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 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) 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,Jak2, as well as 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 to a variety of oncogenes, 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 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-
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/Erk-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, SHP1, 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 (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 (22), 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-BglII fragment, containing nucleotides 536 to the 3′ end of the CrkL cDNA, from pBS-CrkL to replace the phosphatase reaction-amplified region to give pGEX-CrkL-F. The expression plasmids were transformed into Escherichia coli DH5α, 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 32D/EpoR-Wt cells were lysed in the lysis buffer described 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× SDS sample buffer and examined by immunoblotting with indicated antibodies. For competition assays with described previously EpoR-derived phosphorytase peptides (5, 25, 26), 200 μmol/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 containing 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 or 32D/EpoR-Wt cells were subjected to the in vitro kinase reactions using a kinase buffer (HEPES, pH 7.5, 50 mmol/liter NaCl, 5 mmol/liter MgCl2, 5 mmol/liter MnCl2, 100 μmol/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× 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 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-
cell lysates were subjected to affinity purification with GST-CrkL-SH2 (GST-CrkL-SH2) or GST (CrkL-SH2). Eluted proteins as well as an aliquot of total cell lysates were prepared from Epo-stimulated (32D/EpoR-Wt) or unstimulated (32D/EpoR-Wt) 32D/EpoR-Wt cells, as described under Methods. 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 (αPY). 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.

**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 (αPY). 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.

**FIG. 2.** In vitro binding studies of the CrkL SH2 domain and the tyrosine-phosphorylated EpoR. A, denatured (D) or non-denatured (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 electrophorosed onto a polyvinylidene difluoride membrane. The membrane was probed with GST-CrkL-SH2 (GST) followed by detection with anti-GST immunoblotting. The membrane was then sequentially reprobed with GST, anti-EpoR (αEpoR), and anti-phosphotyrosine (αPY), 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 μmol/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 μmol/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 μmol/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/Epo-R-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 probing 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 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/Epo-R-Wt cells stably overexpressing the wild-type A and B forms of Lyn, 32D/Epo-R-LynAB or that expressing a Lyn mutant lacking the significant portion of the catalytic domain, 32D/Epo-R-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
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
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 32D/EpoR-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 demonstrations 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 results 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...
Involvement of CrkL and Lyn in EpoR-mediated signaling in UT-7 cells. A, UT-7 cells were transfected with 25 μg of an empty plasmid (−), a mixture of pXM-LynA and pXM-LynB (W), or pXM-LynAN (N), as indicated, along with 25 μg 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 (αPY) immunoblotting, followed by reprobing with anti-HA, as indicated. B, UT-7 cells were transfected with 15 μg of pSG-CrkL (Wt) or pXM-LynAN (N), as indicated, along with 2 μg of pFA-Elk-1, 20 μg of pFR-Luc, and 0.01 μg of pRL-SV40. Total amounts of plasmids transfected were adjusted to be constant by adding an empty vector plasmid.

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 CrkL-mediated 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 Tyr 406 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, Tyr 403 (30), is different from that for CrkL (Tyr 460), 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 activating 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).

FIG. 6. Involvement of CrkL and Lyn in EpoR-mediated signaling in UT-7 cells. A, UT-7 cells were transfected with 25 μg of an empty plasmid (−), a mixture of pXM-LynA and pXM-LynB (W), or pXM-LynAN (N), as indicated, along with 25 μg 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 (αPY) immunoblotting, followed by reprobing with anti-HA, as indicated. B, UT-7 cells were transfected with 15 μg of pSG-CrkL (Wt) or pXM-LynAN (N), as indicated, along with 2 μg of pFA-Elk-1, 20 μg of pFR-Luc, and 0.01 μg 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.
The present study has also demonstrated that CrkL inducibly binds tyrosine-phosphorylated SHP1 in Epo-stimulated cells. SHIP1 is a hematopoietic specific, inositol 5′-phosphatase that becomes tyrosine-phosphorylated in response to hematopoietic cytokines (31, 32). SHP1 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 SHP1 (34). Recently, Mason et al. (35) reported that SHP1 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 SHP1, Shc, and Grb2. Although the enzymatic activity does not change significantly following cytokine stimulation, it has been speculated that SHP1 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). SHP1 has also been postulated to play a negative regulatory role in hematopoietic progenitor cell proliferation/survival through down-modulation of cytokine receptor-mediated Akt activation. SHP1 has also been implicated in regulation of Ca2+ influx in B cells and mast cells (36, 37). It is notable 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 SHP1 has been implicated in regulation of migration of hematopoietic cells (34), thus raising a possibility that SHP1 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/Erk1 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/Erk1 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/Erk1 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/Erk1 at least in these cells. In accordance with our observation, Tilbrook et al. (39) very recently demonstrated that Lyn plays a crucial role in Epo-regulated 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 β1 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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CrkL Is Recruited through Its SH2 Domain to the Erythropoietin Receptor and Plays a Role in Lyn-mediated Receptor Signaling
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