

CrkL Is Recruited through Its SH2 Domain to the Erythropoietin Receptor and Plays a Role in Lyn-mediated Receptor Signaling*

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Ayako Arai‡, Eiichiro Kanda‡, Yurika Nosaka‡§, Nobuyuki Miyasaka§, and Osamu Miura‡¶

From the Departments of ‡Hematology and Oncology and §Bioregulatory Medicine and Rheumatology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113, Japan

The erythropoietin (Epo) receptor transduces its signals by activating physically associated tyrosine kinases, mainly Jak2 and Lyn, and thereby inducing tyrosine phosphorylation of various substrates including the Epo receptor (EpoR) itself. We previously demonstrated that, in Epo-stimulated cells, an adapter protein, CrkL, becomes tyrosine-phosphorylated, physically associates with Shc, SHP-2, and Cbl, and plays a role in activation of the Ras/Erk signaling pathway. Here, we demonstrate that Epo induces binding of CrkL to the tyrosine-phosphorylated EpoR and SHP1 in 32D/EpoR-Wt cells overexpressing CrkL. *In vitro* binding studies showed that the CrkL SH2 domain directly mediates the EpoR binding, which was specifically inhibited by a synthetic phosphopeptide corresponding to the amino acid sequences at Tyr⁴⁶⁰ in the cytoplasmic domain of EpoR. The CrkL SH2 domain was also required for tyrosine phosphorylation of CrkL in Epo-stimulated cells. Overexpression of Lyn induced constitutive phosphorylation of CrkL and activation of Erk, whereas that of a Lyn mutant lacking the tyrosine kinase domain attenuated the Epo-induced phosphorylation of CrkL and activation of Erk. Furthermore, Lyn, but not Jak2, phosphorylated CrkL on tyrosine in *in vitro* kinase assays. Together, the present study suggests that, upon Epo stimulation, CrkL is recruited to the EpoR through interaction between the CrkL SH2 domain and phosphorylated Tyr⁴⁶⁰ in the EpoR cytoplasmic domain and undergoes tyrosine phosphorylation by receptor-associated Lyn to activate the downstream signaling pathway leading to the activation of Erk and Elk-1.

Erythropoietin (Epo)¹ is a hematopoietic growth factor that regulates the growth and differentiation of erythroid progenitor cells through activation of its specific receptor expressed on the cell surface. The receptor for Epo (EpoR), a member of the cytokine receptor family, exists as a homodimer and, upon binding of Epo, induces activation of receptor-associated tyrosine kinases. Jak2, a member of the JAK family of tyrosine kinases, physically associates with the EpoR membrane-proximal domain, which contains the Box1 and Box2 motifs conserved in the cytokine receptor family, and has been shown in

in vitro and *in vivo* studies to play a crucial role in EpoR-mediated signaling (1–3). Lyn, a Src family tyrosine kinase, has also been shown to associate with the EpoR and may play a role in Epo-mediated differentiation of erythroid progenitor cells (4, 5). Our previous *in vitro* binding studies have suggested that Lyn binds the EpoR constitutively through its tyrosine kinase domain as well as inducibly through the interaction between the Lyn SH2 domain and phosphorylated Tyr⁴⁶⁴ or Tyr⁴⁷⁹ in the EpoR cytoplasmic domain (5). Upon Epo binding, the EpoR undergoes phosphorylation on tyrosine residues in the cytoplasmic domain and thereby recruits various SH2-containing signaling molecules, such as Stat5, Shc, SHP-2, and the p85 regulatory subunit of phosphatidylinositol 3'-kinase, to activate various signal transduction pathways (6–9).

CrkL is a member of the Crk family of adaptor proteins originally identified as homologues of the product of the *v-crk* oncogene and is most abundantly expressed in hematopoietic cells (10). CrkL has the domain structure SH2-SH3-SH3 and has been shown to bind through its N-terminal SH3 domain with various signaling molecules including Sos1 and C3G, two guanine nucleotide exchange proteins for the Ras family of small GTP-binding proteins. Intriguingly, CrkL is physically associated with the BCR-ABL fusion protein and is phosphorylated on Tyr²⁰⁷ in chronic myeloid leukemia cells, which implicates CrkL in the leukemogenesis (10). We and others have previously demonstrated that CrkL also becomes tyrosine-phosphorylated in hematopoietic cells in response to stimulation with Epo or interleukin (IL)-3 and forms complexes with several tyrosine-phosphorylated signaling molecules, such as Cbl, Shc, and SHP-2 (11). The induction of tyrosine phosphorylation of CrkL and its association with Cbl have also been reported in hematopoietic cells stimulated with stem cell factor (12), thrombopoietin (13), and IL-2 (14). We and others have also demonstrated that CrkL activates the integrin-mediated adhesion of hematopoietic cells to fibronectin (15–17). In addition, we have also shown that CrkL is involved through its interaction with C3G in activation of the Ras/Erk signaling pathway leading to the induction of *c-fos* gene expression in response to Epo or IL-3 (18). These observations indicate that CrkL plays roles in cytokine receptor signaling and in modulation of integrin activity in hematopoietic cells. However, molecular mechanisms for the involvement of CrkL in signaling through cytokine receptors have not been precisely defined.

In the present study, we directly demonstrate that Epo induces physical association of CrkL with the tyrosine-phosphorylated EpoR as well as SHP1 in 32D/EpoR-Wt cells overexpressing CrkL. *In vitro* binding studies further indicate that the CrkL SH2 domain directly binds the tyrosine-phosphorylated EpoR most likely through phosphorylated Tyr⁴⁶⁰ in its cytoplasmic domain. The CrkL SH2 domain also plays a crucial role in induction of tyrosine phosphorylation of CrkL in Epo-

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¶ To whom correspondence should be addressed. Tel.: 81-3-5803-5952; Fax: 81-3-5803-0131; E-mail: miura.med1@med.tmd.ac.jp.

¹ The abbreviations used are: Epo, erythropoietin; EpoR, Epo receptor; IL, interleukin; HA, hemagglutinin; GST, glutathione *S*-transferase; PAGE, polyacrylamide gel electrophoresis.

stimulated cells. Studies using 32D/EpoR-Wt clones overexpressing Lyn and its mutant defective in the tyrosine kinase domain suggest that Lyn phosphorylates CrkL on tyrosine, which is supported by results of *in vitro* kinase assays. We also demonstrate that Lyn synergizes with CrkL to activate the Erk/Elk-1 signaling pathway, which implies that the Lyn-mediated phosphorylation of CrkL may play a role in activation of the downstream signaling pathways.

EXPERIMENTAL PROCEDURES

Cells and Reagents—A clone of IL-3-dependent 32D cells expressing the wild-type murine EpoR (32D/EpoR-Wt) was described previously and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 1 unit/ml Epo (19). A human leukemic cell line expressing the endogenous EpoR, UT-7 (20), was kindly provided by Dr. Norio Komatsu (Jichi Medical School, Tochigi, Japan). Recombinant human Epo was kindly provided by Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan).

A rabbit antiserum raised against a glutathione S-transferase (GST) fusion protein containing amino acids 257–441 of the EpoR cytoplasmic domain was described previously and used for immunoprecipitation (21). Rabbit antibodies against CrkL, Cbl, SHIP1, Lyn, and the N-terminal region of EpoR (M-20) as well as a monoclonal antibody against GST were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody against phosphotyrosine (4G10) and a rabbit antibody against Jak2 were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). A mouse monoclonal antibody against the influenza virus hemagglutinin (HA) epitope, used for immunoblotting, and an immobilized anti-HA high affinity rat monoclonal antibody, used for immunoprecipitation, were purchased from Roche Molecular Biochemicals. Anti-phospho-Erk (Thr²⁰²/Tyr²⁰⁴), specific for the activated forms of Erk, was purchased from New England Biolabs (Beverly, MA).

Expression Plasmids—An expression plasmid for human CrkL, pSG-CrkL (22), was kindly provided by Dr. John Groffen (Childrens Hospital Los Angeles, Los Angeles, CA). Expression plasmids for various CrkL mutants were described previously (16). Expression plasmids for alternatively spliced isoforms of murine Lyn (pXM-LynA and pXM-LynB) have been described previously (5, 23). An expression plasmid for a Lyn mutant (pXM-LynAN) in which a C-terminal half-portion of the kinase domain (amino acids 397–512) is deleted was constructed by introducing a stop codon by the polymerase chain reaction method, as described previously (5). An expression plasmid for HA-tagged ERK2 (S3H-ERK2) was kindly provided by Dr. Michael Karin (University of California at San Diego).

Transfection—Transfection for stable expression was carried out essentially as described previously (19). In brief, 32D/EpoR-Wt cells were transfected with 10 μ g of pSG-CrkL, pXM-LynAN, or a mixture of pXM-LynA and pXM-LynB along with 1 μ g of pSV-Zeo (Invitrogen) by electroporation at 960 microfarads and 300 V, followed by selection in medium containing Zeocine (Invitrogen). For each transfection, six resistant clones were isolated by limiting dilution and examined for the expression level of CrkL or Lyn by immunoblotting of cell lysates. The clone expressing the highest level of CrkL (32DE/CrkL), LynAN (32DE/LynAN), or both LynA and LynB (32DE/LynAB) was selected for the subsequent studies.

For transient expression experiments, 32D/EpoR-Wt cells were electroporated at 960 microfarads and 300 V with 50 μ g of an expression plasmid or an empty vector as a control. After being starved overnight in Epo-free RPMI medium containing 5% fetal calf serum, the transfectants were subjected to immunoprecipitation and immunoblotting studies.

Cell Stimulation, Immunoprecipitation, and Immunoblotting—For stimulation of cells with Epo, cells were washed free of Epo, cultured overnight, and left unstimulated as a negative control or stimulated with Epo at a saturating concentration (100 units/ml) unless otherwise described (24). The cells were then solubilized with a lysis buffer composed of 1% Triton X-100, 20 mmol/liter Tris-HCl, pH 7.5, 150 mmol/liter NaCl, 1 mmol/liter EDTA, 1 mmol/liter sodium orthovanadate, 1 mmol/liter phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin. Cell lysates were subjected to immunoprecipitation as described previously. For immunoblot analysis of total cell lysates, an aliquot of the clarified supernatant was directly mixed with equal volumes of 2 \times Laemmli's sample buffer and heated at 100 °C for 5 min. Samples 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 Pharmacia Biotech). For reprobing of the membranes, the membranes were treated with stripping buffer composed of 100 mmol/liter 2-mercaptoethanol, 2% SDS, and 62.5 mmol/liter Tris-HCl, pH 6.7, at 50 °C for 30 min and subsequently probed with a different antibody.

In Vitro Binding Studies Using GST-CrkL Fusion Proteins—To prepare a GST-CrkL fusion protein containing the SH2 domain of CrkL, GST-CrkL-SH2, a 5' portion of the CrkL cDNA (nucleotides 514–844), coding for amino acid residues 1–110, was amplified by polymerase chain reaction as described previously (5) and subcloned between the *EcoRI* and *BamHI* sites of pGEX-4T-3 (Amersham Pharmacia Biotech) to give pGEX-CrkL-SH2. To construct an expression plasmid for a GST-CrkL protein containing full-length CrkL, GST-CrkL-F, pGEX-CrkL-SH2 was digested with *CpoI* and *SmaI* to subclone the *CpoI*-*Bg/II* fragment, containing nucleotides 536 to the 3' end of the CrkL cDNA, from pSG-CrkL to replace the polymerase chain reaction-amplified region to give pGEX-CrkL-F. The expression plasmids were transformed into *Escherichia coli* DH5a, and the recombinant fusion proteins were purified by affinity chromatography on glutathione-Sepharose beads (Amersham Pharmacia Biotech).

In vitro binding of cellular proteins to GST-CrkL-SH2 was examined essentially as described previously (5). In brief, 32D/EpoR-Wt or 32DE/CrkL cells were lysed in the lysis buffer described above, mixed with GST-CrkL-SH2 on glutathione-Sepharose beads, and incubated at 4 °C for 2 h. After being washed twice, the proteins bound to the beads were eluted by boiling in 1 \times SDS sample buffer and examined by immunoblotting with indicated antibodies. For competition assays with described previously EpoR-derived phosphotyrosine peptides (5, 25, 26), 200 μ mol/liter or indicated concentrations of a synthetic peptide in phosphate-buffered saline was added to cell lysate from Epo-stimulated cells before subjected to binding analysis using GST-CrkL-SH2.

For Far Western blotting, GST-CrkL-SH2 was eluted from glutathione-Sepharose beads with a buffer composed of 50 mmol/liter Tris-HCl, pH 8.0, and 5 mmol/liter reduced glutathione. After immunoprecipitates were separated by SDS-PAGE and electrotransferred to Immobilon P membranes, the membrane was first incubated overnight at 4 °C with GST-CrkL-SH2 followed by detection with anti-GST immunoblotting.

In Vitro Kinase Assays—The *in vitro* kinase assays of anti-Lyn and anti-Jak2 immunoprecipitates were performed as described previously (2). In brief, anti-Jak2 and anti-Lyn immunoprecipitates from Epo-stimulated 32D/EpoR-Wt cells were subjected to the *in vitro* kinase reaction in kinase buffer (10 mmol/liter HEPES, pH 7.5, 50 mmol/liter NaCl, 5 mmol/liter MgCl₂, 5 mmol/liter MnCl₂, 100 μ mol/liter sodium orthovanadate) containing GST-CrkL-F, as a substrate, in the presence or absence of 1 mmol/liter cold ATP for 30 min at room temperature. After the kinase reaction, the reaction products were mixed with equal volumes of 2 \times Laemmli's sample buffer, heated at 100 °C for 5 min, and subjected to anti-phosphotyrosine immunoblotting followed by reprobing with anti-CrkL.

Luciferase Reporter Assays—Luciferase reporter assays of transiently transfected cells were performed by using the PathDetect *in vivo* reporting system, essentially as described previously (5). In brief, 32D/EpoR-Wt cells were electroporated with the indicated plasmids and starved overnight in medium without Epo. The cells were then incubated for 5 h in medium with or without Epo, as indicated, and harvested for the luciferase assay using a dual luciferase reporter assay system (Promega) according to the manufacturer's instructions. The total amounts of plasmids transfected were adjusted to be constant in each experiment by adding an empty vector plasmid. The luciferase activity was normalized by the *Renilla* luciferase activity and expressed in arbitrary units.

RESULTS

CrkL Physically Associates with the Tyrosine-phosphorylated Form of EpoR in Epo-stimulated Cells—We previously demonstrated that, in Epo-stimulated 32D/EpoR-Wt cells, CrkL is very rapidly recruited to the EpoR signaling complex by binding with various tyrosine-phosphorylated signaling molecules, including Cbl, Shc, and SHP-2 (5). However, the domain of CrkL that was involved in physical association with the EpoR complex was not examined in our previous study. Furthermore, we could not directly demonstrate the binding of CrkL with the EpoR by anti-EpoR immunoblotting of anti-CrkL immunopre-

precipitates, most likely because of the low sensitivity of this method. Thus, in the present study, we first examined the mechanisms involved in binding of CrkL to the EpoR complex in 32DE/CrkL cells, a clone of 32D/EpoR-Wt cells that stably overexpresses CrkL. As shown in Fig. 1, anti-phosphotyrosine blotting of anti-CrkL immunoprecipitates demonstrated that

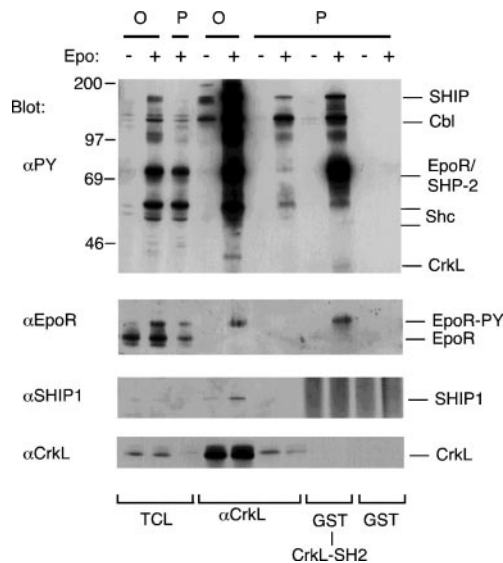


FIG. 1. Physical interaction of CrkL with the erythropoietin receptor in 32D cells and *in vitro*. Parental 32D/EpoR-Wt cells (P) or 32DE/CrkL cells (O), a clone of 32D/EpoR-Wt overexpressing CrkL, were starved overnight from Epo and left unstimulated (-) or stimulated with 100 units/ml of Epo for 5 min (+) at 37 °C before solubilization. The cell lysates were immunoprecipitated with anti-CrkL or affinity purified with GST or GST-CrkL-SH2, as indicated. Aliquots of cell lysates (TCL) or eluted proteins were resolved by SDS-PAGE and subjected to immunoblotting with an anti-phosphotyrosine monoclonal antibody, 4G10 (α PY). The membrane was stripped and reprobed sequentially with antibodies against the EpoR, SHIP1, and CrkL, as indicated. The positions of various signaling molecules including the tyrosine-phosphorylated EpoR (EpoR-PY) are indicated. The molecular mass markers are indicated and given in kilodaltons.

various tyrosine-phosphorylated proteins, including those corresponding in sizes to Cbl (110 kDa), SHP-2 (72 kDa), and Shc (54 kDa), were associated with CrkL in Epo-stimulated 32DE/CrkL cells. Anti-EpoR blotting of the anti-CrkL immunoprecipitates further revealed that CrkL was associated specifically with the tyrosine-phosphorylated 72-kDa form of EpoR, but not with the more abundant, unphosphorylated 67-kDa form in Epo-stimulated 32DE/CrkL cells. In our previous study, a 145-kDa phosphotyrosyl protein was also observed to associate with CrkL in Epo-stimulated 32D/EpoR-Wt cells (5), although its identity has remained to be determined. By anti-SHIP1 blotting of the CrkL immunoprecipitates, the 145-kDa protein was identified as SHIP1 (Fig. 1, *third panel*). Finally, anti-CrkL blotting of the CrkL immunoprecipitates as well as total cell lysates showed that CrkL was overexpressed at a very high level in 32DE/CrkL cells.

The CrkL SH2 Domain and Tyr⁴⁶⁰ in the EpoR Are Involved in the Direct Binding of CrkL with the EpoR *In Vitro*—To examine the mechanisms involved in CrkL binding to the tyrosine-phosphorylated EpoR, we next examined the *in vitro* binding of cellular proteins to a fusion protein containing the CrkL SH2 domain (GST-CrkL-SH2). Anti-phosphotyrosine blotting of the proteins bound to GST-CrkL-SH2 demonstrated that most of the tyrosine-phosphorylated signaling molecules that associate with CrkL in Epo-stimulated 32D/EpoR cells bind GST-CrkL-SH2, but not GST, *in vitro* (Fig. 1). Anti-EpoR blotting further showed that the tyrosine-phosphorylated form, but not the unphosphorylated form, of EpoR was bound to GST-CrkL-SH2 *in vitro* (Fig. 1, *second panel*). We could not determine whether the CrkL SH2 domain is also responsible for the SHIP1 binding, because immunoblotting of the GST-CrkL-SH2-bound proteins with anti-SHIP1, raised against a GST-SHIP1 fusion protein, gave significant background smears (Fig. 1, *third panel*).

To further explore the mechanism by which the CrkL SH2 domain binds to the EpoR, 32D/EpoR-Wt cell lysates were denatured by heating at 100 °C for 5 min in the presence of 1% SDS and subsequently subjected to the binding study with the

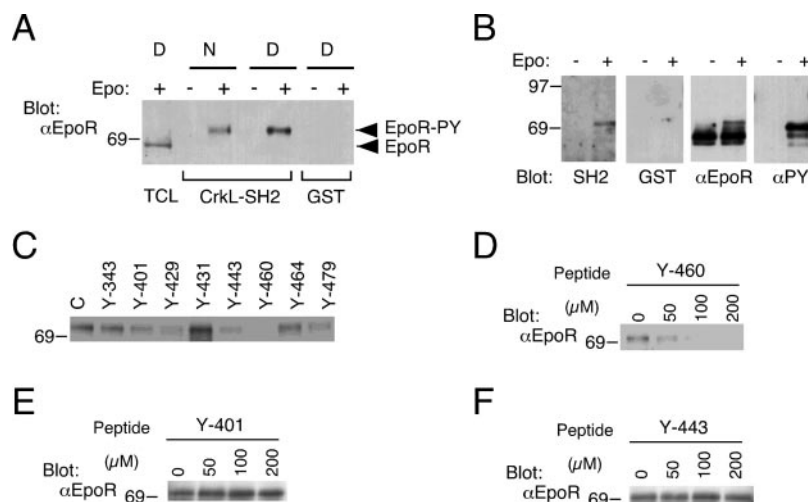


FIG. 2. *In vitro* binding studies of the CrkL SH2 domain and the tyrosine-phosphorylated EpoR. A, denatured (D) or nondenatured (N) cell lysates were prepared from Epo-stimulated (+) or unstimulated (-) 32D/EpoR-Wt cells, as described under "Experimental Procedures." The cell lysates were subjected to affinity purification with GST-CrkL-SH2 (CrkL-SH2) or GST (GST). Eluted proteins as well as an aliquot of total cell lysate (TCL) were then analyzed by immunoblotting with anti-EpoR. B, the EpoR was immunoprecipitated with anti-EpoR from Epo-stimulated (+) or unstimulated (-) 32D/EpoR-Wt cell lysate, resolved by SDS-PAGE, and electrotransferred onto a polyvinylidene difluoride membrane. The membrane was then sequentially probed with GST, anti-EpoR (α EpoR), and anti-phosphotyrosine (α PY), as indicated. C, cell lysate from Epo-stimulated 32D/EpoR-Wt was mixed with phosphate-buffered saline (lane C) or phosphate-buffered saline containing synthetic phosphopeptides (200 μ mol/liter) corresponding to potential tyrosine phosphorylation sites of the EpoR, as indicated, and incubated with GST-CrkL-SH2. The proteins bound to GST-CrkL-SH2 were then analyzed by immunoblotting with anti-EpoR. D-F, cell lysate from Epo-stimulated 32D/EpoR-Wt was mixed with indicated concentrations of the Tyr⁴⁶⁰ (D), Tyr⁴⁰¹ (E), or Tyr⁴⁴³ (F) phosphopeptide, as indicated, and analyzed as described in C.

GST-CrkL-SH2 protein. As shown in Fig. 2A, the tyrosine-phosphorylated EpoR was bound to GST-CrkL-SH2 under the denatured condition as well as under the nondenatured condition, which suggests that the CrkL-SH2 domain directly binds the tyrosine-phosphorylated EpoR *in vitro*. To confirm this, the EpoR was immunoprecipitated from 32D/EpoR-Wt cells and examined by Far Western blot analysis using the GST-CrkL-SH2 protein as a probe. As shown in Fig. 2B, the CrkL-SH2 protein specifically bound the tyrosine-phosphorylated form of EpoR immunoprecipitated from Epo-stimulated cells, thus confirming that the CrkL-SH2 domain directly binds the EpoR *in vitro*. The EpoR tyrosine residues involved in CrkL binding were then examined by using the previously described synthetic phosphotyrosyl peptides corresponding to the possible EpoR phosphorylation sites (5, 25, 26). When added at 200 $\mu\text{mol/liter}$ to lysate from Epo-stimulated 32D/EpoR-Wt cells, the phosphopeptide containing Tyr⁴⁶⁰ completely inhibited the binding of CrkL-SH2 to the tyrosine-phosphorylated EpoR, whereas the other peptides at the same concentration did not show any consistent inhibitory effects in repeated experiments (Fig. 2C and data not shown). The concentration dependence of inhibition was then examined by adding the Tyr⁴⁶⁰ peptide at various concentrations to the *in vitro* binding reaction. The inhibitory effect of Tyr⁴⁶⁰ peptide was shown to be dose-dependent, with the half-inhibitory concentration estimated to be less than 50 $\mu\text{mol/liter}$ (Fig. 2D), which is comparable with those of the Tyr³⁴³ and Tyr⁴⁶⁴ peptides on the Stat5-SH2 and Lyn-SH2 binding, respectively, to the tyrosine-phosphorylated EpoR previously examined using the same reagents (5, 26). In contrast, the Tyr⁴⁰¹ and Tyr⁴⁴³ peptides, which also exhibited moderate inhibitory effects in that particular experiment shown in Fig. 2C, did not show any inhibitory effects in similar titration experiments (Fig. 2, E and F). Taken together, these results suggest that the binding of CrkL with the EpoR in Epo-stimulated cells is mediated, at least partly, through the direct interaction between the CrkL SH2 domain and phosphorylated Tyr⁴⁶⁰ in the EpoR cytoplasmic domain.

The CrkL SH2 Domain Is Required for Epo-induced Tyrosine Phosphorylation of CrkL—We previously demonstrated that CrkL transiently binds various tyrosine-phosphorylated signaling molecules and becomes tyrosine-phosphorylated in 32D/EpoR-Wt cells stimulated with Epo or IL-3 (11). To explore the mechanisms involved in cytokine-induced tyrosine phosphorylation of CrkL, we next determined the region of CrkL that is required for its tyrosine phosphorylation by using previously characterized CrkL mutants shown in Fig. 3A. 32D/EpoR-Wt cells transiently overexpressing wild-type or mutant CrkL were stimulated with Epo for 5 or 30 min, and anti-CrkL immunoprecipitates were examined by anti-phosphotyrosine blotting. In vector-transfected control cells, Epo induced tyrosine phosphorylation of endogenous CrkL, which peaked at 5 min after stimulation and significantly declined at 30 min (Fig. 3B, upper panel). This is in accordance with our previous observation. In cells transiently overexpressing wild-type CrkL, Epo-induced tyrosine phosphorylation of CrkL was significantly augmented and prolonged. In cells expressing the dSH3N mutant, which has a deletion in the N-terminal SH3 domain, the mutant was also inducibly tyrosine-phosphorylated by Epo stimulation. On the other hand, the dSH3C mutant, which lacks the C-terminal SH3 domain as well as an adjacent region encompassing Tyr²⁰⁷, was not tyrosine-phosphorylated by Epo stimulation. The dSH2 mutant, which lacks a significant portion of the SH2 domain, also failed to undergo tyrosine phosphorylation by Epo stimulation, although the phosphorylation site, Tyr²⁰⁷, is intact in this mutant. The expression of wild-type or mutant CrkL was confirmed by rep-

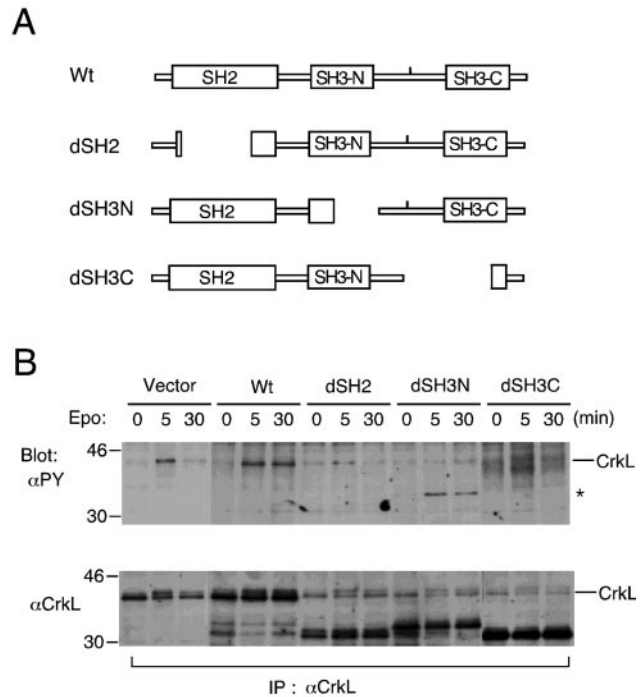


FIG. 3. Epo-induced tyrosine phosphorylation of CrkL mutants in 32D/EpoR-Wt cells. A, schematic representation of CrkL mutants. The thick vertical short line represents the tyrosine phosphorylation site, Tyr²⁰⁷. The SH2 domain (SH2), and the N-terminal or C-terminal SH3 domain (SH3-N and SH3-C, respectively) are indicated. B, an empty plasmid (Vector) or expression plasmids for wild-type and various mutants of CrkL, as indicated, were transfected into 32D/EpoR-Wt cells. After overnight starvation, the cells were stimulated with 100 units/ml Epo for indicated times before solubilization. The cell lysates were immunoprecipitated with anti-CrkL and analyzed by anti-phosphotyrosine (αPY) immunoblotting, as indicated. The membrane was then reprobbed with anti-CrkL, as indicated. The positions of endogenous CrkL are indicated. The position of tyrosine-phosphorylated dSH3N mutant is indicated with an asterisk. Wt, wild type.

robing with anti-CrkL (Fig. 3B, lower panel). These results indicate that the CrkL SH2 domain, involved in binding with the tyrosine-phosphorylated EpoR and other signaling molecules, is also required for the Epo-induced tyrosine phosphorylation of CrkL in hematopoietic cells.

CrkL Is Constitutively Tyrosine-phosphorylated in Lyn-overexpressing Cells and Is a Substrate for Lyn in *In Vitro* Phosphorylation Reaction—We have previously demonstrated that Lyn, as well as Jak2, physically associates with the EpoR in 32D/EpoR-Wt cells to mediate the EpoR signaling (2, 5). To examine possible involvement of Lyn in CrkL-mediated signaling from the EpoR, we established a clone of 32D/EpoR-Wt cells stably overexpressing the wild-type A and B forms of Lyn, 32D/EpoR-LynAB or that expressing a Lyn mutant lacking the significant portion of the catalytic domain, 32D/EpoR-LynAN, as described under "Experimental Procedures." In our previous report, the Lyn SH2 domain was demonstrated to bind directly with the tyrosine-phosphorylated EpoR as well as with Jak2 *in vitro* (5). In addition, the Lyn catalytic domain was implicated in constitutive binding of Lyn with the EpoR. Nevertheless, it was not possible, most likely because of the technical difficulties, to directly demonstrate the physical interaction of Lyn with the EpoR or with Jak2 in 32D/EpoR-Wt cells. In 32D/EpoR-LynAB cells, however, anti-Jak2 blotting of anti-Lyn immunoprecipitates demonstrated that Jak2 was constitutively associated with Lyn (Fig. 4A). Anti-phosphotyrosine blotting of anti-Jak2 immunoprecipitates further showed that Jak2 was constitutively tyrosine-phosphorylated in 32D/EpoR-LynAB cells (Fig. 4B). Jak2 was also found to associate with Lyn in

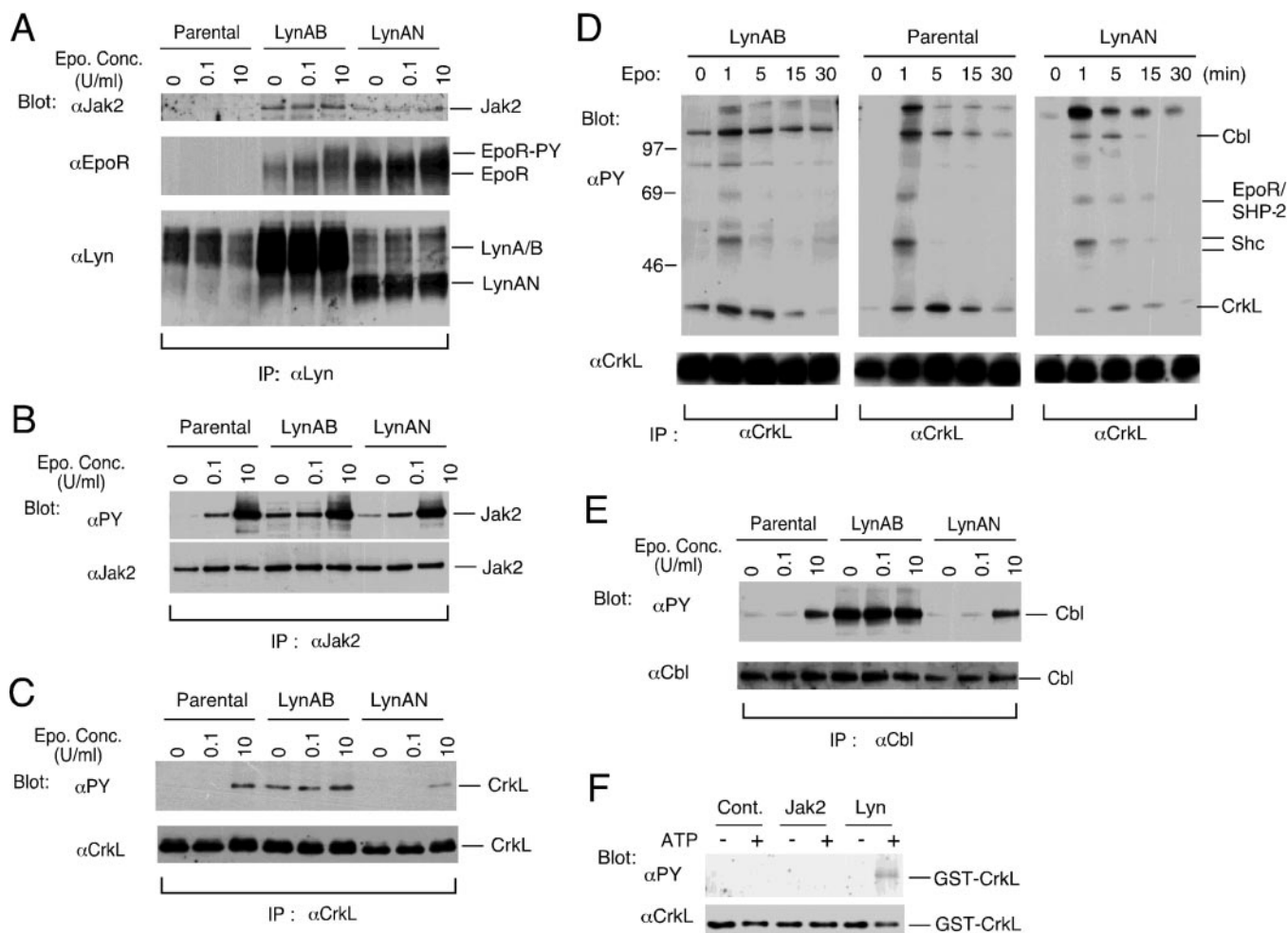


FIG. 4. CrkL is constitutively tyrosine-phosphorylated in Lyn-overexpressing cells and is an *in vitro* substrate for Lyn. A–C and E, parental 32D/EpoR-Wt cells (*Parental*) or 32D/EpoR-Wt cells stably overexpressing the wild-type A and B forms of Lyn (*LynAB*) or a mutant of Lyn-A lacking the tyrosine kinase domain (*LynAN*) were stimulated with indicated concentrations of Epo for 5 min before solubilization. The cell lysates were immunoprecipitated with anti-Lyn (A), anti-Jak2 (B), anti-CrkL (C), or anti-Cbl (E), as indicated, and analyzed by immunoblotting with anti-Jak2 (A) or anti-phosphotyrosine (B, C, and E), as indicated. The membranes were subsequently reprobbed with the indicated antibodies. D, parental 32D/EpoR-Wt cells (*Parental*) or 32D/EpoR-Wt cells stably overexpressing Lyn (*LynAB*) or its mutant (*LynAN*) were stimulated with 100 units/ml Epo for the indicated times before solubilization. The cell lysates were immunoprecipitated (IP) with anti-CrkL and analyzed by immunoblotting with anti-phosphotyrosine (α PY), followed by reprobbed with anti-CrkL, as indicated. F, cell lysate from Epo-stimulated 32D/EpoR-Wt cells was immunoprecipitated with anti-Jak2 (*Jak2*), anti-Lyn (*Lyn*), or normal rabbit serum (*Cont.*), as indicated. Immunoprecipitates were subjected to the *in vitro* kinase assay using GST-CrkL as a substrate in the presence or absence of 1 mM ATP as indicated. The reaction products were analyzed by anti-phosphotyrosine blotting (α PY) followed by reprobbed with anti-CrkL, as indicated. *Conc.*, concentration.

32D/EpoR-LynAN cells (Fig. 4A), in which Jak2 was faintly tyrosine-phosphorylated without Epo stimulation (Fig. 4B). These results are in agreement with our previous observations and raise the possibility that the Lyn SH2 domain may stabilize the tyrosine phosphorylation of Jak2. The physical association of Lyn with the EpoR was also directly demonstrated in 32D/EpoR-LynAB and 32D/EpoR-LynAN cells by anti-EpoR blotting of the anti-Lyn immunoprecipitates (Fig. 4A, middle panel). As expected from the previous results of *in vitro* binding studies (5), Lyn was constitutively associated, most likely through its kinase domain, with the unphosphorylated, 66-kDa form of the EpoR in these cells. After Epo stimulation, Lyn also bound with the tyrosine-phosphorylated, 72-kDa form of EpoR, which also agrees with the previous *in vitro* binding study that demonstrated the binding of Lyn SH2 domain to the tyrosine-phosphorylated EpoR (5). These results extend our previous observations and further support the idea that Lyn is involved in EpoR signaling through its physical interaction with the EpoR complex.

The Epo-induced tyrosine phosphorylation of CrkL was then examined in 32D/EpoR-LynAB and 32D/EpoR-LynAN cells. As

shown in Fig. 4C, CrkL was constitutively tyrosine-phosphorylated in 32D/EpoR-LynAB cells. On the other hand, the Epo-induced tyrosine phosphorylation of CrkL was significantly reduced in 32D/EpoR-LynAN cells. The time courses of Epo-induced tyrosine phosphorylation of CrkL and its association with tyrosine-phosphorylated proteins were also examined in these cells, as shown in Fig. 4D. In parental 32D/EpoR-Wt cells, CrkL very rapidly and transiently associated with the tyrosine-phosphorylated proteins corresponding in size to Cbl (110 kDa), the EpoR (72 kDa), Shp-2 (72 kDa), and Shc (54 kDa) and became tyrosine-phosphorylated after Epo stimulation, in agreement with our previously study (11). Similarly, CrkL transiently associated with these tyrosine-phosphorylated signaling molecules at 1 min after Epo stimulation in 32DE/LynAB cells. However, in these cells, the tyrosine phosphorylation of CrkL and its association with a tyrosine-phosphorylated, 110-kDa protein, corresponding in size to Cbl, were observed before Epo stimulation. Anti-phosphotyrosine blotting of anti-Cbl immunoprecipitates further showed that Cbl was constitutively and remarkably tyrosine-phosphorylated in 32D/EpoR-LynAB cells (Fig. 4E). As shown in Fig. 4D, the

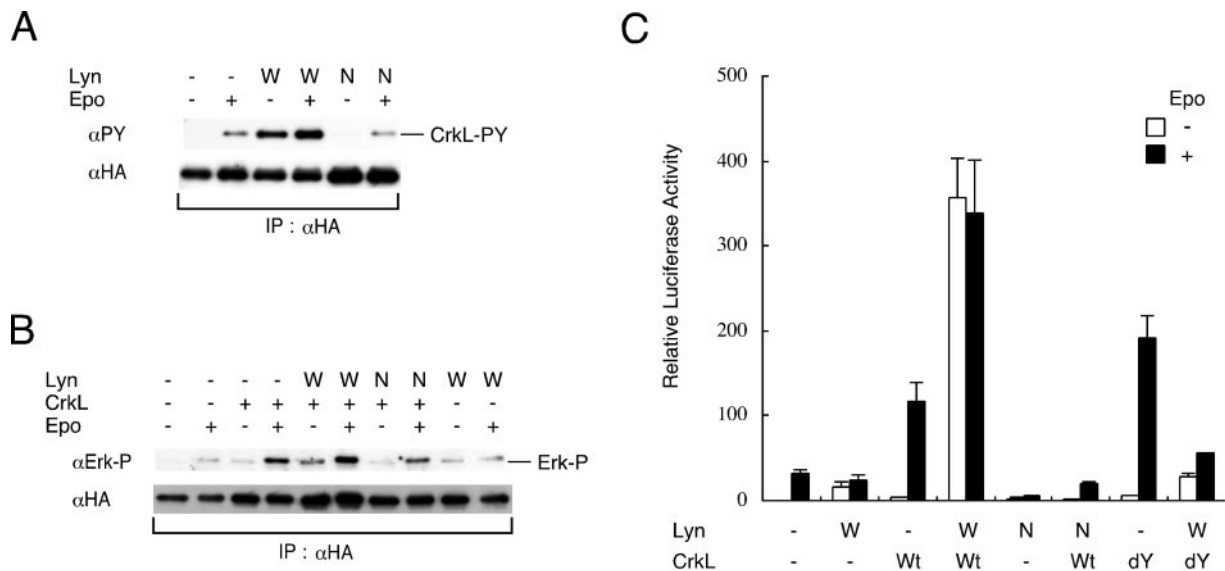


FIG. 5. Effects of Lyn on CrkL-mediated activation of Erk and Elk-1. *A*, 32D/EpoR-Wt cells were transfected with 25 μ g of an empty plasmid (–), a mixture of pXM-LynA and pXM-LynB (W), or pXM-LynAN (N), as indicated, along with 25 μ g of an expression plasmid for HA-tagged CrkL, pSG-CrkL-H. After overnight starvation, the cells were stimulated with 10 units/ml Epo for 5 min or left unstimulated, as indicated, before solubilization. The cell lysates were immunoprecipitated with anti-HA and analyzed by anti-phosphotyrosine (α PY) immunoblotting, as indicated. The membrane was then reprobed with anti-HA, as indicated. A position of tyrosine-phosphorylated CrkL (CrkL-PY) is indicated. *B*, 32D/EpoR-Wt cells were transfected with 15 μ g of a mixture of pXM-LynA and pXM-LynB (W), pXM-LynAN (N), or pSG-CrkL, as indicated, along with 25 μ g of HA-Erk. Total amounts of plasmids transfected were adjusted to be constant by adding an empty vector plasmid. After overnight starvation, the cells were stimulated with 10 units/ml Epo for 5 min or left unstimulated, as indicated, before solubilization. The cell lysates were immunoprecipitated with anti-HA and analyzed by anti-phospho-Erk (α Erk-P) immunoblotting, as indicated. The membrane was then reprobed with anti-HA, as indicated. A position of phosphorylated Erk (Erk-P) is indicated. *C*, 32D/EpoR-Wt cells were transfected with 15 μ g of pSG-CrkL-H (Wt), pSG-CrkL-dY (dY), a mixture of pXM-LynA and pXM-LynB (W), or pXM-LynAN (N), as indicated, along with 2 μ g of pFA-Elk-1, 20 μ g of pFR-Luc, and 0.01 μ g of pRL-SV40. Total amounts of plasmids transfected were adjusted to be constant by adding an empty vector plasmid. After overnight starvation, the cells were incubated for 5 h in medium supplemented with 10 units/ml of Epo (+) or left untreated (–), as indicated, and harvested for the dual luciferase assay. The data represent the averages \pm S.D. of two independent experiments. *IP*, immunoprecipitation; *Wt*, wild type.

Epo-induced tyrosine phosphorylation of CrkL in 32D/EpoR-LynAN cells was significantly reduced as compared with that in 32D/EpoR-Wt cells. However, the physical association of CrkL with tyrosine-phosphorylated proteins corresponding in size to the EpoR, Shp-2, and Shc was prolonged in 32D/EpoR-LynAN cells. Intriguingly, a tyrosine-phosphorylated protein that coimmunoprecipitates with CrkL and migrates more slowly than Cbl was observed more conspicuously in 32DE/LynAN cells than in parental 32D/EpoR-Wt or 32DE/LynAB cells. Although corresponding in size to SHIP1, this protein failed to be recognized by anti-SHIP1 immunoblotting and thus remains to be identified. Because it was strongly suggested that Lyn is involved in Epo-induced tyrosine phosphorylation of CrkL as well as Cbl, we next examined whether Lyn has the ability to phosphorylate CrkL on tyrosine *in vitro*. As shown in Fig. 4F, Lyn or Jak2 immunoprecipitated from Epo-stimulated 32D/EpoR-Wt cells was incubated with a GST fusion protein containing full-length CrkL in the presence or absence of ATP. Anti-phosphotyrosine blotting of reaction products showed that GST-CrkL was tyrosine-phosphorylated only in the presence of both Lyn and ATP, thus demonstrating that Lyn has the ability to phosphorylate CrkL *in vitro*.

Effects of Lyn on CrkL-mediated Activation of the Erk/Elk-1 Pathway—We next examined the effects of CrkL phosphorylation by Lyn on CrkL-mediated signaling from the EpoR. First, CrkL tagged with the HA epitope was transiently coexpressed with the wild-type or dominant negative form of Lyn in 32D/EpoR-Wt cells, and the transfected cells were stimulated with Epo. As shown in Fig. 5A, transfected CrkL was constitutively and remarkably phosphorylated on tyrosine in cells coexpressing the wild-type forms of Lyn, whereas the Epo-induced phosphorylation of transfected CrkL was moderately inhibited by coexpression of the dominant negative form of Lyn. These re-

sults are in agreement with those in the 32D/EpoR-Wt clones stably overexpressing the wild-type or dominant negative form of Lyn shown in Fig. 4.

We then examined the effect of transient overexpression of Lyn on CrkL-mediated signaling leading to the activation of Erk by anti-phospho-Erk blotting of HA-tagged Erk that was transiently expressed in 32D/EpoR-Wt cells and immunoprecipitated by anti-HA. As shown in Fig. 5B, the CrkL overexpression modestly increased the background Erk activity and significantly enhanced the Epo-induced Erk activation, which is in agreement with our previous report (18). On the other hand, the Lyn overexpression significantly increased the background Erk activity, but it did not enhance the Epo-induced Erk activation. Moreover, the coexpression of Lyn with CrkL synergistically increased the background Erk activity, which was further increased by Epo stimulation. In contrast, the cotransfection of the dominant negative form of Lyn moderately inhibited the CrkL-induced enhancement of Erk activation by Epo (Fig. 5B). These data indicate that the effects of Lyn on Erk activation correlate with those on CrkL phosphorylation and thus strongly suggest that Lyn augments the CrkL-mediated EpoR signaling pathway leading to Erk activation, most likely by phosphorylating CrkL.

To confirm that Lyn augments the CrkL-mediated signaling pathway leading to Erk activation, we next examined the effects of Lyn and CrkL on the Epo-induced activation of Elk-1, a downstream effector of Erk, as described under "Experimental Procedures." As shown in Fig. 5C, overexpression of Lyn significantly increased the background Elk-1 activity, which, however, did not significantly increase after Epo stimulation. On the other hand, overexpression of CrkL significantly enhanced the Epo-induced activation of Elk-1 in accordance with our previous report (18). Importantly, overexpression of both CrkL

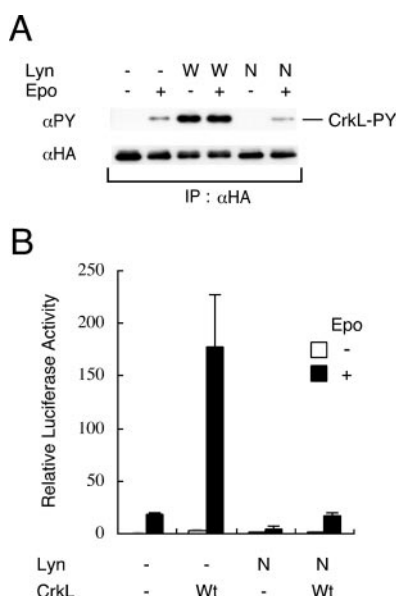


FIG. 6. Involvement of CrkL and Lyn in EpoR-mediated signaling in UT-7 cells. *A*, UT-7 cells were transfected with 25 μ g of an empty plasmid (–), a mixture of pXM-LynA and pXM-LynB (W), or pXM-LynAN (N), as indicated, along with 25 μ g of an expression plasmid for HA-tagged CrkL, pSG-CrkL-H. After overnight starvation, the cells were stimulated with 10 units/ml Epo for 5 min or left unstimulated, as indicated, before solubilization. The cell lysates were immunoprecipitated with anti-HA and analyzed by anti-phosphotyrosine (α PY) immunoblotting, followed by reprobing with anti-HA, as indicated. *B*, UT-7 cells were transfected with 15 μ g of pSG-CrkL (Wt) or pXM-LynAN (N), as indicated, along with 2 μ g of pFA-Elk-1, 20 μ g of pFR-Luc, and 0.01 μ g of pRL-SV40. Total amounts of plasmids transfected were adjusted to be constant by adding an empty vector plasmid. After overnight starvation, the cells were incubated for 5 h in medium supplemented with 10 units/ml Epo (+) or left untreated (–), as indicated, and harvested for the dual luciferase assay.

and Lyn drastically increased the Elk-1 activity, which was independent of Epo stimulation. In contrast, the dominant negative form of Lyn significantly inhibited the Epo-induced activation of Elk-1 when overexpressed alone or in combination with CrkL. These data on Elk-1 activation agree with those on Erk activation, shown in Fig. 5B, and, together, strongly support the idea that the CrkL phosphorylation by Lyn plays a significant role in activation of the downstream signaling pathway leading to the activation of Erk and Elk-1.

In accordance with our previous report (18), the overexpression of a CrkL mutant lacking the tyrosine phosphorylation site, CrkL-dY, also significantly enhanced the Epo-induced Elk-1 activation (Fig. 5C). However, coexpression of Lyn with this mutant failed to induce the synergistic Elk-1 activation observed when Lyn was coexpressed with wild-type CrkL, which is in agreement with the idea that Lyn activates the CrkL-mediated signaling pathway leading to the activation of Erk and Elk-1 by phosphorylating CrkL.

Involvement of CrkL and Lyn in Signaling from the EpoR in Human Pluripotent Hematopoietic UT-7 Cells—Because 32D/EpoR-Wt cells, which were utilized in the present studies to examine the involvement of CrkL and Lyn in EpoR signaling, represent a myeloid hematopoietic cell line heterologously expressing the EpoR, we next examined a human pluripotent hematopoietic cell line, UT-7, which expresses the endogenous EpoR and shows erythroid phenotypes when cultured with Epo (20, 27). We have previously shown that Epo induces tyrosine phosphorylation of CrkL in UT-7 cells (28). When transiently coexpressed, Lyn induced a strong and constitutive tyrosine phosphorylation of CrkL, whereas the dominant negative Lyn moderately inhibited the Epo-induced phosphorylation of CrkL

in UT-7 cells (Fig. 6A). These findings are quite similar to those observed in 32D/EpoR-Wt cells (Fig. 5A). In UT-7 cells, transient overexpression of Lyn induced a drastic increase in Elk-1 activity, which could not be enhanced by stimulation with Epo or by coexpression of CrkL (data not shown). Overexpression of CrkL, however, significantly enhanced the Epo-induced activation of Elk-1 in UT-7 cells, thus suggesting that CrkL is involved in the EpoR-mediated Elk-1 activation pathway in UT-7 cells (Fig. 6B). Furthermore, the dominant negative Lyn mutant significantly inhibited the Epo-induced activation of Elk-1 in UT-7 cells as well as the CrkL-enhanced activation of Elk-1 in cells overexpressing CrkL. These results are in agreement with those obtained with 32D/EpoR-Wt cells (Fig. 5C) and strongly suggest that Lyn is involved in tyrosine phosphorylation of CrkL as well as CrkL-mediated activation of Elk-1 in the signaling mechanisms downstream from the EpoR in UT-7 cells.

DISCUSSION

We previously reported that CrkL physically associates with tyrosine-phosphorylated signaling molecules, including Cbl, SHP-2, and Shc, and becomes tyrosine-phosphorylated in Epo- or IL-3-stimulated hematopoietic cells (11). We also reported that CrkL forms a complex with the EpoR, because the tyrosine-phosphorylated EpoR was present in anti-CrkL immunoprecipitate obtained from Epo-stimulated cell lysate (11). However, it was not elucidated how CrkL interacts with the EpoR as well as with other signaling molecules and undergoes tyrosine phosphorylation in Epo-stimulated cells.

The present study has thus extended our previous study by demonstrating a pivotal role the CrkL SH2 domain plays in forming complexes with various tyrosine-phosphorylated signaling molecules in Epo-stimulated cells (Fig. 1). Moreover, we directly demonstrated that CrkL specifically binds the tyrosine-phosphorylated form of EpoR in Epo-stimulated 32D cells overexpressing CrkL. It was further revealed that the CrkL-SH2 domain directly binds the EpoR *in vitro*, most likely through phosphorylated Tyr⁴⁶⁰ in the EpoR, which is contained in the consensus binding sequence YXXP for the CrkL SH2 domain (29), as demonstrated by the competition assays using synthetic phosphopeptides (Fig. 2). We previously speculated that CrkL interacts with the EpoR mainly through Shc and SHP-2, because anti-CrkL failed to coimmunoprecipitate the EpoR from cell lysate that had been preclarified by using both anti-Shc and anti-SHP-2 (11). However, the docking site in the EpoR cytoplasmic domain for SHP-2, Tyr⁴⁰¹ (30), is different from that for CrkL (Tyr⁴⁶⁰), and that for Shc has not been determined. Therefore, CrkL, SHP-2, and possibly Shc may simultaneously bind the tyrosine-phosphorylated EpoR through the different docking sites, which should explain the inability in our previous study to demonstrate the EpoR in anti-CrkL immunoprecipitate after depleting the EpoR by using both anti-SHP-2 and anti-Shc (11). It is therefore strongly suggested that CrkL is recruited to the activated EpoR complex through interaction between the CrkL SH2 domain and the phosphorylated docking site in the EpoR as well as those in the other signaling molecules.

The demonstration that CrkL is recruited to the activated EpoR complex strongly supports our hypothesis that the EpoR activates the Ras/Erk signaling pathway by bringing the CrkL-C3G complex as well as the Grb2/Sos1 complex to the vicinity of Ras at the plasma membrane (18). In addition, the crucial role demonstrated for the CrkL SH2 domain in CrkL recruitment agrees with our previous observation that the CrkL-mediated activation of Ras/Erk pathway by the EpoR is dependent on the CrkL SH2 domain as well as the N-terminal SH3 domain that binds C3G (18).

The present study has also demonstrated that CrkL inducibly binds tyrosine-phosphorylated SHIP1 in Epo-stimulated cells. SHIP1 is a hematopoietic specific, inositol 5'-phosphatase that becomes tyrosine-phosphorylated in response to hematopoietic cytokines (31, 32). SHIP1 has also been reported to form a complex with CrkL after Fc- α receptor ligation in U937 cells (33) and in BCR/ABL transformed cells overexpressing SHIP1 (34). Recently, Mason *et al.* (35) reported that SHIP1 binds to the EpoR in an SH2-dependent fashion through multiple phosphotyrosine residues, including Tyr⁴⁰¹, Tyr⁴²⁹, and Tyr⁴³¹, and that Epo stimulates the formation of a ternary complex consisting of SHIP1, Shc, and Grb2. Although the enzymatic activity does not change significantly following cytokine stimulation, it has been speculated that SHIP1 exerts its downstream effects via binding to different proteins, including Shc and SHP-2, and translocating to the sites of synthesis of its substrates (31, 32). SHIP1 has been postulated to play a negative regulatory role in hematopoietic progenitor cell proliferation/survival through down-modulation of cytokine receptor-mediated Akt activation. SHIP1 has also been implicated in regulation of Ca²⁺ influx in B cells and mast cells (36, 37). It is notable in this regard that the putative CrkL docking site identified in the EpoR cytoplasmic domain, Tyr⁴⁶⁰, has recently been shown to play a crucial role in Epo-dependent increase in Ca²⁺ influx (38). It is also notable that SHIP1 has been implicated in regulation of migration of hematopoietic cells (34), thus raising a possibility that SHIP1 may play a role in the CrkL-mediated signaling that regulates cell adhesion.

Lyn was strongly implicated in Epo-induced tyrosine phosphorylation of CrkL in 32D/EpoR-Wt cells, because both stable and transient expression experiments showed that overexpression of Lyn induces the constitutive tyrosine phosphorylation of CrkL, whereas that of the dominant negative Lyn mutant inhibited the Epo-induced tyrosine phosphorylation of CrkL (Figs. 4 and 5). Lyn was also demonstrated to be involved in Epo-induced tyrosine phosphorylation of CrkL in UT-7 cells expressing the endogenous EpoR (Fig. 6). The present study also indicated that the recruitment of CrkL to the EpoR is required for the tyrosine phosphorylation of CrkL, because a CrkL mutant lacking the SH2 domain failed to undergo tyrosine phosphorylation in Epo-stimulated cells (Fig. 3). Our previous *in vitro* binding studies suggested that Lyn binds the EpoR through interaction between the Lyn catalytic domain and the membrane-proximal region of EpoR as well as through interaction between the Lyn SH2 domain and the putative docking sites, Tyr⁴⁶⁴ and Tyr⁴⁷⁹, in the EpoR (5). In accordance with this, the present study has demonstrated that Lyn binds the EpoR in 32D/EpoR-Wt cells overexpressing Lyn. We previously demonstrated that Jak2 also physically interacts with the EpoR in 32D/EpoR-Wt cells stimulated with Epo (2). However, Lyn, but not Jak2, phosphorylated CrkL on tyrosine in *in vitro* kinase assays. Therefore, these results strongly suggest that, upon recruitment through its SH2 domain to the tyrosine-phosphorylated EpoR in Epo-stimulated cells, CrkL undergoes phosphorylation on tyrosine by Lyn, which also binds the EpoR.

The transient expression experiments in 32D/EpoR-Wt cells as well as in UT-7 cells (Figs. 5 and 6) have indicated that tyrosine phosphorylation of CrkL by Lyn may play a significant role in Epo activation of the Erk/Elk-1 pathway mediated through CrkL, because the effects of Lyn and its mutant on CrkL phosphorylation correlated with those on activation of Erk and Elk-1. However, this is apparently discordant with the observation that the CrkL-dY mutant lacking the tyrosine phosphorylation site also enhanced Epo activation of the Erk/Elk-1 pathway. One possible explanation for this discrepancy is that Lyn may enhance the CrkL-mediated signaling not by

phosphorylating CrkL but by phosphorylating signaling molecules that form complexes with CrkL. This is, however, unlikely because coexpression of Lyn showed no enhancing effect on the Elk-1 activity in cells overexpressing CrkL-dY, which is in sharp contrast to the synergistic activation of Elk-1 in cells coexpressing Lyn and wild-type CrkL (Fig. 5C). It is thus indicated that Lyn-induced tyrosine phosphorylation of CrkL *per se* should play a role in downstream signaling. Another more likely explanation is that CrkL-dY, with a 20-amino acid internal deletion, may have an alteration in conformation that abrogates the requirement of tyrosine phosphorylation for transducing downstream signals. Intriguingly, Senechal *et al.* (15) also reported that a mutation at Tyr²⁰⁷ enhanced CrkL function in fibroblasts as measured by complex formation with SH2-binding proteins, signal transduction to Jun kinase, and cell transformation and thus suggested that the mutation of Tyr²⁰⁷ activates CrkL function. It is therefore speculated that the phosphorylation of Tyr²⁰⁷ may abrogate the negative regulatory function of this region to activate downstream signaling, although the precise biochemical mechanisms responsible for the effect of tyrosine phosphorylation remain to be explored.

Importantly, the Lyn mutant lacking the tyrosine kinase domain inhibited the Epo-mediated tyrosine phosphorylation of CrkL and activation of Erk/Elk-1 in 32D/EpoR-Wt and UT-7 cells (Figs. 5 and 6). The dominant negative effects of this mutant indicate that without overexpression Lyn plays a role in Epo activation of the CrkL-mediated signaling pathway leading to the activation of Erk/Elk-1 at least in these cells. In accordance with our observation, Tilbrook *et al.* (39) very recently demonstrated that Lyn plays a crucial role in EpoR-mediated activation of Erk in erythroleukemic cells and has a significant impact on the maturation of normal erythroid progenitor cells. In addition to playing a role in activation of the Ras/Erk signaling pathway, CrkL plays a role in cytokine activation of β_1 integrin-mediated hematopoietic cell adhesion (16, 17), most likely through C3G-mediated activation of Rap1 (28). In our previous studies, the CrkL mutant with the defective SH2 domain, which showed a dominant negative effect on cytokine activation of the Ras/Erk signaling pathway, retained the ability, although partially impaired, to activate integrin-mediated cell adhesion (16, 18). Therefore, the recruitment of CrkL to the EpoR and its tyrosine phosphorylation by Lyn may not play a crucial role in CrkL-mediated signaling leading to stimulation of cell adhesion. Studies are currently underway in our laboratory to explore the molecular mechanisms for CrkL-mediated activation of the integrin activation pathway.

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