Erythropoietin Induces Tyrosine Phosphorylation of the Interleukin-3 Receptor β Subunit (βIL3) and Recruitment of Stat5 to Possible Stat5-Docking Sites in βIL3

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The receptors for erythropoietin (Epo) and interleukin-3 (IL-3) both induce the ligand-dependent activation of the Jak2 tyrosine kinase. Activated Jak2 then phosphorylates these receptors and thereby recruits various signaling molecules containing the Src homology (SH)-2 domain, including Stat5, to the tyrosine phosphorylated receptors. In the present study, we demonstrate that Epo stimulation induces unidirectional cross-phosphorylation of the IL-3 receptor β subunit (βIL3) on tyrosines and its rapid and transient association with Stat5 in murine IL-3-dependent cell lines engineered to express the Epo receptor (EpoR). Using cell lines expressing various EpoR mutants, it was demonstrated that the Epo-induced tyrosine phosphorylation of βIL3 is dependent on the membrane-proximal EpoR cytoplasmic region involved in the activation of Jak2, but not on the extracellular and transmembrane regions or on the carboxy-terminal 145 amino acid region containing all the intracellular tyrosine residues. It was also shown that IL-3 induces rapid and dose-dependent association of Jak2 with βIL3. However, Epo failed to induce any detectable association of βIL3 with Jak2 or the EpoR. The present study also demonstrates that in IL-3-stimulated cells, an ovine Stat5 mutant harboring a substitution of Tyr694 to Phe, which abolishes the tyrosine phosphorylation required for activation, fails to dimerize with endogenous Stat5, shows sustained binding with tyrosine-phosphorylated βIL3, and inhibits the tyrosine phosphorylation of endogenous Stat5. These results suggest that βIL3 may have Stat5 docking sites, similar to those found in the EpoR, that facilitate the activation of Stat5 by Jak2 and raise the possibility that Epo may cross-activate or transmodulate the IL-3 receptor signaling pathways.

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ligand-induced receptor tyrosine phosphorylation should play significant roles in signaling through these receptors. Initially purified and cloned as a mediator of prolactin-induced transcription of the β-casein gene, Stat5 has been shown to be activated by a variety of other cytokines, including IL-3, Epo, growth hormone, GM-CSF, IL-5, IL-2, and thrombopoietin. Two murine homologues, Stat5A and Stat5B, sharing 96% amino acid sequence identity, have been cloned, although no functional differences have yet been observed between these two molecules. Very recently, we have demonstrated that the recruitment of Stat5 is recruited, very rapidly and transiently, to the tyrosine-phosphorylated receptors for Epo and IL-3. The recruitment of Stat5 to the EpoR was shown to be mediated through the interaction between the Stat5 SH2 domain and specific phosphorylated tyrosine residues, including Tyr343, in the EpoR cytoplasmic domain. Furthermore, it was demonstrated that the recruitment of Stat5 plays a significant role in the specific activation of Stat5 by the EpoR, although the significance of Stat5 activation on the EpoR-mediated growth signaling has remained controversial. On the other hand, the mechanism and significance of the IL-3-induced binding of Stat5 to β_{H3L} remain unknown.

To address the possibility that the receptors for Epo and IL-3 may cross-activate each other, we examined whether Epo and IL-3 may induce cross-phosphorylation of their receptors. The present study demonstrates that Epo induces unidirectional cross-phosphorylation of β_{H3L}, which is dependent on the membrane-proximal EpoR cytoplasmic region involved in the activation of Jak2. In addition, Epo induced a very rapid and transient binding of Stat5 to tyrosine-phosphorylated β_{H3L}. We also examined the mechanisms of binding of Stat5 to β_{H3L} by introducing an ovine Stat5 mutant, Stat5-Y694F, with a substitution of Tyr694 with Phe, which abolishes the phosphorylation site required for activation of Stat5, into an IL-3-dependent cell line. Stat5-Y694F failed to dimerize with endogenous Stat5, stably associated with tyrosine phosphorylated β_{H3L}, and inhibited the tyrosine phosphorylation of endogenous Stat5. This is consistent with the hypothesis that β_{H3L} has Stat5 docking sites that facilitate the activation of Stat5.

MATERIALS AND METHODS

Cells and reagents. IL-3-dependent 32D cell clones expressing the wild-type and mutant EpoRs were previously described. An IL-3-dependent murine cell line, BaF3, was obtained through the Riken Gene Bank (Ibaraki, Japan). These cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10% WEHI-3 conditioned medium as a source of IL-3. Reconstituted human Epo was kindly provided by Chugai Pharmaceutical Co Ltd (Tokyo, Japan). Recombinant murine IL-3 was purchased from PeproTech Inc. (Rocky Hill, NJ).

A rabbit antiserum against the EpoR cytoplasmic domain was previously described. A rabbit antiserum against Stat5A was kindly provided by Dr J.N. Ihle (St. Jude Children’s Research Hospital, Memphis, TN). An anti-Jak2 antibody raised against a synthetic peptide corresponding to amino acids 1110-1129 mapping at the carboxy terminus of murine Jak2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antibodies against Stat5B and β_{H3L} (K-19) were also from Santa Cruz Biotechnology. Monoclonal antibodies against phosphotyrosine, 4G10, and against the epidermal growth factor (EGF) receptor (EGFR), LA22, were purchased from Upstate Biotechnology, Inc (Lake Placid, NY). A monoclonal antibody, 12CA5, raised against the influenza virus hemagglutinin (HA) epitope was from Boehringer Mannheim (Indianapolis, IN). A glucocorticoid-inducible expression vector, pMAM2-BSD, conferring resistance to blastidicin S, was purchased from Funakoshi (Tokyo, Japan).

Construction of expression plasmids and transfection into BaF3 cells. To construct an expression plasmid for a chimeric receptor containing the EpoR intracellular domain linked to the extracellular and transmembrane regions of the EGFR, a portion of the EGFR cDNA coding for amino acids –24 to 647 was amplified by the polymerase chain reaction (PCR) and subcloned into the expression plasmid pcDNA3, as described previously. A portion of the murine EpoR cDNA containing the region coding for amino acid 252 to the stop codon and the 3′ noncoding region was amplified by the PCR. The 5′ primer used was 5′-CCGATATCACTCTGCAGCAGAAGA-TCT-3′, designed to add the EcoRV recognition sequence at the 5′-end of the amplified fragment, while the 3′ primer was derived from a sequence in the pXM vector. The PCR fragment was digested with both EcoRV and Avr II, whose recognition site is in the 3′-noncoding region, and subcloned in frame into the EcoRV-Xba I site of the pcDNA3 clone containing the 5′ portion of EGFR cDNA to create pcD/EGF-EpoR. The structure of pcD/EGF-EpoR was confirmed by digestion with multiple restriction enzymes. The chimeric receptor encoded by pcD/EGF-EpoR contains the signal peptide, extracellular domain, and transmembrane region of the EGFR followed by an Asp residue resulting from the artificially added EcoRV recognition sequence and the EpoR cytoplasmic region lacking the membrane proximal 4 amino acid residues.

BaF3 clones expressing the wild-type EpoR were isolated as described previously. Transfection of pcD/EGF-EpoR into BaF3 cells and isolation of clones expressing the EGFR/EpoR chimeric receptor were also performed essentially as described previously. In brief, BaF3 cells were transfected with 10 μg of pcD/EGF-EpoR by electroporation and selected in medium containing G418. Four clones were isolated by limiting dilution and examined for the growth response to Epo. All the clones proliferated in response to EGF and one clone was arbitrarily chosen for subsequent studies.

To construct inducible expression plasmids for wild-type ovine Stat5 and its mutant with a substitution of Tyr694 with Phe, the Sal I fragment coding for wild-type or mutant Stat5 tagged with the HA epitope at the amino-terminus was subcloned between the Sal I and Not I sites within the multiple cloning region of the eukaryotic expression vector pMAM2-BSD to create pMB/Stat5-Wt or pMB/Stat5-Y694F, respectively. The inserted Stat5 cDNAs were expressed under the control of the mouse mammary tumor virus long-terminal repeat promoter, which is inducible by dexamethasone.

Immunoprecipitation and immunoblotting. Cells were washed free of IL-3, cultured overnight, and left unstimulated as a negative control or stimulated with Epo, EGF, or IL-3 at indicated concentrations. Cells were solubilized at 4 × 10^5 cells/mL with a lysis buffer composed of 1% Triton X-100, 20 mM NaCl, 1 mM EDTA, 10 mM sodium orthovanadate, 1 mM phenylmethylsulfonil fluoride (PMSF), 10 μg/mL aprotinin, and 10 μg/mL leupeptin. For detection of the association of Jak2 with the cytokine receptors, Triton X-100 in the lysis buffer
was replaced with 1% digitonin. Cell lysates were subjected to immuno precipitation as described previously. Immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred to Immobilon P membranes (Millipore, Bedford, MA). The membranes were probed with a relevant antibody followed by detection using enhanced chemiluminescence Western blotting detection system (Amersham, Buckinghamshire, UK). For reprobing of the membranes, they were treated with stripping buffer composed of 100 mmol/L 2-mercaptoethanol, 2% SDS, and 62.5 mmol/L Tris-HCl (pH 6.7) at 50°C for 30 minutes and subsequently probed with a different antibody.

RESULTS

Epo rapidly induces tyrosine phosphorylation of \( \beta_{\text{IL3}} \). To address the possibility that the receptors for Epo and IL-3 may cross-activate each other, we stimulated 32D/EpoR-Wt, a clone of IL-3- dependent 32D cells expressing the transfected murine EpoR, with Epo or IL-3 for 1 or 5 minutes and examined the tyrosine phosphorylation status of these receptors. As shown in Fig 1A, antiphosphotyrosine blotting of anti-\( \beta_{\text{IL3}} \) immunoprecipitates showed that Epo stimulation induced tyrosine phosphorylation of \( \beta_{\text{IL3}} \), albeit to a lesser extent than IL-3 stimulation. The Epo-induced tyrosine phosphorylation of \( \beta_{\text{IL3}} \) occurred rapidly and transiently, as it peaked at 1 minute after stimulation and significantly decreased at 5 minutes. As shown in Fig 1C, the tyrosine phosphorylation of \( \beta_{\text{IL3}} \) was faintly induced when stimulated at as low as 0.01 U/mL of Epo and gradually increased in intensity as the concentration of Epo increased. In contrast to the observation that Epo induced the tyrosine phosphorylation of \( \beta_{\text{IL3}} \), IL-3 stimulation did not induce any detectable tyrosine phosphorylation of the EpoR (Fig 1B).

Epo-induced tyrosine phosphorylation of \( \beta_{\text{IL3}} \) depends on the EpoR membrane-proximal domain involved in activation of Jak2. To investigate the mechanisms by which the EpoR mediates the Epo-induced tyrosine phosphorylation of \( \beta_{\text{IL3}} \), IL-3-dependent clones expressing various EpoR mutants shown in Fig 2 were examined. We first examined BaF3/EGF-EpoR cells expressing a chimeric receptor, EGF-EpoR, in which the extracellular and transmembrane regions of the EpoR were replaced by those of the EGFR and compared these cells with BaF3/EpoR cells expressing the wild-type EpoR. As shown in Fig 3A, Epo and EGF similarly induced tyrosine phosphorylation of \( \beta_{\text{IL3}} \) in BaF3/EpoR-Wt and BaF3/EGF-EpoR cells, respectively. In BaF3/EGF-EpoR cells, EGF also induced tyrosine phosphorylation of the 160-kD chimeric receptor, which was reactive with antibodies against the EGFR extracellular domain and the EpoR cytoplasmic domain (Fig 3B). On the other hand, IL-3 failed to induce tyrosine phosphorylation of the chimeric receptor in
Fig 2. Schematic representation of mutant EpoRs. Mutant EpoRs employed are schematically shown along with the wild-type EpoR and EGFR. The membrane-proximal cytoplasmic domain of EpoR containing the Box 1 and Box 2 sequences is represented by a striped box. Thick horizontal lines represent tyrosine residues in the EpoR cytoplasmic region. The four conserved cysteine residues in the EpoR extracellular domain are represented by thin horizontal lines. Numbers in parentheses denote the carboxy-terminal amino-acid numbers. Abbreviations used are: WSXWS, the WSXWS motif; W282R, the mutation of Trp282 to Arg; CRD, the cysteine-rich domain; TKD, the tyrosine kinase domain.

BaF3/EGF-EpoR cells or the EpoR in BaF3/EpoR cells (Fig 3B and data not shown). These results agree with those obtained with 32D/EpoR-Wt cells and further indicate that only the intracellular domain, but not the extracellular and transmembrane domains, of the EpoR is required for the induction of tyrosine phosphorylation of βIL-3.

To further define the EpoR cytoplasmic region involved in induction of the tyrosine phosphorylation of βIL-3, we next examined previously characterized two clones of 32D cells expressing mutant EpoRs shown in Fig 2. 32D/EpoR-S expresses the S-mutant EpoR in which the carboxy-terminal 145 amino acids, including all the cytoplasm tyrosines, were deleted by carboxy-terminal truncation,12 and 32D/EpoR-PM4 expresses the PM4-mutant EpoR in which a mutation, Trp282 to Arg, in the membrane-proximal cytoplasmic region abolished the abilities of EpoR to associate with Jak2 and to activate its kinase activity.19,29 In 32D/EpoR-S cells, Epo induced the tyrosine phosphorylation of βIL-3, which was observed when cells were stimulated with as low as 0.01 U/mL of Epo (Fig 4A). In contrast, Epo failed to induce any detectable tyrosine phosphorylation of βIL-3 in 32D/EpoR-PM4 cells (Fig 4B). These results indicate that the Epo-induced tyrosine phosphorylation of βIL-3 depends on the membrane-proximal cytoplasmic region of the EpoR, which is critical for coupling with Jak2, but not on the carboxy-terminal 145 amino-acid region containing all the tyrosine phosphorylation sites.

IL-3 induces physical association of Jak2 with βIL-3. To further investigate the mechanisms of Epo-induced tyrosine phosphorylation of βIL-3, we first examined the physical association of βIL-3 with Jak2, the tyrosine kinase implicated in tyrosine phosphorylation of the EpoR, as well as βIL-3.13,19,33 For this purpose, 32D/EpoR-Wt cells were stimulated with various concentrations of IL-3 and solubilized with a lysis buffer containing digitonin instead of Triton X-100. The cell lysates were subjected to immunoprecipitation with anti-Jak2 and data not shown). These results agree with those obtained with 32D/EpoR-Wt cells and further indicate that followed by immunoblotting with anti-Jak2. As shown in Fig 5A, the association of Jak2 with βIL-3 was readily observed when cells were stimulated with IL-3 at 1 ng/mL and increased remarkably at 10 ng/mL, although low background levels of Jak2 association with βIL-3 were faintly visible in cells stimulated with lower concentrations of IL-3 or even in unstimulated cells. To examine the time course of Jak2 association with βIL-3, 32D/EpoR-Wt cells were then stimulated with 10 ng/mL of IL-3 for various times and examined in the same way. The IL-3–induced association of Jak2 with βIL-3 was observed as early as 1 minute after IL-3 simulation, increased to a plateau level at 5 minutes, and was stably observed for as long as 2 hours after stimulation (Fig 5B).

We next examined whether Epo induces the association of Jak2 or the EpoR with βIL-3. As shown in Fig 5C, Epo and IL-3 rapidly induced the association of Jak2 with their cognate receptors. However, neither Epo nor IL-3 induced any significant increase in binding of Jak2 to βIL-3 nor the EpoR, respectively. Reprobing of the membrane with anti-phosphotyrosine confirmed that tyrosine-phosphorylated 130-kD Jak2 associated with the EpoR in stimulated cells, although the presence of Jak2 in the anti-βIL-3 immunoprecipitates from IL-3–stimulated cells could not be confirmed because of its similar size with tyrosine-phosphorylated βIL-3 (Fig 5C). Further reprobing of the membrane with anti-EpoR and anti-βIL-3 failed to show any physical association between the EpoR and βIL-3, as shown in Fig 5C.

Epo induces binding of Stat5 to βIL-3. IL-3, as well as Epo, induces tyrosine phosphorylation and activation of Stat5. We previously demonstrated that Stat5 transiently binds to the tyrosine phosphorylated receptors for Epo or IL-3 in cells stimulated with Epo or IL-3, respectively.23 To
in (A), the membranes were stripped and reprobed with antiphosphotyrosine immunoblotting, as indicated, followed by antiphosphotyrosine immunoblotting (anti-EGF-EpoR) was starved for 12 hours and left unstimulated (C) or stimulated with 100 U/mL of Epo, 25 ng/mL of IL-3, or 100 ng/mL of IL-3 for the indicated times (Fig 7). Stat5-Wt and Stat5-Y694F, both tagged with the HA epitope, were then immunoprecipitated with anti-HA and subjected to immunoblotting with antiphosphotyrosine. As shown in Fig 7A, IL-3-induced tyrosine phosphorylation of Stat5-Wt and its rapid and transient association with a tyrosine-phosphorylated protein of 140-kD, pp140, corresponding in size to tyrosine-phosphorylated βIL3. This is consistent with our previous findings with murine Stat5. On the other hand, Stat5-Y694F was only faintly tyrosine phosphorylated, but its association with pp140 was much more conspicuous and prolonged as compared with that of Stat5-Wt. Reprobing with anti-Stat5B, which does not cross-react with ovine Stat5 (data not shown), further showed that Stat5-Wt, but not Stat5-Y694F, dimerized with Stat5B in IL-3-stimulated cells (Fig 7A, middle panel). Further reprobing with anti-HA showed that the expression level of Stat5-Wt was moderately higher than that of Stat5-Y694F. Because βIL3 was tyrosine phosphorylated to equivalent levels in these two clones (data not shown), these data suggest that the binding of Stat5-Y694 to tyrosine phosphorylated βIL3 was sustained in contrast to the transient binding of Stat5-Wt. Anti-βIL3 blotting of the anti-HA immunoprecipitates, however, failed to demonstrate unambiguously that pp140 corresponded to tyrosine phosphorylated βIL3 because of a technical difficulty due to high backgrounds. We thus immunoprecipitated Stat5-Wt and Stat5-Y694F with anti-Stat5A, which cross-reacts with ovine Stat5 (data not shown), and examined their physical association with βIL3. As shown in Fig 7B, anti-βIL3 blotting of the anti-Stat5A immunoprecipitates obtained from the BaF3 clone expressing Stat5-Y694F confirmed the sustained binding of Stat5-Y694F to βIL3. These data indicate that the phosphorylation of Tyr694 of Stat5 is critical for the formation of Stat5 dimers, as well as for the dissociation of Stat5 from tyrosine-phosphorylated βIL3. These data thus strongly support a hypothetical model of Stat5 activation in which Stat5 is recruited to tyrosine-phosphorylated docking sites in βIL3 and, after phosphorylated by Jak2, rapidly dissociate from βIL3 to form a homodimer through the stable reciprocal interaction between the phosphorylated Tyr694 and the SH2 domain.

To examine the effect of sustained binding of Stat5-Y694F to βIL3 on the IL-3-mediated activation of Stat5, we next selectively immunoprecipitated endogenous Stat5B with anti-Stat5B and analyzed its phosphorylation status by antiphosphotyrosine immunoblotting. As shown in Fig 7C, the IL-3-induced tyrosine phosphorylation of Stat5B was sig-
Fig 4. Induction of tyrosine phosphorylation of β₃ is dependent on the membrane-proximal EpoR cytoplasmic region involved in the activation of Jak2. A clone of 32D cells expressing the S-mutant EpoR with carboxy-terminal deletion of 145 amino acids (A) or the PM4-mutant EpoR, which harbors a mutation, Trp282 to Arg, in the membrane-proximal cytoplasmic region that abolishes the ability to couple with Jak2 (B) were examined as described in the legend to Fig 1.

In the present study, we have demonstrated that Epo induces rapid and transient unidirectional cross-phosphorylation of β₃ on tyrosine(s) and its transient association with Stat5. Although we could not demonstrate any detectable Epo-induced binding of β₃ with Jak2 or the EpoR, the Epo-induced tyrosine phosphorylation of β₃ was dependent on the membrane-proximal EpoR cytoplasmic region involved in the activation of Jak2. It was also demonstrated that a Stat5 mutant, Stat5-Y694F, shows a sustained binding to tyrosine-phosphorylated β₃ and inhibits the IL-3-induced activation of endogenous Stat5. The results of the present study support a model for IL-3R-mediated Stat5 activation in which the Stat5-docking site(s) in β₃ transiently recruits Stat5 to facilitate its tyrosine phosphorylation and raise a possibility that Epo may cross-activate the IL-3R-signaling pathways.

The present study has also shown that IL-3 induces the association of Jak2 with β₃ in a dose- and time-dependent manner in IL-3-dependent hematopoietic cells. Consistent with this, Cattaneo et al has very recently reported that Jak2 physically associates with β₃ in a ligand-dependent manner in a central nervous system progenitor cell line, ST14A, transfected with both β₃ and the α chain of the murine IL-3R. These observations are, however, inconsistent with a previous report by Quelle et al, which showed that Jak2 constitutively associated with human βc when both were expressed in insect cells using the baculovirus expression vector. It is thus possible that the interaction of Jak2 with human βc may be different from that with murine β₃. However, in contrast to observations in hematopoietic cells, Jak2 was constitutively activated when expressed in insect cells, and the EpoR was constitutively tyrosine phosphorylated and associated with Jak2 in these cells.
Fig 5. IL-3, but not Epo induces the physical association of Jak2 with \( \beta_{IL3} \). After starvation, 32D/EpoR-Wt cells were stimulated with various concentrations of IL-3 for 1 minute (A) or for the indicated times with 10 ng/mL IL-3 (B). Cells were lysed in a lysis buffer containing 1% digitonin instead of 1% Triton X-100, immunoprecipitated with anti-\( \beta_{IL3} \), and subjected to anti-Jak2 immunoblotting. The membranes were then reprobed with anti-\( \beta_{IL3} \) (C) 32D/EpoR-Wt cells were treated as described in the legend to Fig 1, except that the cells were lysed in the digitonin lysis buffer. The cell lysates were immunoprecipitated with anti-EpoR or anti-\( \beta_{IL3} \), as indicated, and subjected to anti-Jak2 immunoblotting. The membrane was reprobed sequentially with antiphosphotyrosine (\( \alpha_{PY} \)), anti-\( \beta_{IL3} \), and anti-EpoR, as indicated. The size markers are indicated and given in kD.

is thus more likely, as suggested by Quelle et al\(^{33}\) that the very high concentrations that were achieved in insect cells precluded the detection of subtle changes in affinities that would affect associations in mammalian cells. In agreement with this idea, Brizzi et al\(^{35}\) have recently reported that Jak2 physically associates with \( \beta_c \) only upon GM-CSF stimulation in human polymorphonuclear leukocytes, thus suggesting that, under physiological conditions, the association between Jak2 and human \( \beta_c \) may also be ligand-dependent. Although the exact mechanisms of how IL-3 and GM-CSF induce the Jak2 binding to the \( \beta \) subunit of their receptors remain unknown, it is speculated that the ligand-dependent heterodimerization of the \( \beta \) subunit with the \( \alpha \) subunit or homodimerization of the \( \beta \) subunits may be involved in increasing the affinity of Jak2 binding to \( \beta_c \) or \( \beta_{IL3} \).

The induction of tyrosine phosphorylation of \( \beta_{IL3} \) by Epo suggests that Jak2 activated by the EpoR may physically interact with \( \beta_{IL3} \) to cause its tyrosine phosphorylation upon Epo stimulation. However, in cotransfection experiments, neither Jak2 nor the EpoR was demonstrated to associate with \( \beta_{IL3} \) upon Epo stimulation of cells. This could be due to a low stoichiometry of the association. It is also possible that the association is very transient or unstable to be detected by the method employed. Consistent with the

Fig 6. Epo induces binding of Stat5 to \( \beta_{IL3} \). 32D/EpoR-Wt cells were starved for 12 hours and left unstimulated (C) or stimulated with 100 U/mL of Epo or 10 ng/mL of IL-3 for 1 minute. The cells were lysed and immunoprecipitated with anti-Stat5B. The immunoprecipitates were subjected to immunoblotting with anti-\( \beta_{IL3} \), followed by reprobing with antiphosphotyrosine (\( \alpha_{PY} \)) and anti-Stat5B, as indicated.
idea that Jak2 activated by the EpoR may phosphorylate β_{IL3}, the ability of the EpoR to induce the tyrosine phosphorylation of β_{IL3} was abrogated by a mutation in the membrane-proximal cytoplasmic region of the EpoR, W282R, that abolishes the ability of the receptor to activate Jak2.\(^{15,19}\) The extracellular and transmembrane regions of the EpoR, on the other hand, was not required for the induction of β_{IL3} phosphorylation, thus indicating that the EpoR does not interact with β_{IL3} through these regions. This finding is also against the possibility that a hypothetical second subunit that has been shown to bind Epo in \(^{36-38}\) 125I-Epo binding studies \(^\text{36-38}\) may be involved in induction of the tyrosine phosphorylation of β_{IL3}. The carboxy-terminal 145 amino acid region of the EpoR containing all the intracellular tyrosines was also dispensable for the induction of β_{IL3} phosphorylation. Therefore, neither the phosphorylated tyrosines in the EpoR nor the various signaling molecules recruited to the phosphotyrosines are involved in the possible interaction of the EpoR with β_{IL3}.

Unidirectional cross-phosphorylation of the β subunits of the IL-3/GM-CSF receptor by Epo\(^{39}\) or G-CSF\(^{40}\) stimulation has previously been reported, although the mechanisms for the cross-phosphorylation or the possible effects on downstream signaling events from the IL-3R was not examined in these studies. Hanazono et al\(^{39}\) recently reported that Epo-extracellular and transmembrane regions of the EpoR, on the other hand, was not required for the induction of β_{IL3} phosphorylation in human leukemic cell line, UT-7. We could not, however, demonstrate Epo-induced tyrosine phosphorylation of murine β_{IL3} in the 32D and BaF3 transfectants nor human β_{IL3} in human leukemic cell lines, TF-1 and F36E (data not shown). This discrepancy might be due to differences in the cell lines and reagents used in the two studies or due to other differences in experimental design. Previously, Pan et al\(^{40}\) reported that granulocyte colony-stimulating-factor (G-CSF) induces unidirectional cross-phosphorylation of both β_{IL3} and β_{ILc} on tyrosines in 32D cells expressing the transfected murine G-CSF receptor. Unlike the Epo-induced phosphorylation in the present study, G-CSF induced the tyrosine phosphoryla-
tion of $\beta_{IL3}$ to the degree comparable with that induced by IL-3. This difference may reflect the difference in the numbers of the receptors expressed on the cell surface; whereas the G-CSF receptor was expressed at around 10,000 molecules/cell, 40 the EpoR is inefficiently transported to the cell surface and expressed at around a 10-fold lower level, as compared with the G-CSF receptor, in transfected IL-3-dependent cell lines. 12,19,29,41,42

In a previous report, we demonstrated that Stat5 binds very rapidly and transiently to $\beta_{IL3}$ after IL-3 stimulation. 23 The time course of this binding was very similar to that of the Epo-dependent Stat5 binding to the EpoR, which was shown to have specific phosphotyrosyl Stat5 docking sites that facilitate the activation of Stat5. Therefore, we speculated that $\beta_{IL3}$ may also have Stat5 docking sites similar to those demonstrated in the EpoR. 23 In the present study, it was demonstrated that Stat5-Y694F failed to dimerize with endogenous Stat5, showed a sustained binding to tyrosine phosphorylated $\beta_{IL3}$, and inhibited the activation of endogenous Stat5 in cells stimulated with IL-3. These results suggest that Stat5-Y694F may inhibit the activation of Stat5 by the IL-3R in a dominant-negative manner by stably binding to tyrosine-phosphorylated $\beta_{IL3}$ and thereby inhibiting the recruitment of endogenous Stat5. These findings thus agree with the idea that $\beta_{IL3}$ has the Stat5 docking sites that facilitate its activation and strongly support a hypothetical model for Stat activation by cytokines in which Stats are recruited, through their SH2 domains, to phosphotyrosyl docking sites in receptors and, after tyrosine phosphorylated by receptor-associating Jak5, rapidly dissociate from receptors to form homo- or heterodimers through reciprocal SH2 domain-phosphotyrosine interactions. 6 Notably, the intracellular domain of murine $\beta_{IL3}$ has 5 tyrosine residues conforming to a YXXL motif that has been proposed as a putative motif for Stat5 docking sites in the EpoR. 27 Further studies are, however, required to identify the Stat5 docking sites in $\beta_{IL3}$ and to determine its significance on activation of Stat5 by the IL-3R.

The present study has raised the possibility that Epo may cross-activate the IL-3R to induce activation of Stat5, because Stat5 was shown to be recruited to the possible Stat5-docking sites in $\beta_{IL3}$ upon Epo stimulation. A possible recruitment of other SH2-containing signaling molecules, such as those involved in activation of the Ras/MAP kinase pathway, to tyrosine-phosphorylated $\beta_{IL3}$ upon Epo stimulation also needs to be examined. In this regard, it should be noted that, unlike the carboxy-terminal truncation of $\beta_c$, 44 the truncation of the EpoR cytoplasmic domain severely impairs, but does not completely abrogate the ability of EpoR to activate the Ras/MAP kinase pathway in IL-3-dependent cells. 45 It is thus possible that the truncated mutant EpoR may activate the Ras/MAP kinase pathway at a very low level by cross-activating the IL-3R. Alternatively, Epo may downregulate the IL-3R-mediated signaling, because the tyrosine phosphorylation of $\beta_{IL3}$ causes an intrinsic change, which greatly increases its susceptibility to proteolysis both in vitro and in vivo. 46 The present study, however, could not determine the functional significance of the Epo-induced tyrosine phosphorylation of $\beta_{IL3}$, because the EpoR and the IL-3R activate strikingly similar signaling events and cellular responses in the hematopoietic cell lines examined. These two receptors, however, elicit different biological responses when expressed on hematopoietic progenitor cells, including burst-forming units erythroid (BFU-E). Further studies using purified erythroid progenitor cells will be required to elucidate the functional significance of Epo-induced tyrosine-phosphorylation of $\beta_{IL3}$ on the regulation of the hematopoiesis.

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Erythropoietin Induces Tyrosine Phosphorylation of the Interleukin-3 Receptor β Subunit (β_{IL3}) and Recruitment of Stat5 to Possible Stat5-Docking Sites in β_{IL3}

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