RAPID COMMUNICATION

Physical and Functional Interactions Between Stat5 and the Tyrosine-Phosphorylated Receptors for Erythropoietin and Interleukin-3

By Hiroshi Chin, Norihiko Nakamura, Ryuichi Kamiyama, Nobuyuki Miyasaka, James N. Ihle, and Osamu Miura

Erythropoietin (Epo) and interleukin-3 (IL-3) stimulate activation of the Jak2 tyrosine kinase and induce tyrosine phosphorylation and activation of Stat5. In the present study, we have shown that Epo or IL-3 stimulation induces binding of Stat5 to the tyrosine-phosphorylated Epo receptor (EpoR) or IL-3 receptor β subunit (β3), respectively, in IL-3-dependent 32D cells expressing the EpoR. The binding of Stat5 to these cytokine receptors was shown to be rapid and transient, occurring within 1 minute of stimulation of cells and significantly decreasing after 5 minutes of cell treatment. In vivo binding experiments in COS cells showed that binding of Stat5 to the EpoR was mediated through the STAT SRC homology 2 (SH2) domain. In vitro binding studies further showed that Stat5, but not other Stats examined, bound specifically to tyrosine-phosphorylated recombinant EpoR proteins. In these in vivo and in vitro binding studies, Stat5 bound, albeit to a lesser degree, to truncated EpoR mutants in which all the intracellular tyrosines except Y-343 were removed. Furthermore, EpoR-derived synthetic phosphotyrosine peptides corresponding to Y-343, Y-401, Y-431, and Y-479 inhibited the in vitro binding of Stat5. When expressed in 32D cells, a mutant EpoR in which all the intracellular tyrosines were removed by carboxy-terminal truncation showed a significantly impaired ability to induce tyrosine phosphorylation of Stat5, particularly at low concentrations of Epo, but exhibited an increased sensitivity to Epo for growth signaling as compared with the wild-type EpoR. These results indicate that Stat5 specifically and transiently binds to the EpoR through the interaction between the Stat5 SH2 domain and specific phosphorylated tyrosines, including Y-343, in the EpoR cytoplasmic domain. It was implied that β3 may also have similar Stat5 docking sites. The Stat5 docking sites in the EpoR were shown to facilitate specific activation of Stat5, which, however, may not be required for the EpoR-mediated growth signaling. © 1996 by The American Society of Hematology.
of cellular proteins. Recent studies further showed that Epo and IL-3 induce tyrosine phosphorylation and activation of Stat5, which was initially purified and cloned as a mediator of prolactin-induced transcription of the β-casein gene. Two murine homologues, Stat5A and Stat5B, sharing 96% amino acid sequence identity, have been identified, although no functional differences have yet been observed between these two molecules. A variety of other cytokines, including growth hormone, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-5, IL-2, and thrombopoietin, have also been shown to activate Stat5.

Mun et al previously reported that tyrosine residues in the intracellular region of the β component of the human IL-3R/GM-CSF receptor were not required for induction of tyrosine phosphorylation and activation of Stat5. On the other hand, very recent studies showed that Y-343, based on nomenclature for the mature protein, of the EpoR plays an essential role in Stat5 activation, because mutations of this tyrosine in truncated EpoR mutants that had lost all the other tyrosine residues significantly diminished or eliminated the ability of the receptors to activate Stat5. Furthermore, synthetic tyrosine phosphopeptides derived from the EpoR sequence containing Y-343 disrupted Stat5 DNA binding activity. It was thus proposed that Y-343 and other tyrosines in the EpoR cytoplasmic region may function as Stat5 docking sites. However, in these studies, binding of Stat5 with the EpoR was not shown. Therefore, the exact mechanisms by which Stat5 specifically interacts with the EpoR to become tyrosine phosphorylated and activated by Jak2 have remained elusive. In addition, a possible role Stat5 has remained controversial, because conflicting results were reported in these studies.

In the present study, we provide the first direct evidence that Stat5 binds to the receptors for Epo and IL-3 after ligand stimulation. We further investigated the mechanisms of interaction of Stat5 with the EpoR and also evaluated the significance of the Stat5 binding on Epo-induced Stat5 phosphorylation and growth signaling.

MATERIALS AND METHODS

Cells and reagents. IL-3–dependent 32D cell clones that express equivalent numbers, as determined by 125I-Epo binding assays, of the wild-type EpoR and the truncated S-mutant EpoR were previously described and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10% WEHI-3 conditioned medium as a source of IL-3. Recombinant human Epo was kindly provided by Chugai Pharmaceutical Co Ltd (Tokyo, Japan). Recombinant murine IL-3 was purchased from PeproTech Inc (Rocky Hill, NJ). COS-7 cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% FCS.

Expression vectors for the murine wild-type or truncated EpoRs, the murine Stat5A cDNA, and EpoR-derived synthetic phosphopeptides were described previously. A eukaryotic expression vector, pJ3M, was obtained from American Type Culture Collection (Rockville, MD).

A rabbit antiserum raised against a glutathione S-transferase (GST) fusion protein containing amino acids 257 to 441 of the EpoR cytoplasmic domain was described previously. Rabbit antibodies against Jak2, Stat3, Stat4, Stat5A, and Stat6 were also described previously. Rabbit antibodies against Stat5B and β1/β3 (K-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies against phosphotyrosine (4G10), c-Myc (9E10), and Stat1 were purchased from Upstate Biotechnology, Inc (Lake Placid, NY), Berkeley Antibody Co (Richmond, CA), and Transduction Laboratories (Lexington, KY), respectively.

Immunoprecipitation and immunoblotting. Cells were washed free of IL-3, cultured overnight, and left unstimulated as a negative control or stimulated with Epo or IL-3 at a saturating concentration, unless otherwise described. Cells were solubilized at 4 °C with lysis buffer composed of 1% Triton X-100, 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonfluoride (PMSF), 10 μg/mL aprotinin, and 10 μg/mL leupeptin. Cell lysates were subjected to immunoprecipitation as described previously. For immunoblot analysis of total cell lysates, aliquots of the clarified supernatant were directly mixed with equal volumes of 2× Laemmli’s sample buffer and heated at 100°C for 5 minutes. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-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, the proteins were treated with stripping buffer composed of 100 mmol/L 2-mercaptoethanol, 2% SDS, and 0.25 mmol/L Tris-HCl (pH 6.7) at 50°C for 30 minutes and subsequently probed with a different antibody.

Transient expression in COS-7 cells. For construction of an expresion plasmid for the Stat5 SH2 domain, a portion of the murine Stat5A cDNA coding for amino acids 589 to 677 was amplified by the polymerase chain reaction (PCR) using 5′ and 3′ primers of 5′-CCCGATCC TGGAAATGATGGGGCTATCCT-3′ and 5′-CCGAAT TCACGTCTCAGGCTCGCCCTC-3′, respectively. The primer sequences were designed to add the BamHII and EcoRI recognition sites at the 5′ and 3′ ends, respectively, of the amplified fragment. These sites were then used for directional subcloning of the amplified fragment into an expression plasmid, pJ3M, which is designed to add an epitope tag from c-Myc to the N-terminus of protein expressed. Transfection of expression plasmids into COS-7 cells was performed using the Lipofectamine reagent (HBCO-BRL, Grand Island, NY) according to the manufacturer’s instructions. Two days after transfection, cells were stimulated with Epo or left unstimulated, solubilized, and subjected to immunoprecipitation and immunoblotting as described above.

Preparation of tyrosine-phosphorylated GST-EpoR recombinant proteins and in vitro binding assays. Bacterial expression plasmids containing the cytoplasmic domains of the wild-type and S-mutant EpoRs were generated by subcloning the Bgl II-EcoRI fragments from the eukaryotic expression vectors pXM-EpoR and pXM-EpoR-S, respectively, into the BamHII-EcoRI site of pGEX-3 (Pharmacia, Uppsala, Sweden). To generate an expression plasmid for the Stat5 SH2 domain, a portion of the murine Stat5A cDNA coding for amino acids 589 to 677 was amplified by the polymerase chain reaction (PCR) using 5′ and 3′ primers of 5′-CCCGATCC TGGAAATGATGGGGCTATCCT-3′ and 5′-CCGAAT TCACGTCTCAGGCTCGCCCTC-3′, respectively. The primer sequences were designed to add the BamHII and EcoRI recognition sites at the 5′ and 3′ ends, respectively, of the amplified fragment. These sites were then used for directional subcloning of the amplified fragment into an expression plasmid, pJ3M, which is designed to add an epitope tag from c-Myc to the N-terminus of protein expressed. Transfection of expression plasmids into COS-7 cells was performed using the Lipofectamine reagent (HBCO-BRL, Grand Island, NY) according to the manufacturer’s instructions. Two days after transfection, cells were stimulated with Epo or left unstimulated, solubilized, and subjected to immunoprecipitation and immunoblotting as described above.

Preparation of tyrosine-phosphorylated GST-EpoR recombinant proteins and in vitro binding assays. Bacterial expression plasmids coding for GST fusion proteins containing the cytoplasmic domains of the wild-type and S-mutant EpoRs were generated by subcloning the Bgl II-EcoRI fragments from the eukaryolic expression vectors pXM-EpoR and pXM-EpoR-S, respectively, into the BamHII-EcoRI site of pGEX-3 (Pharmacia, Uppsala, Sweden). To generate an expression plasmid for the Stat5 SH2 domain, a portion of the murine Stat5A cDNA coding for amino acids 589 to 677 was amplified by the polymerase chain reaction (PCR) using 5′ and 3′ primers of 5′-CCCGATCC TGGAAATGATGGGGCTATCCT-3′ and 5′-CCGAAT TCACGTCTCAGGCTCGCCCTC-3′, respectively. The primer sequences were designed to add the BamHII and EcoRI recognition sites at the 5′ and 3′ ends, respectively, of the amplified fragment. These sites were then used for directional subcloning of the amplified fragment into an expression plasmid, pJ3M, which is designed to add an epitope tag from c-Myc to the N-terminus of protein expressed. Transfection of expression plasmids into COS-7 cells was performed using the Lipofectamine reagent (HBCO-BRL, Grand Island, NY) according to the manufacturer’s instructions. Two days after transfection, cells were stimulated with Epo or left unstimulated, solubilized, and subjected to immunoprecipitation and immunoblotting as described above.
on a large scale by affinity chromatography on glutathione-Sepharose beads (Pharmacia). To prepare tyrosine-phosphorylated fusion proteins, E. coli cells were further cultivated for 2 hours in Trp starvation medium in the presence of indoleacrylic acid to induce expression of the Elk tyrosine kinase. Purified proteins were resolved by SDS-PAGE and subjected to evaluation with Coomassie Blue staining or antiphosphotyrosine blotting.

In vitro binding of various Stat proteins to the GST-EpoR fusion proteins was examined essentially as described previously. In brief, 32D/EpoR-Wt cells were lysed in the lysis buffer described above at 2 × 10⁸ cells/mL. Lysates were mixed with 1 μg of GST fusion proteins on glutathione-Sepharose beads and incubated at 4°C for 2 hours. After being washed three times with the lysis buffer, proteins bound to the beads were eluted by boiling in Laemmli’s SDS sample buffer and examined by immunoblotting with antibodies against various Stat proteins. For competition of Stat5 binding to EpoR with EpoR-derived phosphotyrosine peptides, cell lysates were incubated for 2 hours at 4°C with 200 μmol/L or the indicated concentrations of a synthetic peptide before incubation with beads containing the tyrosine-phosphorylated wild-type EpoR fusion protein.

Cell growth assay. For measurement of the cell number increase, cells were seeded into a 96-well plate at a density of 1 × 10⁵/mL in 10% FCS-containing RPMI 1640 medium supplemented with the indicated concentrations of Epo or with 25 U/mL of IL-3. After 2 days, the number of viable cells was measured by the sodium 3'-[1-(phenylaminocarbonyl)-3, 4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) colorimetric assay (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer’s recommendation.

RESULTS

Epo and IL-3 induce binding of Stat5 to their tyrosine-phosphorylated receptors and stimulate its tyrosine phosphorylation. To explore the mechanisms for Epo- or IL-3-induced activation of Stat5, we stimulated 32D/EpoR-Wt, a clone of IL-3-dependent 32D cells expressing the transfected wild-type murine EpoR cDNA, were washed out of IL-3 for 12 hours and left unstimulated (−) or stimulated with Epo (Ep) or IL-3 (Il3) for 1 minute at 37°C before solubilization. The cell lysates were immunoprecipitated with antiserum specifically reactive with Stat5A or Stat5B, as indicated. Immunoprecipitates were resolved by 6% SDS-PAGE and subjected to immunoblotting with an antiphosphotyrosine monoclonal antibody, 4G10 (αPY). The relevant portion of the membrane was stripped and reprobed sequentially with anti-Stat5A and anti-Stat5B antisera, as indicated. The molecular weight markers are indicated and given in kilodaltons.

![Figure 1](https://example.com/figure1.png)

**Fig 1.** Epo and IL-3 induce tyrosine phosphorylation of Stat5 and its association with tyrosine-phosphorylated 72- and 140-kD proteins, respectively. 32D/EpoR-Wt cells, a clone of an IL-3-dependent 32D cell line expressing the transfected wild-type murine EpoR cDNA, were washed out of IL-3 for 12 hours and left unstimulated (−) or stimulated with Epo (Ep) or IL-3 (Il3) for 1 minute at 37°C before solubilization. The cell lysates were immunoprecipitated with antisera specifically reactive with Stat5A or Stat5B, as indicated. Immunoprecipitates were resolved by 6% SDS-PAGE and subjected to immunoblotting with an antiphosphotyrosine monoclonal antibody, 4G10 (αPY). The relevant portion of the membrane was stripped and reprobed sequentially with anti-Stat5A and anti-Stat5B antisera, as indicated. The molecular weight markers are indicated and given in kilodaltons.

thereafter. Immunoblotting with anti-EpoR showed that the tyrosine-phosphorylated 72-kD form of EpoR, which corresponds to only a small fraction of the whole EpoR, physiologically associated with Stat5 and comigrated with pp72. The association of Stat5 with the EpoR was dependent on Epo stimulation, peaked at 1 minute after stimulation, and became barely detectable after 10 minutes. Reprobing of the filter with anti-Stat5B confirmed equal loading of Stat5B immunoprecipitates. The association of Stat5 with β₅,₃ was similarly examined, as shown in Fig 2B. Antiphosphotyrosine and anti-β₅,₃ blotting of Stat5B immunoprecipitates showed that IL-3 stimulation of 32D/EpoR-Wt induced a transient association of Stat5B with tyrosine-phosphorylated β₅,₃, which peaked at 1 minute after stimulation. Slower migration of Stat5B, run on a 5% gel, was conspicuously observed at 5 or 30 minutes after IL-3 stimulation and corresponded to a prominent tyrosine phosphorylation of Stat5 observed at these times. For the results shown in Fig 2A and B, comparable results were also observed by using anti-Stat5A (data not shown).

The SH2 domain of Stat5 is involved in binding to the EpoR. To further explore the mechanisms of Stat5 binding to the EpoR, we then examined whether the Stat5 SH2 domain is involved in binding to the tyrosine-phosphorylated EpoR, because it has been established that a variety of intra-
cellular signaling molecules bind through their SH2 domains to tyrosine-phosphorylated receptors for various growth factors and cytokines. For this purpose, we expressed the Stat5 SH2 domain, which was tagged with a c-Myc epitope, in COS-7 cells and examined its physical association with coexpressed EpoR or its truncated mutants (Fig 3A) by anti-Myc immunoblotting of anti-EpoR immunoprecipitates. As shown in Fig 3B, the association of the Stat5 SH2 domain with the wild-type EpoR was barely detectable when only these two proteins were expressed in COS cells. However, the association increased significantly when the Jak2 kinase was coexpressed, with a further increase in binding observed when cells were stimulated with Epo. The Stat5 SH2 domain also bound to the truncated H mutant, which retained Y-343 as the only tyrosine in the intracellular domain when the mutant was expressed with Jak2. In contrast, the Stat5 SH2 domain failed to associate with the further truncated S mutant, in which all the intracellular tyrosines were removed. Reprobing of the membrane with anti-EpoR antibody confirmed that the wild-type EpoR and the mutants were expressed at comparable levels (Fig 3B). Further reprobing with antiphosphotyrosine antibody showed that the wild-type EpoR was constitutively tyrosine phosphorylated, whereas the tyrosine phosphorylation of the two mutants was not detectable (Fig 3B). These results indicate that the Stat5 SH2 domain is involved in binding with the EpoR. However, it remained to be determined whether the phosphorylated tyrosine residues in the EpoR cytoplasmic region are also involved in the binding, because the tyrosine phosphorylation status of the wild-type EpoR or the H mutant, as examined by antiphosphotyrosine blotting, did not correlate with the binding of the Stat5 SH2 domain in COS cells.

Involvement of phosphorylated tyrosine residues in the EpoR cytoplasmic domain in binding with Stat5. To examine whether phosphorylated tyrosine residues in the EpoR cytoplasmic domain are required for binding with Stat5, we prepared tyrosine-phosphorylated GST-EpoR fusion proteins (Fig 4A) by coexpressing the Elk tyrosine kinase with these fusion proteins in E. coli, as described in the Materials and Methods. The tyrosine-phosphorylated and unphosphorylated fusion proteins, bound to glutathione Sepharose beads, were then used for in vitro binding assays with Stat5 and other Stat proteins in cell lysates. As shown in Fig 4B, Stat1, Stat3, Stat4, or Stat6 failed to associate with the fusion proteins, irrespective of their tyrosine phosphorylation status. Stat5A and Stat5B, on the other hand, specifically bound to the tyrosine-phosphorylated forms of GST-EpoR fusion proteins. Importantly, binding of Stat5A and Stat5B was observed, albeit to a lesser extent, with an GST-EpoR protein (−91) in which the carboxy-terminal 91 amino acids, including all the tyrosine residues except for Y-343, were removed by truncation. These results indicate that the phosphorylation of Y-343 or other tyrosines in the EpoR cytoplasmic domain is required for specific binding with Stat5. In accordance with the failure of other Stats to associate with the EpoR, antiphosphotyrosine blotting of immunoprecipitates obtained with specific antibodies failed to detect any tyrosine phosphorylation of these Stats in 32D/EpoR cells stimulated with Epo for 1 minute (data not shown). This implies that the specificity of Stat activation by EpoR depends on the specific recruitment of Stat5 to the phosphotyrosines in the EpoR cytoplasmic domain.

To define tyrosine residues in the EpoR that are involved in binding of Stat5, in vitro binding competition assays were performed using synthetic phosphopeptides corresponding to the potential phosphorylation sites of the EpoR (Table I). Cell lysates from 32D/EpoR-Wt cells were incubated with each of these phosphopeptides and subsequently subjected to the in vitro binding assay with the tyrosine-phosphorylated GST-EpoR-Wt protein. As shown in Fig 4C, the phosphopeptide containing Y-343, Y-431, or Y-479 almost completely inhibited binding of Stat5 to the EpoR, whereas the dephosphorylated forms of these peptides did not show any significant inhibition. The phosphopeptide containing Y-401
Also significantly inhibited the Stat5 binding in two repeated experiments, although the inhibition by this peptide was only moderate in the experiment shown in Fig 4C. Various concentrations of phosphopeptides containing Y-343, Y-401, Y-431, and Y-479 were then tested to determine the relative efficiency of each peptide to inhibit Stat5 binding (Fig 4D). Densitometric scanning of immunoblots showed that the Y-479 peptide most efficiently inhibited the binding, whereas the Y-401 peptide was least effective (Table 1).

Epo-induced Stat5 phosphorylation and cell growth in 32D cells expressing a mutant EpoR that lacks the Stat5 docking sites. To explore the significance of Stat5 docking sites of the EpoR, we examined the Epo dose response of tyrosine phosphorylation of Stat5 in 32D/EpoR-S, a clone of 32D cells expressing the S-mutant EpoR in which all the 8 tyrosine residues were removed by carboxy-terminal truncation of 145 amino acids (Fig 3A). As shown in Fig 5A, tyrosine phosphorylation of Stat5 was readily detectable in 32D/EpoR-Wt cells stimulated with 0.01 U/mL of Epo. In contrast, Epo-induced Stat5 phosphorylation in 32D/EpoR-S cells was hardly discernible when stimulated with a dose as high as 1 U/mL of Epo, although it was observed at an almost comparable level with that in 32D/EpoR-Wt at 100 U/mL of Epo. The ability of the S-mutant EpoR to induce tyrosine phosphorylation of Stat5 was thus impaired most significantly when stimulated at low concentrations of Epo. On the other hand, Epo-induced tyrosine phosphorylation of Jak2 was comparably observed in 32D/EpoR-Wt or 32D/EpoR-S when stimulated at 10 to 100 U/mL of Epo. These results indicate that the recruitment of Stat5 to the tyrosine-phosphorylated EpoR may be required for tyrosine phosphorylation of Stat5 at physiological concentrations of Epo, whereas Jak2 may directly interact and phosphorylate Stat5 when prominently autophosphorylated by stimulation with higher concentrations of Epo.

To evaluate a possible role that Stat5 may play in the EpoR-mediated growth signaling, we next compared the Epo dose responses of proliferation of 32D/EpoR-Wt and 32D/EpoR-S. As shown in Fig 5B, 32D/EpoR-S exhibited an increased sensitivity to Epo as compared with 32D/EpoR-Wt and proliferated significantly in response to as low as 10 mU/mL of Epo. This is in agreement with previous studies that showed the hypersensitivity of various truncated EpoR mutants to Epo in BaF3 and 32D cell lines.38,52,53 Because Epo-induced tyrosine phosphorylation of Stat5 was distinctly diminished and hardly discernible in 32D/EpoR-S stimulated at 10 to 100 mU/mL of Epo, the increased mitogenic response of 32D/EpoR-S as compared with 32D/EpoR-Wt at
A

Intracellular

GST

S -91 -42

B

Blot:

C

aStat1 97-
aStat3 97-
aStat4 97-
aStat5A 97-
aStat5B 97-
aStat6 97-

D

Y-343  Y-401

Peptide (μM)

0 25 50 100 200 0 25 50 100 200

Y-431  Y-479

Peptide (μM)

0 25 50 100 200

Fig 4. Binding of Stat5 to the tyrosine-phosphorylated intracellular domain of EpoR in vitro. (A) Schematic representation of GST-EpoR fusion proteins used in the binding studies. The intracellular domain of wild-type EpoR is represented by a hatched area. The positions of truncation for three mutants (S, -91, and -42) are indicated. Vertical thick lines represent tyrosine residues in the EpoR cytoplasmic domain. (B) In vitro binding assays of various Stats to GST-EpoR fusion proteins. Cell lysates from 32D/EpoR-Wt cells were incubated with beads containing GST or various GST-EpoR fusion proteins indicated above the panels. GST-EpoR fusion proteins that were tyrosine phosphorylated in E. coli by the Elk tyrosine kinase, as described in the Materials and Methods, are denoted with -PY. Proteins bound to beads were resolved, along with total cell lysate (TCL), by 6% SDS-PAGE and immunoblotted with antibodies against various Stats as indicated. (C) Inhibition of in vitro binding of Stat5 to the tyrosine-phosphorylated EpoR by EpoR-derived tyrosine-phosphorylated synthetic oligopeptides. Cell lysates from 32D/EpoR-Wt were preincubated at 4°C for 2 hours with synthetic phosphopeptides (200 μmol/L) corresponding to potential tyrosine phosphorylation sites in EpoR, as indicated above the panel, or without any peptide as a control (C). The unphosphorylated equivalents of Y-343 (Y-343-DP) and Y-479 (Y479-DP) polypeptides were also used in competition experiments, as indicated. Preincubated cell lysates were then subjected to the in vitro binding assay, as described in (B), using the tyrosine-phosphorylated GST-EpoR-Wt protein and immunoblotted with anti-Stat5B antibody. (D) Concentration dependence of inhibition of Stat5 binding with EpoR-derived phosphopeptides. Cell lysates were preincubated with various concentrations of EpoR-derived phosphopeptides, as indicated, and subjected to the in vitro binding assay as described above.

these low concentrations of Epo suggests that Epo-induced Stat5 activation may be dispensable for the EpoR-mediated growth signaling.

DISCUSSION

The present study provides the first direct evidence that Stat5 binds to the tyrosine-phosphorylated cytokine receptors. The ligand-dependent binding of Stat5 to the receptors for Epo and IL-3 in vivo was found to be rapid and transient, occurring within 1 minute of stimulation of cells and significantly decreasing after 5 minutes of cell treatment. This is in contrast to the previously reported binding of Stat3 or Stat2 to the IFNα receptor, which was ligand-dependent but stable, and strongly supports a hypothetical model for Stat activation in which Stats are recruited to the tyrosine-

Table 1. Inhibition of Stat5 Binding to the EpoR by EpoR-Derived Phosphotyrosine Peptides

<table>
<thead>
<tr>
<th>Amino Acid Sequence</th>
<th>Inhibition of Stat5 Binding</th>
<th>Half-Inhibitory Concentration (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QDT Y-343 LVLDKWL</td>
<td>+</td>
<td>25-50</td>
</tr>
<tr>
<td>SFE Y-401 TILDPSS</td>
<td>+</td>
<td>50-100</td>
</tr>
<tr>
<td>HLK Y-429 LYLVVSD</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>KYL Y-431 LYYSDS</td>
<td>+</td>
<td>25-50</td>
</tr>
<tr>
<td>STD Y-443 SSSSGQ</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>DGP Y-480 SHPYENS</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>SHP Y-464 ENSLVPD</td>
<td></td>
<td>ND</td>
</tr>
<tr>
<td>HPG Y-479 VACS</td>
<td>+</td>
<td>&lt;25</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.
Fig 5. Epo dose responses of Stat5 phosphorylation and cell growth in a clone of 32D cells expressing a mutant EpoR without Stat5 docking sites. (A) Epo dose response of tyrosine phosphorylation of Stat5 and Jak2 in 32D clones expressing the wild-type or S-mutant EpoR. 32D cells expressing the wild-type or S-mutant EpoR, as indicated above panels, were starved for 12 hours and then stimulated with the indicated concentrations of Epo for 5 minutes before solubilization. Stat5 and Jak2 were immunoprecipitated, as indicated, and subjected to antiphosphotyrosine (aPY) immunoblotting. The membranes were then reprobed with the antibody used for immunoprecipitation to show equal loading. (B) Epo dose responses of proliferation of 32D clones expressing the wild-type and S-mutant EpoRs. 32D/EpoR-Wt (●) or 32D/EpoR-S (■) cells were cultured in the presence of indicated concentrations of Epo for 2 days. The numbers of viable cells were then measured by the XTT colorimetric assay. Each data point represents the mean ± SD of triplicate determinations and is expressed as a percentage of maximal stimulation with IL-3. The results shown are representative of three repeated experiments.
phosphorylated cytokine receptors through the SH2 domain and, after phosphorylated by Jak2, rapidly dissociate from the receptor to form homodimers through reciprocal SH2 domain-phosphotyrosine interactions (Fig 6).

In vivo binding studies in COS cells showed that the Stat5 SH2 domain is involved in binding with the EpoR. In vitro binding studies using GST-EpoR fusion proteins further showed that tyrosine phosphorylation of the EpoR cytoplasmic region is required for binding with Stat5. Importantly, other Stats examined, including Stat1, Stat3, Stat4, and Stat6, failed to associate with the tyrosine-phosphorylated GST-EpoR. Because none of these Stats was tyrosine phosphorylated in Epo-stimulated cells (data not shown), specific binding of Stat5 to the tyrosine-phosphorylated GST-EpoR proteins support the idea that the specificity of Stat activation by cytokine receptors is dependent on the specific interaction between the Stat SH2 domains and the phosphotyrosine sequences in cytokine receptor cytoplasmic regions.

Competition studies using synthetic tyrosine phosphopeptides derived from possible tyrosine phosphorylation sites in the EpoR further showed that Y-343, Y-401, Y-431, and Y-479, when phosphorylated, may be involved in the recruitment of Stat5. Of these tyrosine residues, Y-343 most likely plays an important role in binding with Stat5, because the in vivo binding study in COS cells showed that a truncated EpoR retaining Y-343 as the only intracellular tyrosine residue bound the Stat5 SH2 domain (Fig 3B). Furthermore, another truncated EpoR that also retains only Y-343 bound Stat5 in vitro when tyrosine phosphorylated (Fig 4B). In accordance with these results indicating that Y-343 should play a role in binding with Stat5, previous studies showed that this tyrosine residue plays an important role in activation of Stat5, because substitutions of Y-343 in the context of truncated EpoRs retaining only this tyrosine severely impaired or abrogated the ability of the receptors to activate Stat5. In these studies, EpoR-derived tyrosine-phosphorylated peptides containing Y-343 also disrupted the DNA binding activity of activated Stat5 dimer. However, it should be noted that Epo-induced phosphorylation of Y-343 has not been observed in previous studies or in the present study (Fig 3B), although a truncated EpoR retaining only Y-343 was tyrosine phosphorylated when expressed in Sf9 cells using a baculovirus vector or when coexpressed with the Elk tyrosine kinase in E coli (data not shown). It is thus possible that phosphorylated Y-343 may be highly susceptible to protein tyrosine phosphatases in mammalian cells. In accordance with this idea, the tyrosine phosphorylation of the H-mutant EpoR was observed in COS transfectants pretreated with a tyrosine phosphatase inhibitor, pervanadate, before Epo stimulation (H.C., N.N., and O.M., unpublished observation).

The other three tyrosine residues shown to inhibit Stat5 binding to the EpoR in the present study were also implicated in EpoR-mediated activation of Stat5 in previous studies. Using the same synthetic phosphopeptides used in this study, Quelle et al reported that the phosphopeptide containing Y-431 or Y-479 disrupted the DNA binding activity of activated Stat5 dimer. On the other hand, using different synthetic peptides, Gobert et al showed that a phosphopeptide containing Y-401 efficiently inhibited Stat5 binding to the EpoR, whereas the Y-401 phosphopeptide exhibited the most inefficient inhibitory activity among the four peptides, as judged by the concentrations of phosphopeptides required to reduce the Stat5 binding by half (Table 1). The differences in the synthetic peptides used or the assay systems employed may be responsible for the discrepancies observed. In this regard, it should be noted that the Elk-induced tyrosine phosphorylation pattern of the GST-EpoR fusion protein may be different from the Jak2-induced phosphorylation pattern of endogenous EpoRs in erythroid progenitors. Gobert et al also showed that Y-401 plays a role in EpoR-mediated activation of Stat5 by using a mutant receptor having Y-401 as the sole tyrosine residue in the cytoplasmic region, whereas Y-431 was found to have a marginal effect on Stat5 activation. However, it is noteworthy that the amino acids following Y-431 (Y-431 LVV) most closely resemble those following Y-343 (Y-343 LVL), which suggests that the Stat5 SH2
domain may bind to both Y-343 and Y-431. Although binding of Stat5 to the EpoR was most efficiently inhibited by the Y-479 phosphopeptide in the present study, the functional significance of Y-479 on EpoR-mediated Stat5 activation has remained to be determined.

The functional significance of the Stat5 docking sites on Stat5 activation was shown by the truncated S-mutant EpoR, in which all the intracellular tyrosine residues were removed by carboxy-terminal truncation, because this mutant exhibited a severely impaired ability to induce tyrosine phosphorylation of Stat5 (Fig 5A). The impairment was most significant at low Epo concentrations, whereas the tyrosine phosphorylation of Stat5 was readily detectable in the mutant-expressing cells stimulated at higher Epo concentrations. These observations are consistent with those of previous studies on a mutant EpoR in which all eight of the intracellular tyrosines of EpoR were mutated or the seven carboxy-terminal tyrosines were removed by truncation and Y-343 was mutated. Because the association of Stat5 with the truncated EpoR was undetectable in vitro (Fig 4B) or in vivo (data not shown), as expected from the absence of all the Stat5 docking sites, induction of tyrosine phosphorylation of Stat5 at high concentrations of Epo might have been mediated through a direct interaction between Stat5 and Jak2, which was conspicuously tyrosine phosphorylated at high concentrations of Epo (Fig 5A). This is in accordance with our previous study in which an epidermal growth factor (EGF) receptor/Jak2 tyrosine kinase domain chimera induced EGF-dependent tyrosine phosphorylation of Stat5 without activating any cytokine receptors. Taken together, these observations indicate that the Stat5 docking sites of the EpoR may function to facilitate Stat5 activation at physiological concentrations of Epo, whereas Stat5 may be also activated through direct binding and phosphorylation by Jak2 when cells were stimulated at higher concentrations of Epo.

In previous studies, conflicting results have been reported on whether activation of Stat5 may play a role in the EpoR-mediated growth signaling. Using IL-3–dependent DA3 cells expressing a truncated EpoR mutant in which the only one remaining tyrosine residue, Y-434, was mutated, Quelle et al reported that the impairment in Stat5 activating ability did not affect the ability of the mutant to transduce a mitogenic signal. This suggested that Epo-induced activation of Stat5 may not play a role in growth signaling from the EpoR. In apparent contradiction to this report, Damen et al showed, also using the DA3 cells, that the impairment in Stat5 activating abilities of mutant EpoRs in which all the intracellular tyrosines were mutated or removed by carboxy-terminal deletion correlated with the impairment in mitogenic abilities. It was thus suggested that Stat5 may play a role in the EpoR-mediated growth signaling. In accordance with this finding, Gobert et al observed, in IL-3–dependent FDCP-1 cells, a decrease in mitogenic ability of a mutant EpoR that is quite similar to the mutant EpoR that Quelle et al examined in DA3 cells. It has thus remained to be determined whether Stat5 may play a role in growth signaling from the EpoR or what accounted for these conflicting results. It should also be noted that truncated EpoR mutants expressed in the DA3 cell line, which was used in the former two studies, do not exhibit hypersensitivity to Epo, which has been observed when these mutant EpoRs were expressed in other IL-3–dependent cell lines such as BAF3 or 32D. Based on the Epo hypersensitivity observed in these cell lines, it has been postulated that the carboxy-terminal region of EpoR has an inhibitory effect in growth signaling. This effect should play a physiologically important role in the growth control of erythroid progenitor cells, because truncated EpoRs have been shown to cause hereditary erythrocytosis. Because the failure to observe this inhibitory effect in DA3 cells implies that the EpoR-mediated growth signaling in DA3 cells may be modulated differently from that in the erythroid progenitors, the effect of Stat5 activation on the EpoR-mediated growth signaling observed in DA3 cells may not be physiologically relevant.

In the present study, a clone of 32D cells expressing the S-mutant EpoR, impaired in its ability to activate Stat5 due to the absence of Stat5 docking sites, exhibited an increased sensitivity to Epo for proliferation as compared with the wild-type EpoR-expressing cells (Fig 5B). This finding is consistent with the previous observation that the carboxy-terminal region of EpoR has the inhibitory effect on growth signaling, as described above. Recent studies suggested that this inhibitory effect may be caused, at least in part, by the recruitment of hematopoietic cell phosphatase (HCP), which dephosphorylates and thus inactivates Jak2, to the tyrosine-phosphorylated carboxy-terminal region of the EpoR through interaction with the HCP SH2 region. However, notably, the hypersensitivity of the S-mutant EpoR to Epo was most distinctly observed at Epo concentrations of 10 to 100 mU/mL, at which the impairment in Stat5 activation was also most significant (Fig 5). It is thus possible that Stat5 may play an inhibitory role in the EpoR-mediated growth signaling. In this regard, it should be noted that Stat5 regulates the transcriptional activation of c-jun, which may have a negative regulatory role in cell growth. Intriguingly, growth inhibitory roles have recently been reported for other Stats, such as Stat1 or Stat3. Alternatively, the absence of Stat5 activation might have been compensated by other downstream signaling pathways from the EpoR, even though Stat5 could play a role in growth signaling. Consistent with this possibility, Muil et al very recently reported a partial inhibitory effect of a dominant negative Stat5 mutant on cell growth induced with high concentrations of IL-3, thus suggesting that Stat5 activation is required for a maximal proliferative response. Further studies on the downstream signaling pathways, particularly on the one leading to the induction of c-myc expression that is not regulated by Stat5, are required to elucidate the exact mechanisms by which the growth of hematopoietic cells are regulated by activated cytokine receptors.

The time course of physical association of Stat5 with βIL3 (Fig 2B), which is quite similar to that with the EpoR, strongly suggests that βIL3 may also have similar Stat5 docking sites. We also observed that the tyrosine-phosphorylated 140-kD protein (pp140), corresponding in size to βc, associated with Stat5 in GM-CSF–stimulated human cell lines, UT-7 and TF-1 (H.C., N.N., and O.M., unpublished observation), thus suggesting that Stat5 may also interact with the
human βc subunit. However, this is inconsistent with the previous observation that tyrosine residues in the βc cytoplasmic region of the human IL-3 and GM-CSF receptors were not required for activation of Stat5, although the dose response of GM-CSF–induced Stat5 activation was apparently not examined.25 Further studies on the physical and functional interactions of Stat5 with the IL-3 and GM-CSF receptors are under way in our laboratory to elucidate the mechanisms of Stat5 activation by these receptors.

ACKNOWLEDGMENT

We thank Drs Y. Ikawa, M. Nakamura, Miyajima, and H. Wakao for helpful discussions and K. Okada for excellent technical assistance.

REFERENCES

33. Wakao H, Gouilleux F, Groner B: Mammary gland factor (MGF) is a novel member of the cytokine regulated transcription factor gene family and confers the prolactin response. EMBO J 13:2182, 1994
44. Miura O, Ihle JN: Subunit structure of the erythropoietin receptor analyzed by 125I-Epo cross-linking in cells expressing wild-type or mutant receptors. Blood 81:1739, 1993
61. Yamakawa Y, Nakajima K, Fukuda T, Hibi M, Hirano T: Differentiation and growth arrest signals are generated through the cytoplasmic region of gp130 that is essential for Stat3 activation. EMBO J 15:1557, 1996
Physical and functional interactions between Stat5 and the tyrosine-phosphorylated receptors for erythropoietin and interleukin-3

H Chin, N Nakamura, R Kamiyama, N Miyasaka, JN Ihle and O Miura