitors from subjects with PFCP exhibit increased sensitivity to exogenous Epo in serum-containing, semi-solid medium erythroid colony formation assays [34–36]. In a number PFCP subjects, mutations in the gene encoding the EPOR have been described where heterozygosity for dominant, gain-of-function EPOR mutations that lead to truncation of the membrane-distal cytoplasmic region of EPOR have been associated with clinical erythrocytosis and increased sensitivity of erythroid progenitors to Epo [35–38]. Previous studies in our laboratory as well as others have shown that cultured hematopoietic cells engineered to express these mutant human EPOR forms exhibit increased Epo sensitivity [35,36,38,39] that is associated with deregulation of the rates of Jak2 and Stat5 inactivation [39,40]. We have been interested in investigating the role of EPOR in the molecular pathogenesis of PFCP with the goal of defining EPOR-mediated signaling responses that relate to changes in the sensitivity of hematopoietic cells to Epo.

In this study, we characterized structural requirements for generation of increased Epo sensitivity associated with truncated human EPOR expression and PFCP. Specifically, we examined the ability of the membrane-proximal domain of a truncated human EPOR mutant and its cytoplasmic tyrosine residues to transduce intracellular signals in response to Epo. We took advantage of a novel EPOR mutation G5881T (EPOR-ME) that is associated with an extensive 110 amino acid carboxy-terminal truncation and autosomal dominantly inherited PFCP [36]. This EPOR mutation results in the deletion of seven of nine intracytoplasmic tyrosine residues of human EPOR. We determined the role of the remaining, membrane-proximal EPOR cytoplasmic tyrosines Y^{285} and Y^{344} in generation of increased Epo sensitivity and PFCP phenotype associated with EPOR-ME and studied Epo-mediated signaling in hematopoietic cells engineered to express mutant human EPOR. The data indicates that introduction of phenylalanine amino acid substitution for Y^{285} in EPOR-ME alone has a relatively minor effect on Epo hypersensitivity whereas mutation of Y^{344} leads to loss of increased Epo sensitivity. A tyrosine-null EPOR-ME mutant eliminating both tyrosines Y^{285} and Y^{344} results in further decrease of Epo-mediated proliferation suggesting that both tyrosine residues may contribute to Epo-induced proliferation signals. Jak2 tyrosine phosphorylation in response to Epo was prolonged in cells expressing truncated EPOR variants, including the tyrosine-null mutant, compared to cells expressing wild type EPOR. We found that in the context of EPOR-ME, Y^{344} was required for Epo-induced Stat5 tyrosine phosphorylation. Furthermore, the positive effects of EPOR tyrosines Y^{285} or Y^{344} on cellular proliferation were associated with Epo-induced tyrosine phosphorylation of Stat1 in hematopoietic cells.

2. Materials and methods

2.1. Cell lines, culture conditions and reagents

Murine interleukin-3 (IL-3)-dependent 32D cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% heat-inactivated fetal bovine serum, (FBS, Hyclone, Logan, UT), 10% conditioned medium from the WEHI-3B cell line as a source of IL-3 and 1% streptomycin–penicillin. Untransfected and transfected 32D cells were maintained at a culture density of less than 5×10^5/ml. Recombinant human Epo (rhEpo) was generously provided by Amgen (Thousand Oaks, CA) and murine IL-3 was purchased from Stem Cell Technologies (Vancouver, BC). rhEpo was conjugated with biotin using procedures described previously [41].

2.2. Plasmids and transfections

Human wild type (WT) and mutant (ME) EPOR complementary DNAs (cDNA) were cloned into the pBabe-puro retroviral vector [36,42]. EPOR-ME lacks seven of nine cytoplamic tyrosine residues. Site-directed mutagenesis of the remaining two tyrosine amino acids to

phenylalanine to generate $Y^{344}F$ (YM1), $Y^{285}F$ (YM3) or both mutations (YM2) within the cytoplasmic region of EPOR-ME was carried out by primer-mediated mutagenesis using polymerase chain reaction and EPOR-ME cDNA as template that contains the G5881T mutation isolated from erythroid progenitors of a PFCP subject [36]. To generate $Y^{344}F$ or $Y^{285}F$ amino acid substitutions in the cytoplasmic domain, oligonucleotides were synthesized which incorporated a single adenine to thymidine mutation at nucleotide position 1208 (5′-GTGAGCATGCCCAGGATACCT**T**TCTGGTGCT-3′) or position 1031 (5′-TTTGAAGGCCTCTTCACCACCCACAAGGGTAACT**T**CCAGCTGTGGCTGTTCCAGAATGAT-3′), respectively, in EPOR-ME cDNA according to numbering in Genbank sequence M60459 [5]. PCR reactions were performed using an antisense primer in 3′ untranslated region of EPOR (5′-CTGAGAGAGGCCTCGCCAT-3′) or Sp6 promoter primer in flanking pGEM4 plasmid sequence. To generate EPOR-ME with double $Y^{285}F$ and $Y^{344}F$ mutations (YM2), a second primer-mediated mutagenesis was carried out using the oligonucleotide primer containing A1031T mutation and mutant EPOR-ME $Y^{344}F$ (YM1) plasmid as template in the PCR reaction. The PCR amplification products were cloned into a plasmid to replace a fragment of EPOR-ME cDNA encoding the cytoplasmic domain and the resultant mutant EPOR cDNAs were cloned into the retroviral vector pBabe-puro. The presence of the introduced mutations and the integrity of the resulting plasmids were confirmed by restriction endonuclease digestion and DNA sequencing. The constructed expression plasmids were transfected into amphotropic retroviral producer cell line PA317 using Lipofectamine reagent (Invitrogen, Carlsbad, CA) and transfected cells were selected by growth in 3.5 μg/ml puromycin (Sigma, St. Louis, MO). Stably transfected, puromycin-resistant PA317 cells grown to 50% confluence in a 6 cm tissue culture dish were co-cultured with 5×10^5 32D cells for 24 h in the presence of tissue culture medium for 32D cells and 4 μg/ml polybrene (Sigma) and 32D cells infected with retrovirus were selected in IMDM/FBS/WEHI containing 1 μg/ml puromycin. Single cell clones of puromycin-resistant 32D cells were isolated by limiting dilution, and multiple clones were expanded for further analysis.

2.3. Analysis of EPOR expression

To confirm EPOR expression and to select clones that express comparable surface EPOR for further studies, cells (1×10^5) were incubated with biotin-labeled Epo for 1 h at 37 °C as described [41]. After incubation, cells were washed and stained with phycoerythrin-conjugated streptavidin (SA-PE) for 1 h at 4 °C. Then, the cells were washed and analyzed by flow cytometry (Becton Dickinson, San Jose, CA). As negative staining controls, cells were incubated with SA-PE only. In addition, untransfected 32D cells and 32D cells transfected with empty pBabe-puro vector were analyzed for biotin-Epo binding as negative controls. In some experiments, to demonstrate specificity of biotin-Epo binding, cells were pre-incubated with 5-fold molar excess of unlabelled Epo for 15 min at 37 °C prior to sequential incubation with biotin-Epo and SA-PE. Three independent clones of each cell line expressing tyrosine-mutant EPOR constructs were selected on the basis of comparable surface EPOR expression.

2.4. Measurement of cell proliferation

Transfected 32D cells were washed free of serum and WEHI-conditioned medium and incubated at an initial density of 1×10^5 cells/ml in medium containing IMDM and 10% FBS supplemented with murine IL-3 (10 ng/ml) or rhEpo at the indicated concentrations or without supplemental growth factors. Cells were cultured in triplicates and viable cells were counted daily for 5 days using trypan-blue exclusion technique. Three independent clones of cells expressing the tyrosine-mutant EPOR constructs were analyzed for Epo-mediated proliferation and the total daily cell count results were expressed as mean ± standard error.

2.5. Antibodies, gel electrophoresis and immunoblotting

Phosphotyrosine-specific antibodies against Stat5 (Tyr694) and Stat1 (Tyr701) were from Cell Signaling Technologies (Beverly, MA), phosphotyrosine-specific antibody against Jak2 (Tyr 1007/1008) was from Biosource International (Camarillo, CA), antibody against total Stat5 that detects Stat5a/b isoforms (N-20, cat. number sc-836) was from Santa Cruz Biotechnology (Santa Cruz, CA), anti-Stat1 (C-terminus) was from BD Biosciences (San Diego, CA), anti-Jak2 antibody to detect total Jak2 was purchased from Upstate Biotechnology (Lake Placid, NY). All antibodies were used in accordance with the manufacturer's recommendations. Horseradish peroxidase (HRP)-conjugated secondary anti-rabbit IgG antibodies were purchased from Pierce (Rockford, IL). In some experiments, cells were washed with PBS and deprived of serum and growth factors as described prior to stimulation with indicated concentrations of Epo or IL-3 [40]. To prepare whole cell lysates, cells were washed twice with ice-cold PBS and lysed in ice-cold buffer containing 1% Triton X-100, 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 5 mM EDTA, 10 mM NaF, supplemented with 1 mM Na_3VO_4, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml aprotinin and 10 μg/ml leupeptin followed by centrifugation at 13,000 ×g for 15 min at 4 °C. The soluble proteins were mixed with 2X Laemmli sample buffer, boiled at 95 °C for 5 min and separated by 8% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and separated proteins were electrophoretically transferred to PVDF (Immobilon-P) membranes (Millipore, Bedford, MA). To block residual binding sites, the membranes were incubated with TBST (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% (v/v) Tween-

20) containing 5% nonfat dry milk for 1–2 h at room temperature. Immunoblots were then incubated overnight at 4 °C with the indicated primary antibodies at a dilution according to the manufacturer's instructions. After washing with TBST, the blots were incubated with appropriate HRP-conjugated secondary antibodies (1:20,000) for 1 h at room temperature, washed 3 times with TBST and the immune complexes were detected using SuperSignal Chemiluminescent Substrate (Pierce, Rockford, IL) and autoradiography. In some experiments, the membranes were stripped in buffer containing 62.5 mM Tris–HCl, [pH 6.7], 2% SDS and 100 mM β-mercaptoethanol at 50 °C for 30 min, reblocked, washed and reprobed.

3. Results and discussion

3.1. Expression of human EPORs in IL-3-dependent hematopoietic cells

We studied the contribution of human EPOR cytoplasmic domain tyrosines Y^{285} and Y^{344} to receptor function in the context of a naturally occurring truncated EPOR associated with PFCP. In addition to the full length, wild-type human EPOR (WT) and truncated EPOR-ME isolated from a patient with PFCP, we analyzed the function of variant EPOR-ME receptors with single Tyr→Phe substitutions retaining either the membrane proximal Y^{285} (YM1) or membrane distal tyrosine Y^{344} (YM3), as well as a tyrosine-null EPOR variant (YM2) generated by site-directed mutagenesis (Fig. 1). Retroviruses encoding these variant receptors were introduced into the murine IL-3-dependent 32D cell line that normally does not proliferate in response to Epo [35,36]. Single cell clones of puromycin-resistant 32D cells were isolated and examined for cell surface expression of transfected EPOR using flow cytometric analysis and clones expressing comparable levels of mutant receptors were selected for further analysis. Results of a representative analysis of surface EPOR expression as determined by the biotinylated-Epo binding assay are illustrated in Fig. 2. No specific binding of biotin–Epo was observed in the parental 32D cells (32D untransfected) or cells transfected with empty pBabe-puro vector (32D vector) as negative controls. Comparable binding of biotin–Epo was observed in cell lines transfected with expression plasmids encoding the WT, ME and each of the variant, truncated EPOR forms. Specificity of biotin–Epo binding was demonstrated by the ability of unconjugated rhEpo to compete for the binding of biotin-conjugated Epo to the cells (Table 1).

3.2. Cells expressing EPOR-ME exhibit increased Epo sensitivity compared to WT cells

We determined the ability of hematopoietic cells engineered to express the different EPOR forms to proliferate in the presence of Epo. Growth curves were generated by culturing the cells in increasing concentrations of Epo (range 0–10 units/ml) instead of WEHI-conditioned medium containing IL-3 (Fig. 3). All 32D cell lines expressing EPORs were growth factor-dependent. As a positive control, all cell lines were cultured in recombinant murine IL-3 (10 ng/ml) and exhibited similar growth curves. As negative controls, untransfected parental 32D cells or 32D cells transfected with empty pBabe-puro retroviral vector were unable to proliferate or survive even in the presence of maximal Epo concentrations (data not shown). Consistent with previous findings in our laboratory as well as others using hematopoietic cells expressing truncated PFCP-associated EPOR forms, we observed that 32D cells that express EPOR-ME could proliferate in the lowest Epo concentrations of 0.01–0.1 units/ml (Fig. 3) and thus exhibited increased Epo sensitivity compared to WT cells that express the full length human EPOR [35,36,38,43].

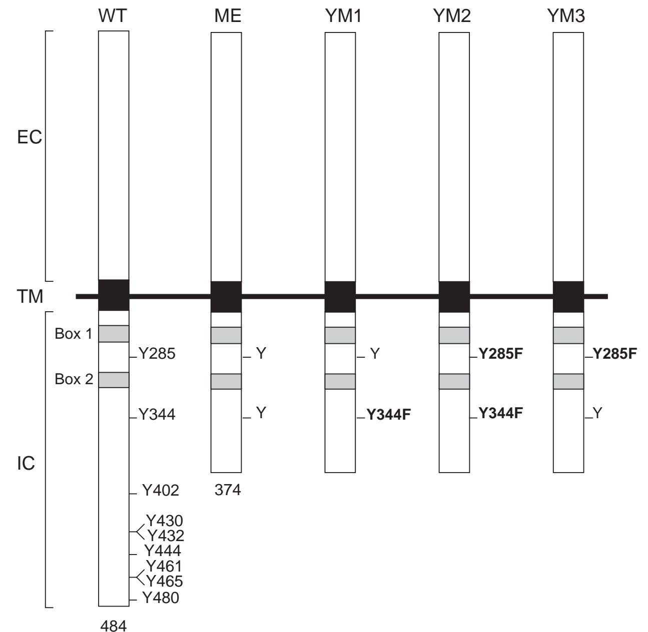


Fig. 1. Structures of the wild type and mutant human EPORs. A schematic diagram illustrates the extracellular (EC), transmembrane (TM) and intracellular (IC) domains of the wild type (WT, 484 amino acids) and truncated ME receptors (374 amino acids). The hydrophobic leader sequence consisting of the first 24 amino acids is not taken into account in the numbering. The cytokine receptor family homologous regions box 1 and 2 and the positions of the nine tyrosine residues in the full-length human EPOR cytoplasmic domain are indicated by "Y". In the YM1 mutant the membrane-distal tyrosine Y^{344} and in YM3 mutant the membrane proximal Y^{285} were replaced with phenylalanine (F). In the YM2 mutant both tyrosines Y^{285} and Y^{344} were replaced with phenylalanines generating a tyrosine-null EPOR-ME.

3.3. EPOR-ME tyrosines Y^{285} and Y^{344} contribute to Epo-induced proliferation signals but they are dispensible for proliferation in high ligand concentrations

The tyrosine residue Y^{344} in the human EPOR corresponds to Y^{343} in the murine EPOR, a tyrosine that

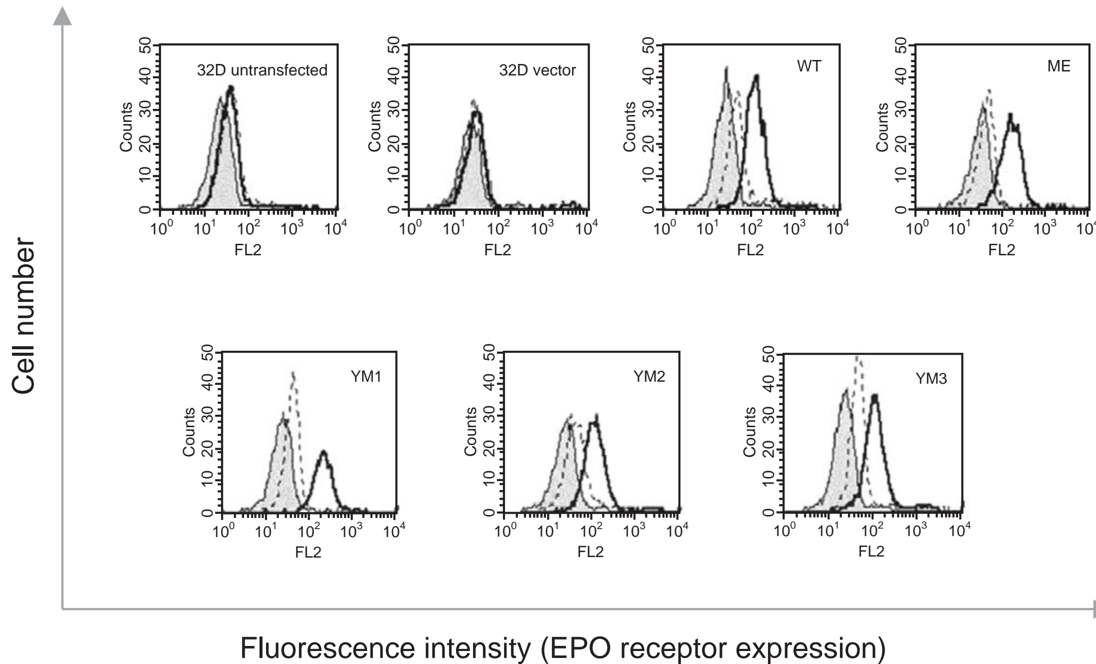


Fig. 2. Expression of cell surface EPORs in 32D cells. Flow cytometric analysis of EPOR expression was performed on parental untransfected 32D cells or cells transfected with empty pBabe-puro vector (negative controls) and the receptor constructs illustrated in Fig. 1. Cells were stained with biotinylated rhEpo followed by phycoerythrin-conjugated streptavidin (filled bold line), without biotinylated-Epo incubation step (gray shaded area) or with brief pre-incubation in a 5-fold molar excess of unconjugated rhEpo (dashed line) and analyzed for fluorescence (FL2). Table 1 illustrates the relative fluorescence intensity for each cell line.

positively modulates Epo-induced proliferation [44]. The function of Y^{285}, a tyrosine that is unique to the human EPOR, has not been previously investigated. To determine the role of membrane-proximal receptor tyrosines in mediating Epo-induced proliferation of cells expressing EPOR-ME, we analyzed the growth of 32D transfectants expressing Tyr→Phe mutants in response to Epo. Mutation of the membrane-distal tyrosine residue Y^{344} to phenylalanine in EPOR-ME (YM1) was associated with reduced Epo sensitivity compared to ME cells in response to 0.01–0.1 units/ml of Epo consistent with a positive effect of Y^{344} on proliferation (Fig. 3). Introduction of Tyr→Phe substitution of the membrane-proximal tyrosine residue Y^{285} in EPOR-ME (YM3 cells) was associated with a minor effect on the proliferation of YM3 cells in comparison to ME cells, as YM3 cells exhibited increased Epo sensitivity in low Epo concentration of 0.01–0.1 units/ml compared to WT cells (Fig. 3). To further investigate the contribution of Y^{285} to Epo-induced proliferation, we determined the proliferation of cells expressing the tyrosine-null EPOR-ME (YM2) and found reduced overall Epo-induced proliferation of YM2 cells compared to YM1 cells at all Epo concentrations, consistent with the ability of Y^{285} in human EPOR to contribute to Epo-mediated proliferation in the context of EPOR-ME. Although the Epo dose–response of cells expressing the tyrosine-null EPOR (YM2) showed decreased Epo responsiveness, this cell line could proliferate in response to highest Epo concentration (10 units/ml), demonstrating that cytoplasmic tyrosine residues in EPOR-ME are dispensable for receptor mitogenic function.

Table 1
Binding of biotin-labeled Epo to untransfected and transfected 32D cells

Relative fluorescence intensity			
Cell line	Control	Biotin–Epo	Epo+biotin–Epo
32D	1.00	1.4	1.6
Vector	1.00	1.2	1.2
WT	1.00	4.8	1.8
ME	1.00	5	1.4
YM1	1.00	7.5	1.7
YM2	1.00	5.6	1.7
YM3	1.00	5.1	2

Flow cytometric analysis of EPOR expression was performed on parental untransfected 32D cells and transfected 32D cells stained with biotin-conjugated rhEpo (biotin–Epo) followed by an incubation with phycoerythrin-conjugated streptavidin (Fig. 2). Specificity of biotin–Epo binding was determined by preincubation of the cells with 5-fold molar excess of unlabelled rhEpo (Epo+biotin–Epo) as a competitor for biotin–Epo binding. Controls represent cells stained with phycoerythrin–streptavidin only, the fluorescence intensity of which was defined as 1.00. The numbers indicate fold change in fluorescence intensity relative to controls.

3.4. Epo-induced tyrosine phosphorylation of Jak2 in 32D cells expressing variant human EPORs

The G5881T mutation in EPOR-ME results in the most extensive naturally occurring EPOR truncation reported to

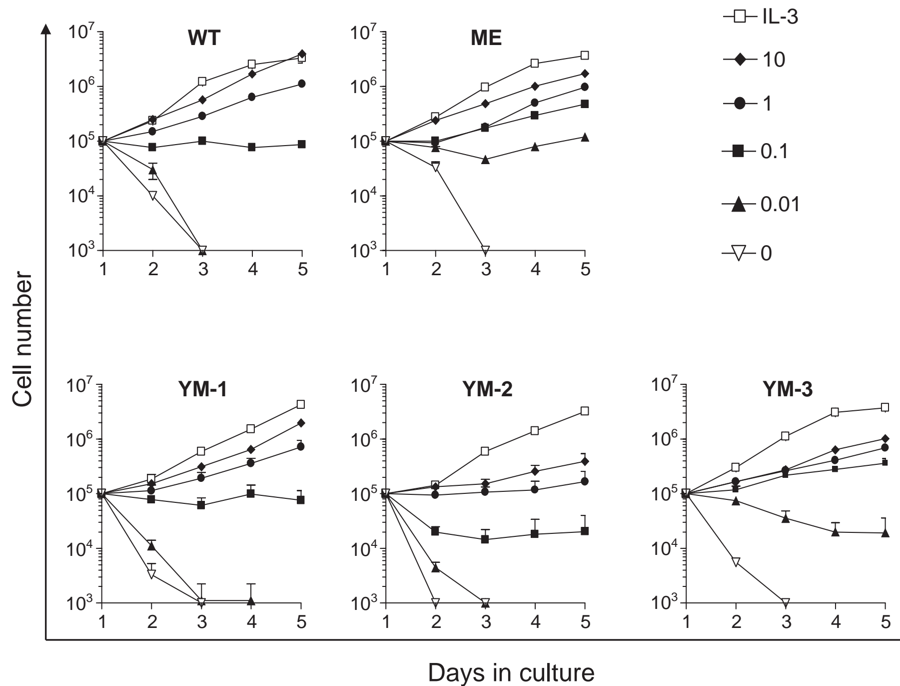


Fig. 3. Epo-dependent proliferation of 32D cells expressing wild type and mutant EPOR forms. Growth curves of transfected 32D cells were generated by counting the cells daily for a period of 5 days. Cells were washed extensively to remove WEHI-conditioned medium and then cultured in growth medium containing IMDM/10% FBS supplemented with either IL-3 (10 ng/ml) or the indicated concentrations of human Epo. A plot of total cell numbers are expressed as mean ± standard error as described in Materials and methods.

date, deleting 110 of the cytoplasmic amino acids in the membrane-distal negative regulatory domain [36]. Prolonged Jak2 tyrosine phosphorylation in transfected hematopoietic cells expressing truncated murine EPOR has been implicated in generation of Epo hypersensitivity, associated with deletion of Y^{429} and Y^{431} required for the binding of the protein tyrosine phosphatase SHP-1 that downregulates Jak2 [25,27]. Consistent with these studies, previous work in our laboratory as well as others demonstrated deregulation of the rate of Jak2 inactivation in hematopoietic cells that express different truncated human EPOR mutants associated with familial polycythemia [35,39,40]. We investigated the ability of EPOR-ME and the Tyr→Phe mutant receptor forms to mediate Jak2 tyrosine phosphorylation and its time course (Fig. 4). After cytokine and serum starvation, transfected cells were stimulated with Epo (10 units/ml) or left unstimulated as indicated and cell lysates were subjected to SDS-PAGE and immunoblotting using anti-phospho-Jak2 antibody. Epo treatment of the cells induced the tyrosine phosphorylation of Jak2 by 10 min in all transfected cell lines (Fig. 4). In WT cells, Jak2 tyrosine phosphorylation returned to basal levels by 30 min whereas in ME cells Jak2 tyrosine phosphorylation remained detectable 120 min following Epo stimulation, consistent with our previous functional studies of a different human EPOR mutant truncated by 59 carboxy-terminal amino acids [40]. In 32D cells expressing the Tyr→Phe mutant EPORs, Epo treatment also induced the tyrosine phosphorylation of Jak2 that was prolonged compared to WT cells (Fig. 4), consistent with decreased rate of Jak2 inactivation in these cells.

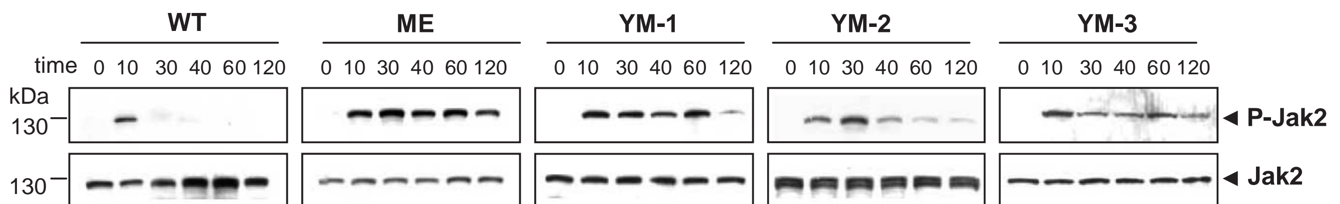


Fig. 4. Epo-induced tyrosine phosphorylation of Jak2 in 32D cells expressing wild type or mutant EPORs. Stably transfected 32D cells expressing the wild-type (WT) or indicated mutant EPORs were deprived of serum and growth factors and then either left unstimulated (0) or stimulated with Epo (10 units/ml) and harvested at the indicated time points (range 10–120 min) as described in Ref. [40]. Cell lysates (100 μg protein) were immunoblotted with anti-phospho-Jak2 antibody to detect tyrosine phosphorylated Jak2 (P-Jak2, upper panels). The blots were then stripped and hybridized to anti-Jak2 antibody to detect total Jak2 protein in each lane (lower panels). Arrows indicate 130 kDa immunoreactivity corresponding to tyrosine phosphorylated Jak2 (P-Jak2) and total Jak2.

3.5. Tyrosine Y^{344} in cytoplasmic domain of EPOR-ME is required for Epo-induced tyrosine phosphorylation of Stat5

Epo-mediated activation of Stat5 plays a role in the regulation of in vivo erythropoiesis as demonstrated by the presence of anemia in mouse embryos that are deficient in Stat5a/b [20,45,46] and in adult mice that exhibit ineffective erythropoiesis [47]. Several distinct tyrosine residues in the cytoplasmic domain of murine EPOR have been implicated in the tyrosine phosphorylation and activation of Stat5, including Y^{343} that corresponds to Y^{344} in the human EPOR [44,48]. We investigated the role of human EPOR cytoplasmic tyrosine residues Y^{285} and Y^{344} in tyrosine phosphorylation of Stat5 in the context of the truncated EPOR-ME. Epo treatment of cells expressing wild-type EPOR (WT) or EPOR-ME resulted in the tyrosine phosphorylation of Stat5 as detected by Western blotting (Fig. 5A). In YM1 cells, introduction of Y^{344}F mutation in EPOR abolished Epo-induced tyrosine phosphorylation of Stat5 demonstrating the inability of Y^{285} to mediate Stat5 tyrosine phosphorylation. In contrast, in YM3 cells that retain Y^{344}, Stat5 was tyrosine phosphorylated following Epo treatment (Fig. 5A). In YM2 cells that express a tyrosine-null truncated EPOR, Stat5 was not tyrosine phosphorylated in response to Epo (Fig.5A). We also examined the time course of Stat5 tyrosine phosphorylation in 32D transfectants and found prolonged Epo-induced tyrosine phosphorylation of Stat5 in ME cells compared to WT cells (Fig. 5B). In WT cells, tyrosine phosphorylation of Stat5 was observed by 10 min following Epo treatment and the phosphorylation was decreased at 40 min and returned to basal levels by 60 min. In contrast, in ME cells, Epo-induced tyrosine phosphorylation of Stat5 persisted for 120 min, consistent with previous results from our laboratory and others demonstrating prolonged Epo-induced tyrosine phosphorylation of Stat5 in 32D cells expressing different human EPOR mutants truncated by 59 or 75 amino acids in the carboxy-terminal domain compared to 32D cells expressing the wild-type EPOR [39,40]. Stat5 tyrosine phosphorylation was also prolonged in YM3 cells that exhibited increased sensitivity to Epo (Fig. 5B).

These data indicate that Y^{344} of human EPOR is required for the tyrosine phosphorylation of Stat5 in the context of the truncated human EPOR-ME. Generation of increased Epo sensitivity in ME and YM3 cells is associated with the ability of Y^{344} to mediate Stat5 activation. The data also indicates that Stat5 activation was not required for Epo-mediated cellular proliferation in response to high Epo concentration because YM1 and YM2 cells could proliferate despite the inability of mutant Y^{344}F EPOR to activate Stat5. Taken together, these findings suggest that Stat5 activation, although not essential for Epo-stimulated cellular proliferation, appears to be required for maximal Epo responsiveness, consistent with findings of in vivo studies demonstrating the requirement for Y^{343} in murine EPOR for induction of erythrocytosis in response to recombinant Epo [20] and the inability of Stat5-null mice to generate high erythropoietic rates in response to stress [47].

3.6. Epo induces the tyrosine phosphorylation of Stat1 in 32D cells expressing truncated human EPOR

A role for Stat1 in regulation of erythropoiesis was suggested by studies of Stat1 knock-out mice demonstrating reduced responsiveness of Stat1-deficient erythroid burst-forming unit progenitors to Epo [49]. In UT-7 leukemia cells, Epo-mediated activation of Stat1 inhibited differentiation and was involved in stimulation of Epo-induced cellular proliferation [50,51]. Another study of UT-7 cells expressing chimeric receptor constructs consisting of the extracellular domain of G-CSFR and intracellular domain of human EPOR suggested that Y^{432} may be required for Stat1 tyrosine phosphorylation in response to G-CSF stimulation [52]. In the next set of experiments, we investigated whether Epo induces the tyrosine phosphorylation of Stat1 in 32D cells expressing truncated EPOR-ME that lacks Y^{432}. Cells were deprived of serum and cytokines and then either left unstimulated or stimulated with the indicated concentrations of Epo or IL-3 as a positive control. Whole cell lysates were subjected to Western blotting using phosphotyrosine-specific anti-Stat1 antibodies. As shown in Fig. 6A, Epo induced the tyrosine phosphorylation of Stat1 in 32D cells expressing full length EPOR (WT) as well as cells expressing truncated EPOR-ME (Fig. 6A). We then investigated the ability of tyrosines Y^{285} and Y^{344} to mediate Stat1 tyrosine phosphorylation in response to Epo (Fig 6A). We observed tyrosine phosphorylation of Stat1 in both YM1 and YM3 cells but not in the tyrosine-null YM2 cells. Similar results were obtained in experiments investigating the time course of Stat1 tyrosine phosphorylation as shown in Fig. 6B. We observed transient tyrosine phosphorylation of Stat1 in WT cells with peak phosphorylation occurring at 30 min and returning to basal levels by 60 min. In contrast, Stat1 tyrosine phosphorylation was prolonged in ME, YM1 and YM3 cells compared to WT cells. In YM2 cells expressing a tyrosine-null truncated EPOR, Stat1 tyrosine phosphorylation was not detected suggesting that cytoplasmic tyrosines Y^{285} or Y^{344} in the context of EPOR-ME facilitate Stat1 tyrosine phosphorylation in response to Epo. These data indicate that 32D cells expressing EPOR-ME or YM3 variants, capable of activating both Stat5 and Stat1, exhibited increased Epo sensitivity whereas YM1 cells that activated Stat1 but not Stat5 did not. Furthermore, tyrosine-null YM2 cells that activated neither Stat1 nor Stat5 exhibited decreased overall proliferation suggesting that Stat1 may contribute to Epo-mediated proliferation in the context of a truncated EPOR in 32D cells. Further

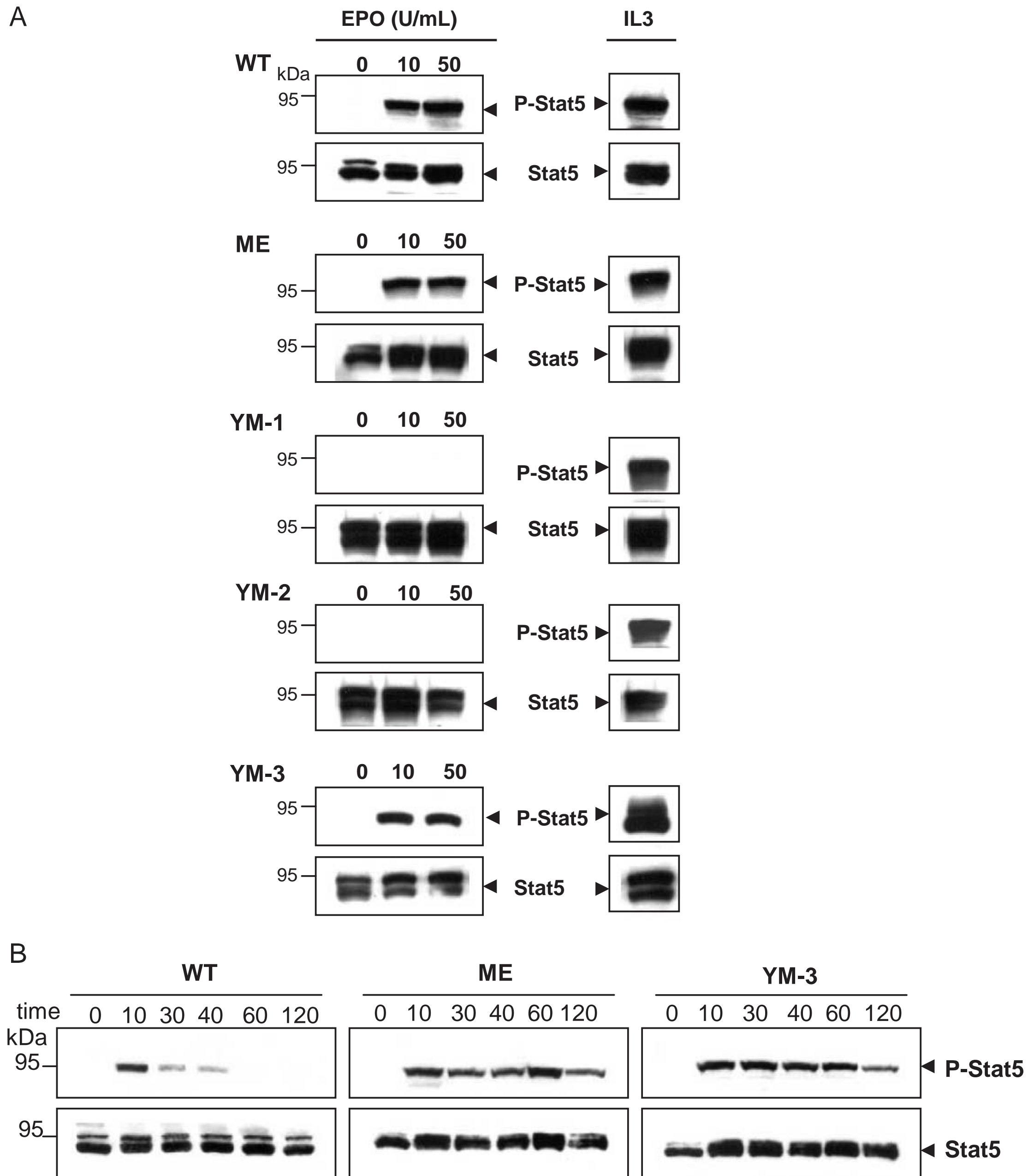
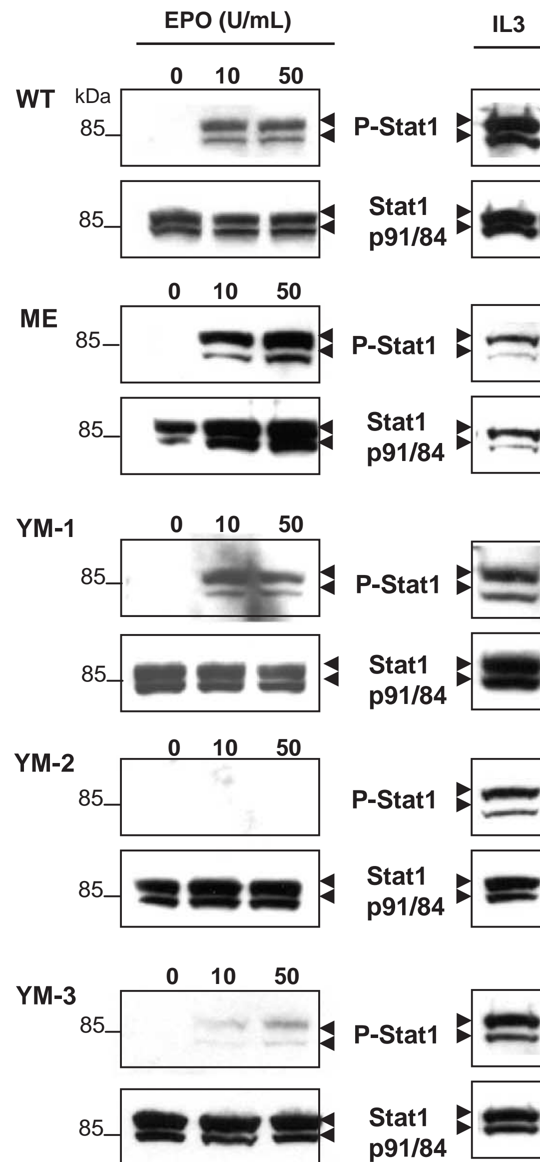


Fig. 5. Epo-induced tyrosine phosphorylation of Stat5 mediated by wild-type (WT) and mutant EPORs. A. Stably transfected 32D cell lines expressing the indicated human EPORs were either left unstimulated (0) or stimulated for 10 min with the indicated concentrations of Epo or 10 ng/ml of IL-3 as positive control. Cells were solubilized and Western blotting was performed using anti-phospho-Stat5 antibody to examine for Epo-induced tyrosine phosphorylation of Stat5 (P-Stat5). In YM1 and YM2 cells that contain $Y^{344}F$ mutation, Stat5 was tyrosine phosphorylated following IL-3 stimulation but not in response to Epo. The integrity of the proteins and comparable loading in each lane was confirmed by reprobing the blots and hybridization to anti-Stat5 antibody that detects total Stat5 including both Stat5a and Stat5b forms. Arrows indicate immunoreactivity for tyrosine phosphorylated Stat5 in upper panels (P-Stat5) and total Stat5 (Stat5a/b) in lower panels (Stat5). B. Time course of Stat5 tyrosine phosphorylation in 32D cells. Cells expressing WT or mutant EPORs (ME and YM3) were deprived of serum and growth factors, stimulated with Epo (10 units/ml), cell lysates prepared at the indicated time points (10–120 min) and immunoblotting was performed as described in Materials and methods. The blots were hybridized sequentially to anti-phospho-Stat5 antibody (upper panels) and anti-Stat5 antibody that detects total Stat5 (lower panels). The position of the molecular weight marker 95 kDA is shown.

studies of the mechanisms of Epo-induced Stat1 activation and its role in proliferation are warranted, particularly given the decreased responsiveness of Stat1-deficient murine erythroid progenitors to Epo [49].

In the present study, we investigated the function of a naturally occurring, truncated human EPOR mutant that confers increased Epo sensitivity to hematopoietic cells. Table 2 summarizes the results of our studies from which



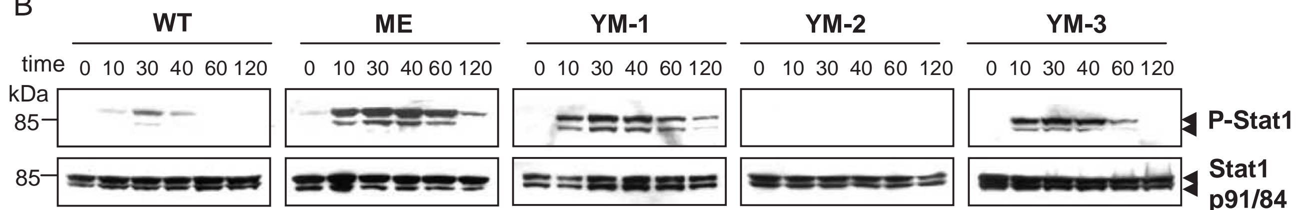


Fig. 6. Epo-induced tyrosine phosphorylation of Stat1 in 32D cells. A. 32D cell lines expressing the indicated human EPORs were either left unstimulated (0) or stimulated for 10 min with the indicated concentrations of Epo or 10 ng/ml of IL-3 as positive control. Whole cell lysates were subjected to Western analysis using anti-phospho-Stat1 antibody to examine for Epo-induced tyrosine phosphorylation of Stat1 (P-Stat1). In the tyrosine-null YM2 cells Stat1 was tyrosine phosphorylated following IL-3 stimulation but not in response to Epo. The integrity of the proteins and comparable loading in each lane was confirmed by reprobing the membranes with anti-Stat1 antibody to detects total Stat1, including both Stat1α and Stat1 β forms. Arrows indicate immunoreactivity for tyrosine phosphorylated Stat1 in upper panels (P-Stat1) and total Stat1 (p91/84) in lower panels. B. Time course of Stat1 tyrosine phosphorylation in 32D cells. Cells expressing WT or mutant EPORs were deprived of serum and growth factors, stimulated with Epo (10 units/ml), cell lysates prepared at the indicated time points and immunoblotting was performed as described in Materials and methods. The blots were hybridized sequentially to anti-phospho-Stat1 antibody (upper panels) and anti-Stat1 antibody that detects total Stat1 (lower panels).

several conclusions can be drawn: 1) Mutation of membrane-proximal tyrosine residue Y^{285} alone in EPOR-ME does not abolish increased Epo sensitivity, however, Y^{285} may contribute to Epo-dependent proliferation signals. 2) In the context of EPOR-ME, cytoplasmic tyrosine residues are dispensable for Epo-mediated pro-

Table 2
Epo sensitivity and signaling in 32D cells expressing wild type and mutant EPORs

Receptor name	Increased Epo* sensitivity	Epo-dependent tyrosine phosphorylation of[a]		
		Jak2	Stat5	Stat1
WT	N/A	+	+	+
ME	+	++	++	++
YM1	–	++	–	++
YM2	–	++	–	–
YM3	+	++	++	++

[a] Phosphorylation not detected (–), detected (+) or prolonged (++).
* Increased Epo sensitivity relative to WT cells was not observed (–) or present (+) based on the ability of the cells to proliferate in low concentrations of Epo (range 0.01–0.1 units/ml) as illustrated in the experiments in Fig. 3. N/A: not applicable.

liferation. 3) The membrane-distal tyrosine residue Y^{344} is required for Stat5 tyrosine phosphorylation and increased Epo sensitivity of ME and YM3 cells. However, Stat5 activation is not required for Epo-mediated cellular proliferation in high Epo concentrations. 4)EPOR intra-cytoplasmic tyrosine residues Y^{285} or Y^{344} facilitate the tyrosine phosphorylation of Stat1 in the context of EPOR-ME.

Our studies show that expression of a tyrosine-null truncated human EPOR is associated with reduced overall Epo-mediated proliferation but that receptor tyrosines are dispensable for EPOR mitogenic function. These results are consistent with the findings of an in vivo study in which the membrane-distal region and cytoplasmic tyrosines of EPOR were found to be non-essential for erythropoiesis in mice carrying a murine EPOR truncated by 108 amino acids [20]. In this mouse model, introduction of Y^{343}F mutation in the truncated murine EPOR, generating a tyrosine-null receptor, was associated with anemia, consistent with decreased efficiency of a receptor devoid of tyrosines. Interestingly, however, expression of the truncated murine EPOR containing intact Y^{343} did not lead to polycythemia in heterozygous mice and resulted in only mild elevation of hematocrits of homozygous animals [20]. This finding was in contrast to the results of another study in which in vivo replacement of the murine EPOR gene by a PFCP-associated, truncated human EPOR gene resulted in dominant polycythemia in heterozygous mice [53]. A significant difference in these models was the extents of the carboxy-terminal truncations of 108 versus 83 amino acids in the murine versus human EPORs, respectively. However, we have shown that a more extensive 110 amino acid truncation in human EPOR (EPOR-ME) is associated with PFCP in heterozygous patients and confers increased Epo sensitivity to transfected hematopoietic cells [36]. Another significant difference in the structures of the murine and human EPORs is the presence in the human EPOR of the additional tyrosine residue Y^{285}. The present studies demonstrate that Y^{285} may contribute to Epo-mediated cellular proliferation in the context of the truncated human EPOR-ME in 32D cells, suggesting a possible role for Y^{285} in generation of increased Epo sensitivity phenotype of hematopoietic cells that is characteristic of PFCP.

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