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Multiple tyrosine residues in the cytosolic domain of the erythropoietin receptor promote activation of STAT5

(erythropoiesis/cytokine receptors/JAK kinases/transcription factors)

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ABSTRACT **Signaling through the erythropoietin receptor (EPO-R) is crucial for proliferation, differentiation, and survival of erythroid progenitor cells. EPO induces homodimerization of the EPO-R, triggering activation of the receptor-associated kinase JAK2 and activation of STAT5. By mutating the eight tyrosine residues in the cytosolic domain of the EPO-R, we show that either Y^{343} or Y^{401} is sufficient to mediate maximal activation of STAT5; tyrosine residues Y^{429} and Y^{431} can partially activate STAT5. Comparison of the sequences surrounding these tyrosines reveals YXXL as the probable motif specifying recruitment of STAT5 to the EPO-R. Expression of a mutant EPO-R lacking all eight tyrosine residues in the cytosolic domain supported a low but detectable level of EPO-induced STAT5 activation, indicating the existence of an alternative pathway for STAT5 activation independent of any tyrosine in the EPO-R. The kinetics of STAT5 activation and inactivation were the same, regardless of which tyrosine residue in the EPO-R mediated its activation or whether the alternative pathway was used. The ability of mutant EPO-Rs to activate STAT5 did not directly correlate with their mitogenic potential.**

Erythropoietin (EPO), a hormone synthesized by the kidney, is the primary regulator of mammalian erythropoiesis. Hormone binding to its cognate receptor is crucial for proliferation, differentiation, and survival of erythroid progenitor cells. The erythropoietin receptor (EPO-R) (1) belongs to the large cytokine receptor superfamily, which is characterized by a WSXWS motif in the extracellular domain and by the absence of enzymatic activity in the cytoplasmic domain. To transmit a signal these receptors couple to protein tyrosine kinases and other signal transduction proteins. Ligand induced homodimerization of the EPO-R (2) triggers the activation of the receptor-bound protein tyrosine kinase JAK2 (3), resulting in a transient increase in tyrosine phosphorylation of several cellular proteins including the EPO-R. Since phosphotyrosine residues are specifically recognized by Src homology 2 (SH2) domains, this event enables the EPO-R to recruit a number of signal transduction proteins to the receptor, including SH-PTP1 (4), SH-PTP-2 (5), and PI3 kinase (6).

Little is known about how signals from the activated receptor complex assembled at the plasma membrane are transmitted to the nucleus and converted into gene activation. One system involves activation of the JAK–STAT pathway (for reviews see refs. 7 and 8). At least six STAT proteins, STAT1–STAT6, have been cloned (for review see ref. 9); all have an SH2 domain that allows it to bind to a phosphorylated tyrosine on a receptor or other protein. Following tyrosine phosphorylation by a Janus kinase or other kinase(s), STAT proteins dissociate from the receptor and form homo- or heterodimers via their SH2 domains. They then move to the nucleus and bind to and presumably activate specific genes. STAT proteins enable a signal to be transduced within minutes from the cell surface to the nucleus. Because several cytokine receptors activate the same Janus kinases it is thought that the specificity of intracellular responses is in part determined by specific STATs being recruited to particular tyrosine residues in receptors where they can be phosphorylated by associated JAKs (for review see ref. 7).

STAT5 was originally isolated as a mammary gland protein that becomes phosphorylated upon prolactin stimulation (10). In mice, several cytokines including EPO activate two closely related isoforms of STAT5, STAT5a, and STAT5b (11). Upon phosphorylation, STAT5 either homo- (STAT5a/STAT5a or STAT5b/STAT5b) or heterodimerizes (STAT5a/STAT5b) and acquires the ability to specifically bind DNA sequences that are localized upstream of several early response genes. The most membrane proximal tyrosine residue in the EPO-R, Y^{343}, was suggested to be critical for EPO mediated STAT5 activation (11, 12). However, these studies suggested that other tyrosines in the EPO-R might also be sufficient for STAT5 activation. The specific motif in the EPO-R recognized by the STAT5/SH2 domain also remains to be determined.

Here we report the properties of mutant EPO-Rs containing only one of the eight cytosolic tyrosines. We show that two tyrosine residues in the EPO-R can support maximal EPO-induced STAT5 activation. We also define an alternative pathway for STAT5 activation that requires no tyrosines in the EPO-R and that presumably involves another tyrosine-phosphorylated protein.

MATERIALS AND METHODS

Plasmids and Mutagenesis. The EPO-R cDNA was introduced into the *Bam*HI and *Eco*RI restriction sites in the retrovirus expression vector pBabe (puro) (13). Mutant EPO-Rs containing no tyrosine residues in the cytoplasmic domain (F8) or only one tyrosine (F7 Y^{xxx}) were generated by overlap extension (14) and cloned into the *Apa*I and *Eco*RI restriction sites in pBabe-EPO-R (puro). Single or double Y to F mutant EPO-Rs were generated by PCR mutagenesis and introduced into the eukaryotic expression vector pXM as described (4).

Selection of Stable Cell Lines. pBabe (puro) DNA encoding the wild-type EPO-R or mutant EPO-Rs F8 and F7 Y^{xxx} was transfected by the calcium-phosphate method into the packaging cell line Bosc 23 (15). Forty-eight hours after transfection viral supernatants were harvested and used to infect 10^6 BaF3 cells. Cell pools expressing the EPO-R in conjunction with the puromycin resistance gene were selected and maintained in the presence of 1.5 μg/ml of puromycin (Sigma) and 10% WEHI conditioned medium as a source of interleukin 3

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Abbreviations: EPO, erythropoietin; EPO-R, EPO receptor; SH2, Src homology 2; EMSA, electrophoretic mobility-shift assay; IL, interleukin.
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(IL-3). Cell pools expressing the Y to F mutant EPO-Rs were generated by electroporation and were selected in the presence of 0.6 mg/ml of G418 and 10% WEHI conditioned medium (4). For each of the cell pools used, equivalent surface expression of the EPO-Rs was validated by immunoblotting with an anti-EPO-R antiserum and confirmed by binding experiments with [^{125}I]EPO (data not shown).

Immunoprecipitation and Immunoblotting. BaF3 cells expressing the wild-type EPO-R or mutant EPO-Rs were starved for 3 hr in serum-free medium in the presence of 1 mg/ml of bovine serum albumin (BSA), stimulated at 37°C with 100 units of Epo/ml (provided by Amgen) for 5 min, and lysed by addition of an equal volume of 2× lysis buffer as described (4). For each immunoprecipitation experiment lysates corresponding to 10^7 cells were used. Immunoprecipitation experiments were carried out as described (4) using 0.5 μg of antiserum raised against JAK2 (Santa Cruz Biotechnology) or 0.5 μg of anti-STAT5b antiserum (Santa Cruz Biotechnology). Analysis was performed on low-bis SDS/10% PAGE followed by immunoblotting with anti-PTyr 4G10 (Upstate Biotechnology) at a dilution of 1:5000 and enhanced chemiluminescence (DuPont/NEN). For reprobing, blots were treated with β-ME/SDS as described (4) and incubated with antibodies as indicated.

Preparation of Nuclear Extracts. Nuclear extracts were prepared using the procedure described by Schreiber *et al.* (16). BaF3 cells expressing the wild-type EPO-R or mutant EPO-Rs were starved for 3 hr in serum-free medium in the presence of 1 mg/ml BSA and stimulated with 100 units of Epo/ml for 10 min at 37°C. Cells were washed with ice-cold phosphate buffered saline and cell pellets were resuspended in 10 mM Hepes (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 1 mM benzamidine, 50 μg/ml leupeptin, and 10 μg/ml pepstatin. Cells were allowed to swell on ice for 15 min, mixed vigorously, and centrifuged at 20,000 × *g* for 1 min. The nuclear pellets were extracted with a solution containing 20 mM Hepes (pH 7.9), 400 mM NaCl, 0.2 mM EDTA, 2 mM PMSF, 1 mM DTT, 1 mM benzamidine, 50 μg/ml leupeptin, and 10 μg/ml pepstatin. The tubes were rocked vigorously for 20 min and then centrifuged for 5 min at 20,000 × *g*. Supernatants were stored at −80°C.

Electrophoretic Mobility-Shift Assay (EMSA). DNA-binding reactions were conducted by the incubation of 10 μg of nuclear extract with 2 × 10^4 cpm of ^{32}P-labeled oligonucleotide probe for 15 min at room temperature in mixtures containing 10 mM Hepes (pH 8.0), 0.1 mM EDTA, 2 mM DTT, 2 μg poly(dI·dC), 4 mM KCl, 0.1% Nonidet P-40, 2 mM spermidine, and 8% glycerol. Complexes were separated on 5% nondenaturating polyacrylamide gels in 0.5× TBE. Gels were dried and visualized by autoradiography. The probe used contains a core sequence corresponding to the interferon γ-activated sequence (GAS) element of the FcγRI promoter (5′-GTATTTCCCAGAAAAGGAAC-3′) (17). For supershift experiments, nuclear extracts were incubated with 1 μg of anti-STAT5, anti-STAT3, or anti-STAT1 antibodies (Santa Cruz Biotechnology) followed by EMSA. For competition experiments, nuclear extracts were incubated with a 100-fold molar excess of an unlabeled oligonucleotide containing the STAT5-binding site from the β-casein promoter (5′-AGATTTCTAGGAATTCAATCC-3′) (18), or a nonspecific interferon α-stimulated response element sequence (5′-AAGTACTTTCAGTTTCATATTACTCTA-3′) (19).

RESULTS

Y343 or Y401 in the EPO-R Is Sufficient for Maximal STAT5 Activation. To identify the role of each tyrosine residue in the EPO-R for activation of STAT5, we generated a mutant

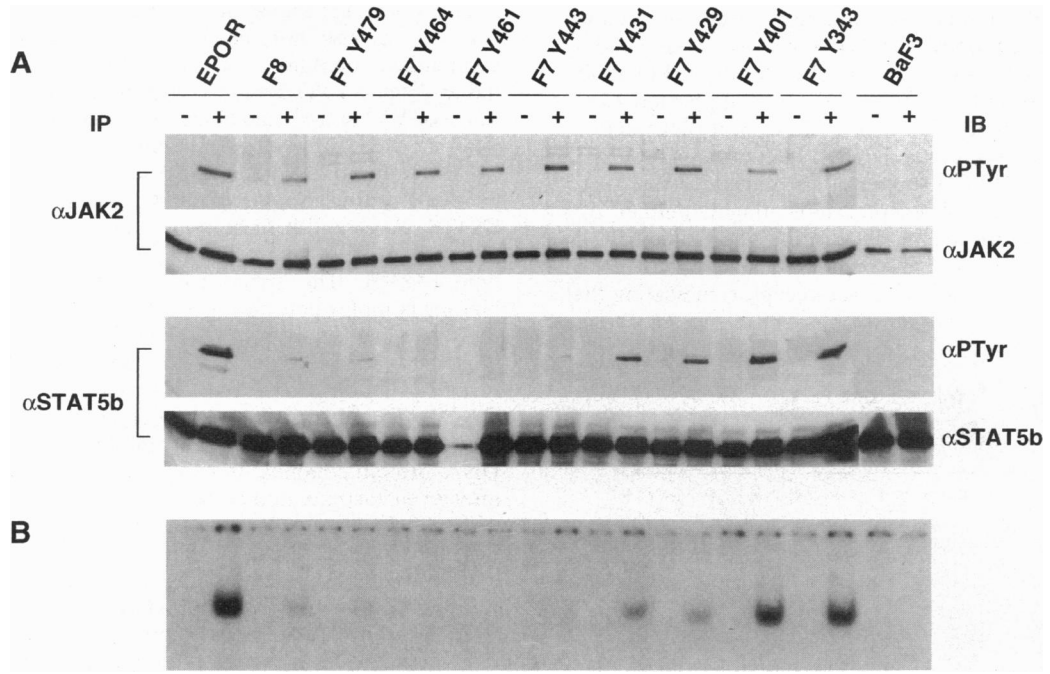

FIG. 1. Either tyrosine 343 or tyrosine 401 of the EPO-R is sufficient for maximal EPO-induced phosphorylation of STAT5 and activation of STAT5 DNA-binding activity. (*A*) EPO-induced tyrosine phosphorylation of JAK2 and STAT5 in BaF3 cells expressing the wild-type EPO-R or mutant EPO-Rs. Pools of BaF3 cells stably expressing the wild-type EPO-R or indicated mutant EPO-Rs were starved and then stimulated with 100 units of EPO/ml for 5 min. Detergent lysates equivalent to 10^7 cells were used for immunoprecipitations (IP) with antiserum directed against STAT5b (lower two panels) or JAK2 (upper two panels). The gels were subjected to immunoblotting (IB) with the anti-phosphotyrosine antibody 4G10 followed by enhanced chemiluminescence (αPTyr). The blots were reprobed with the antisera used for immunoprecipitation, either anti-JAK2 (αJAK2) or anti-STAT5b (αSTAT5b), respectively. (*B*) BaF3 cells stably expressing the wild-type EPO-R or indicated mutant EPO-Rs were starved and then stimulated with 100 units of EPO/ml for 10 min. Nuclear extracts were prepared and analyzed by EMSA as described using a [^{32}P]-FcγRI GAS probe.

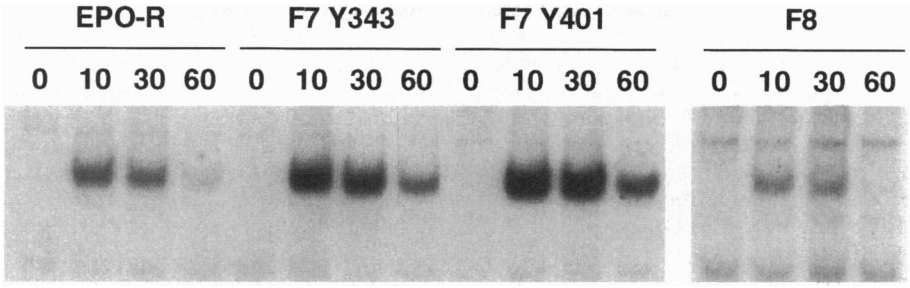

FIG. 2. Kinetics of STAT5 activation and inactivation in BaF3 cells expressing the wild-type EPO-R or mutant EPO-Rs. BaF3 cells stably expressing the wild-type or indicated mutant EPO-Rs were starved and then stimulated with 100 units of EPO/ml for 10, 30, or 60 min. As described in the legend to Fig. 1*B*, nuclear extracts were prepared and analyzed by EMSA. Exposure times were 12 hr for the left panel and 40 hr for the right panel.

EPO-R lacking all eight tyrosine residues in the cytoplasmic domain (F8). We also generated eight mutant receptors containing only one tyrosine; these were named F7 Y^{xxx}, according to the single tyrosine residue present. Wild-type and mutant EPO-Rs were stably expressed in the proB cell line BaF3; pools of transfected cells expressing comparable numbers of cell surface receptors were identified by measuring the binding of [^{125}I]EPO and used for all experiments (data not shown).

Fig. 1*A* (upper two panels) shows that, in cells expressing F8 and each of the F7 Y^{xxx} mutants, EPO stimulation of JAK2 phosphorylation was comparable to that of cells expressing wild-type EPO-R. In this experiment, lysates of cells treated or not with EPO were immunoprecipitated with an anti-JAK2 serum followed by immunoblotting with an antibody against phosphotyrosine (anti-PTyr). Thus, EPO-stimulated activation of JAK2 does not require tyrosine residues in the receptor cytosolic domain.

In contrast, specific tyrosine residues are required for activation of STAT5 (Fig. 1*A* and *B*). Activation of STAT5 was monitored by two approaches—immunoblotting with an anti-PTyr monoclonal antibody (Fig. 1*A*, lower two panels) and by an EMSA (Fig. 1*B*)—with comparable results. EPO-R F8, lacking any cytosolic tyrosines, consistently supported EPO-stimulated STAT5 activation to a level ≈8% of that found in cells expressing the wild-type EPO-R. In contrast, EPO-R F7 Y343 and EPO-R F7 Y401 supported the same level of STAT5 activation as the wild-type receptor, indicating that either Y^{343} or Y^{401} is sufficient for maximal activation of STAT5. EPO-R F7 Y429 and EPO-R F7 Y431 supported ≈30% the level of activation of STAT5 as the wild-type receptor, indicating that both Y^{429} and Y^{431} can mediate activation of STAT5 to a significant extent.

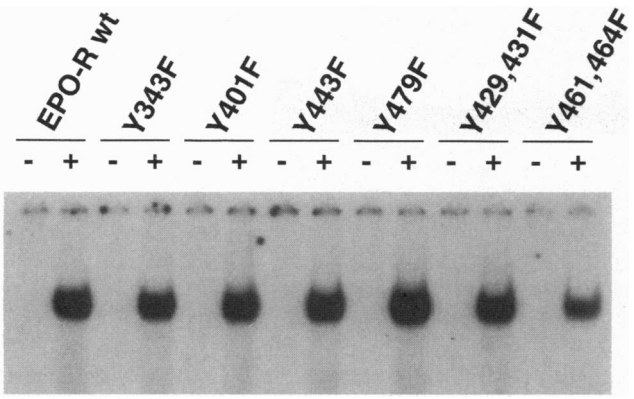

FIG. 3. EPO induction of STAT5 DNA-binding activity in BaF3 cells expressing the wild-type EPO-R or indicated mutant EPO-Rs in which one or two tyrosine residues were changed to phenylalanine. BaF3 cells stably expressing the wild-type EPO-R or mutant EPO-Rs were starved and then stimulated with 100 units of EPO/ml for 10 min. As described in the legend to Fig. 1*B*, nuclear extracts were prepared and analyzed by EMSA.

As analyzed by EMSA, the time course of STAT5 activation and inactivation was the same in cells expressing the wild-type EPO-R and mutants F8, F7 Y343, F7 Y401, F7 Y429, and F7 Y431 (Fig. 2). Maximal activation of STAT5/DNA binding in each cell line was reached 10 min after EPO stimulation. STAT5/DNA-binding activity remained approximately at this level for 30 min and declined thereafter; at 60 min, only ≈10% of the maximal level of STAT5 binding remained.

The conclusion that multiple tyrosine residues in the EPO-R can activate STAT5 to maximal levels is corroborated by the experiment in Fig. 3. A series of mutant EPO-Rs was generated by changing one or two individual tyrosines to phenylalanine. As above, we generated lines of transfected BaF3 cells that express comparable amounts of surface EPO receptors. In each of these lines EPO-induced tyrosine phosphorylation of the EPO-R was comparable to that achieved in BaF3 cells expressing the wild-type EPO-R (data not shown). EPO-induced STAT5 activation in these cell lines, detected by EMSA, was also normal (Fig. 3); in each cell line tested, comparable amounts of STAT5/DNA-binding activity was induced upon EPO stimulation. Importantly, neither the absence of Y^{343} or Y^{401} had a demonstrable effect on the level of EPO induced extent of STAT5-binding activity.

EPO-Induced DNA-Binding Complex Is Specific for the STAT5-Binding Sequence and Contains Predominately STAT5. To establish these points, nuclear extracts were prepared from EPO-stimulated BaF3 cells expressing the wild-type EPO-R. The specificity of the EPO-induced DNA-binding complex detected by EMSA was tested by conducting the assay in the presence of a 100-fold excess of competing unlabeled oligonucleotides. The analysis shown in Fig. 4 revealed that only the addition of oligonucleotides containing the STAT5-binding sites from the GAS/FcγRI (lane 2) or β-casein (lane 3) promoters inhibited the formation of a DNA–protein complex. In contrast, the presence of excess of interferon α-stimulated response element sequences (lane 4) had no influence on binding of STAT proteins to the GAS/FcγRI probe. Furthermore, addition of an anti-STAT5 antibody caused the formation of a supershifted band (lane 6), whereas treatment with antibodies specific for STAT3 or STAT1 had no effect (lanes 7 and 8). Thus, in BaF3 cells, the EPO-induced DNA-binding protein complex is predominantly composed of activated STAT5.

DISCUSSION

Multiple Tyrosine Residues in the EPO-R Enhance STAT5 Activation. Our principal conclusion is that either of two tyrosine residues in the cytosolic domain of the EPO-R—Y^{343} and Y^{401} —are sufficient to promote maximal tyrosine phosphorylation and activation of STAT5/DNA-binding activity.

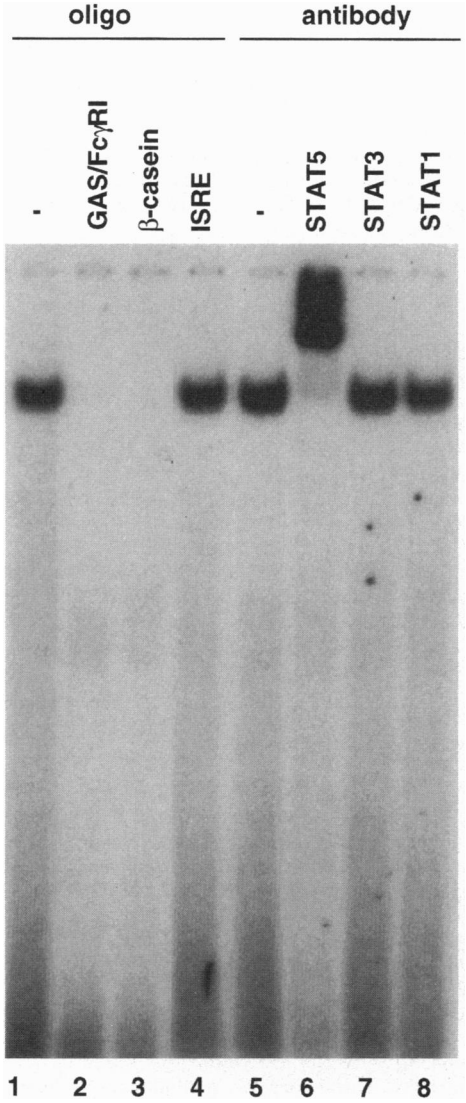

FIG. 4. Specificity of the STAT DNA-binding complex in EPO-stimulated cells. As detailed in the legend to Fig. 1*B*, nuclear extracts were prepared from EPO-induced BaF3 cells expressing wild-type EPO-R. These were preincubated with no additions (lanes 1 and 5) or with a 100-fold molar excess of unlabeled oligonucleotides containing the indicated specific STAT5-binding sites (lanes 2 and 3) or a nonspecific sequence (lane 4) as detailed in *Materials and Methods*. Extracts were also preincubated with antisera specific for STAT5 (lane 6), STAT3 (lane 7), or STAT1 (lane 8). As described, the extracts were analyzed by EMSA using a [^{32}P]-FcγRI GAS probe.

Two other tyrosine residues —Y^{429} and Y^{431}—activate STAT5 to an intermediate level. Expression of a mutant EPO-R lacking all eight tyrosine residues in the cytosolic domain (F8) supported a low but detectable level of EPO-induced STAT5 activation, indicating the existence of an alternative pathway for STAT5 activation independent of any tyrosine in the EPO-R.

By using a panel of EPO-Rs truncated for portions of the cytosolic domain, others have concluded that Y^{343} is one of the residues critical for EPO-induced STAT5 activation (11, 12). However, because large deletion mutations may severely alter the overall structure of the receptor cytosolic domain, it was not clear whether Y^{343} is essential for STAT5 activation or whether other tyrosine residues are involved. We and others (12) have not been able to detect tyrosine phosphorylation of mutant EPO-Rs containing single tyrosine residues in the cytosolic domain and, thus, we cannot be certain that the ability of mutants EPO-R F7-Y343 and EPO-R F7-Y401 to support maximal activation of STAT5 is dependent on phosphorylation of tyrosines 343 and 401, respectively. However, we feel that this is very likely, given the conventional model of activation of STAT proteins, in which phosphorylated tyrosine residues in a receptor are essential for STAT phosphorylation and activation (20).

The ability of multiple phosphotyrosine residues in the cytosolic domain to activate STAT proteins is not unique to the EPO-R. The IL-2 receptor β-chain (21) and gp130 (20) also contain several tyrosine residues that, individually, are sufficient for activation of STAT factors. Such redundancy may have evolved to ensure efficient STAT activation in a situation where several intracellular signal transduction proteins might compete for the same receptor phosphotyrosine residue. Alternatively, it could reflect an adaptation to a situation where particular EPO-R tyrosine residues only become phosphorylated at specific stages during erythroid differentiation.

STAT5 Binds to Phospho-YXXL Motifs in the EPO-R. In general, SH2 domains bind to phosphotyrosine residues that are localized in sequences with specific amino acids at the +1 to +4 positions (22). Both Y^{343} and Y^{401} of the EPO-R, as well as Y^{429}, have a leucine at the +3 position. With valine at the +3 position, the sequence following Y^{431} is similar. By analogy to the YXXQ motif identified in gp130 and the lymphocyte inhibitory factor receptor essential for STAT3 activation (20), a YXXL motif appears to be the determinant specifying STAT5 activation. This is supported by the fact that one of the tyrosine residues required for STAT5 activation by the IL-2 receptor β-chain—Y^{510}—also contains a leucine at the +3 position. However, the sequence surrounding the second tyrosine residue in the IL-2 receptor β-chain sufficient for STAT5 activation—Y^{392}—(YCTF) is only remotely similar (21). Importantly, there is no direct experiment showing that an isolated STAT5/SH2 domain is capable of directly binding to any specific sequence of amino acids containing a phosphotyrosine residue, and we have been unable to show that a glutathione *S*-transferase fusion protein containing the STAT5/SH2-domain can bind *in vitro* to tyrosine-phosphorylated EPO-R (data not shown). Thus, STAT5 may bind by means of an intermediate protein(s) to specific phosphotyrosine residues in the EPO receptor.

Interestingly, neither the YXXQ or YXXL motif in receptors required for STAT activation is homologous to the sequences surrounding the tyrosine residue in STAT proteins that becomes phosphorylated. For example, the sequence in the vicinity of Y^{694} in STAT5—YVKPQ—clearly lacks the conserved leucine at position +3 (23). Importantly, the affinity of the STAT1/SH2 domain for the phosphotyrosine in STAT1 is greater than that for the specific binding site in the α-chain of the interferon γ receptor (24). Thus, the phospho-YXXL motif may not represent the highest affinity-binding site for the STAT5/SH2 domain. Initially, the STAT5/SH2 domain could indeed bind to a phospho-YXXL sequence on a receptor or adapter protein. Once phosphorylated on Y^{694}, however, the STAT5/SH2 domain would bind to the presumably higher affinity site surrounding Y^{694}, resulting in release of the STAT protein from the receptor and STAT5 dimerization. Alternatively, the phospho-YXXL motif could be recognized by the SH2 domain of an adapter protein that, in turn, binds either to the STAT5/SH2 domain or to another segment of STAT5.

Low Levels of STAT5 Activation Are Achieved in the Absence of Tyrosine Residues in the EPO-R. In cells expressing a mutant EPO-R lacking all eight tyrosine residues in the cytoplasmic domain we and others (12) consistently observed low but significant levels of EPO-induced STAT5 activation. The kinetics of STAT5 activation and inactivation were identical to that in a cell line expressing the wild-type EPO-R. Similar to the EPO-R, a mutant of the closely related growth hormone receptor lacking any cytosolic tyrosines activates STAT5 to detectable levels (25). Thus, STAT5 can become

activated by an alternative pathway not involving direct binding to a receptor, presumably by binding to a phosphotyrosine residue on another protein activated as a consequence of receptor activation. This alternative pathway may involve recruitment of STAT5 to JAK2 because both the EPO-R and growth hormone receptor activate JAK2 (3, 26). Also, seven tyrosine residues in JAK2 show similarity to the YXXL motif identified in the EPO-R crucial for STAT5 activation (27). Alternatively, STAT5 could become activated by binding to a receptor tyrosine kinase or a member of the *src* of tyrosine kinase family. Both the epidermal growth factor and platelet-derived growth factor receptors induce tyrosine phosphorylation of STAT1 and STAT3 (28–30). Furthermore, in v-*src* transformed cells there are elevated levels of tyrosine—phosphorylated STAT3 (31, 32). It seems likely that multiple pathways for STAT activation are used in parallel and could be required for optimal function *in vivo*; the exact pathway may depend on the specific receptor and on the specific transduction proteins expressed in the cell.

The Extent of STAT5 Activation by the EPO-R Does Not Directly Correlate with the Proliferative Potential of the Receptor. Conflicting results have been reported regarding the function of EPO-induced STAT5 activation in cell proliferation. We find that mutant EPO-Rs F7-Y343 and F7-Y401 are equally efficient in stimulating STAT5 activation. However, of the two mutant EPO-Rs capable of maximally activating STAT5, EPO-R F7-Y343 supported near maximal cell proliferation of 32D cells and ≈50% of normal proliferation in BaF3 cells. EPO-R F7-Y401 is less efficient in stimulating proliferation in both cell lines (U.K., H. Wu, J.G.H., and H.F.L., unpublished work). Similarly, STAT5 activation mediated by the IL-2 receptor requires a receptor segment that is dispensable for mitogenic signaling (33). Thus, STAT5 may serve no major mitogenic function. The phenotype of STAT1 and STAT6 knock-out mice indicates that the primary function of these STAT proteins is the induction of specific subsets of genes (34–37). During erythroid differentiation, STAT5 may be required mainly for induction of specific genes required for differentiation and/or maintenance of erythroid progenitors.

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