CrkL Mediates Ras-dependent Activation of the Raf/ERK Pathway through the Guanine Nucleotide Exchange Factor C3G in Hematopoietic Cells Stimulated with Erythropoietin or Interleukin-3*

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CrkL is an SH2 and SH3 domain-containing adaptor protein implicated in pathogenesis of chronic myelogenous leukemia. Here, we demonstrate that overexpression of CrkL enhances the erythropoietin (Epo)- or interleukin (IL)-3-induced activation of Elk-1 and the c-fos gene promoter activity in 32D/EpoR-Wt cells. Moreover, the Epo-induced activation of ERK1 and ERK2 was augmented and prolonged in cells inducibly overexpressing CrkL. A moderate increase in Epo-induced activation of JNK was also observed in cells overexpressing CrkL. Overexpression of C3G enhanced the Elk-1 activation synergistically with CrkL, while a C3G mutant lacking the guanine nucleotide exchange domain showed an inhibitory effect. Studies using a dominant negative Ha-Ras mutant demonstrated that the Elk-1 and ERK2 activation enhanced by CrkL and C3G was dependent on Ras. Consistent with this, the Epo-induced activation of Ras was augmented in cells inducibly overexpressing CrkL. Most importantly, a CrkL mutant defective in the SH2 or N-terminal SH3 domain showed an inhibitory effect on the Epo-induced activation of ERK2. These data indicate that the CrkL-C3G complex plays a role in Epo- or IL-3-induced, Ras-dependent activation of the Raf/ERK pathway leading to the activation of Elk-1 and the c-fos gene transcription.

The growth and differentiation of hematopoietic progenitor cells are regulated by several cytokines, such as erythropoietin (Epo) and interleukin (IL)-3, which act through the type I cytokine receptors (1, 2). The hematopoietic cytokine receptors mainly couple with Jak2, a member of the Jak family of tyrosine kinases, to transduce a growth signal in hematopoietic cells (2, 3). Through activation of Jak2 and other tyrosine kinases, the cytokine receptors become tyrosine-phosphorylated and thereby recruit various Src homology domain 2 (SH2)-containing signaling molecules, such as Shc, Shp-2, and Stat5, to activate downstream signaling pathways. One of the main signaling pathways from the cytokine receptors is the Ras/extracellular signal-regulated kinase (ERK) activation cascade involving the small GTP-binding protein Ras and the downstream serine/threonine kinases Raf-1, mitogen-activated/extracellular-signal regulated protein kinase kinase (MEK)1/2, and ERK1/2 (2). Previously, we and others (2, 4) have shown that the activated hematopoietic receptors induce the tyrosine phosphorylation of Shc and thereby recruit the preformed complex of the adaptor protein Grb2 and the guanine nucleotide exchange factor Sos1 to the vicinity of Ras at the plasma membrane to activate this small GTP-binding protein. Activated Ras then recruits, by direct interaction, Raf-1 to the plasma membrane where the catalytic activity of the kinase becomes activated. Subsequently, Raf-1 stimulates the MEK1 and MEK2 kinases by phosphorylation, which then phosphorylate ERK1 and ERK2 on both serine and tyrosine residues to activate their kinase activity (5). The activated ERKs shuttle into the nucleus and induce the activity of various transcription factors including Elk-1. As a member of the transcription factor family of ternary complex factors, Elk-1, when activated by phosphorylation, binds to the c-fos serum response element to induce the transcription of this proto-oncogene (6).

CrkL is a member of the Crk family of adaptor proteins originally identified as homologues of the product of the v-crk oncogene (7). Three forms of cellular Crk proteins, composed mostly of SH2 and SH3 domains, have been found: both Crk II and CrkL have the domain structure SH2-SH3-SH3, while Crk I, the alternatively spliced form of Crk II, lacks the C-terminal SH3 domain (8, 9). The N-terminal SH3 domain of CrkL has been shown to bind Sos1 and C3G, two guanine nucleotide exchange proteins for the Ras family of small GTP-binding proteins (10, 11). Interestingly, recent studies have established that CrkL, which is most abundantly expressed in hematopoietic cells (12), also binds through its N-terminal SH3 domain to the BCR-ABL fusion protein expressed in chronic myelogenous leukemia cells and becomes phosphorylated at Tyr-207 (13–16). It has been, thus, implied that CrkL may play a role in leukemic transformation. We have previously demonstrated that CrkL also becomes tyrosine-phosphorylated in hematopoietic cells in response to stimulation with Epo or IL-3 and forms complexes with several tyrosine-phosphorylated signaling molecules, such as Cbl, Shc, and Shp-2 (17). The induction of tyrosine phosphorylation of CrkL and its association with Cbl have also been reported in hematopoietic cells stimulated with stem cell factor (18), thrombopoietin (19), and IL-2 (20). These observations strongly suggest that CrkL plays a role in cytokine receptor signaling in hematopoietic cells. Previously, overexpression of CrkL was shown to activate c-Jun N-terminal

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The abbreviations used are: Epo, erythropoietin; EpoR, Epo receptor; IL, interleukin; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated/extracellular-signal regulated protein kinase kinase; SH2 and SH3, Src homology 2 and 3, respectively; JNK, c-Jun N-terminal kinase; RBD, Ras-binding domain; GST, glutathione S-transferase; HA, hemagglutinin.
kinase (JNK) (21, 22) and to transform fibroblasts in a Ras-dependent manner (21). In contrast, overexpression of CrkL was demonstrated to activate adhesion of cytokine-dependent hematopoietic cells (23, 24) without showing any effects on the cytokine dependence (23). It is thus speculated that CrkL plays a signaling role in hematopoietic cells that is different from that in fibroblasts. However, the role of CrkL in hematopoietic cytokine receptor signaling and its downstream signaling events have remained largely unknown. Therefore, in the present study we have examined the involvement of CrkL in signaling from the Epo and IL-3 receptors in hematopoietic cells and found 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.

MATERIALS AND METHODS

Cells and Reagents—A clone of IL-3-dependent 32D cells expressing the wild-type murine EpoR (32D/EpoR-Wt) was described previously (25) and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 1 unit/ml human recombinant Epo. 32DE/Tet-CrkL and 32DE/TAT-CrkL were also described previously (24). COS7 cells were cultured in Dulbeccos modified Eagle’s medium supplemented with 10% fetal calf serum.

Recombinant human Epo was kindly provided by Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan). Recombinant murine IL-3 was purchased from PeproTech Inc. (Rocky Hill, NJ). A MEK inhibitor, PD98059, and anisomicyn were purchased from New England Biolabs (Beverly, MA) and Sigma, respectively.

Antibodies against phosphotyrosine (4G10) and ERK1/2 (ERK1/2-CT) were purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies against CrkL, C3G, and Soe1 were from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal pan-Ras antibody (Ras 10) and a monoclonal antibody against the influenza virus hemagglutinin (HA) were purchased from Calbiochem and Roche Molecular Biochemicals (Mannheim, Germany), respectively.

Plasmids—A luciferase reporter plasmid for the c-fos gene, containing a 0.4-kilobase pair c-fos promoter upstream of the initiation site and luciferase coding region (26), was kindly provided by Dr. Sumiko Watanabe (University of Tokyo, Tokyo, Japan). The PathDetect in vivo reporting system for Elk-1, which consists of the pFA-Elk plasmid, encoding the fusion protein consisting of the full-length yeast GAL4 and the activation domain of Elk-1, and the pFR-luc plasmid, in which expression of luciferase is controlled by a promoter containing the GAL4-binding sites, was purchased from Stratagene (La Jolla, CA). A control Renilla luciferase plasmid, pRl-SV40, was purchased from Promega (Madison, WI).

An expression plasmid for human CrkL, pSG-CrkL (27), was kindly provided by Dr. John Groffen (Childrens Hospital Los Angeles, Los Angeles, CA). Expression plasmids for various CrkL mutants were described previously (24). An expression plasmid for C3G (pcDNA-C3G) as well as those for dominant negative mutants of C3G (pcDNA-C3G-DS3, Raf-1 (pcDNA-Raf-dS3), and Ha-Ras (pcDNA-Ha-Ras17N) were also described previously (24). An expression plasmid for C3G lacking the CrkL binding site (10), pcDNA-C3G-DN, was constructed by subcloning the PolII-Xba1 fragment of pcDNA-C3G, coding for leu618 to the C terminus of C3G, in frame into the EcoRV-Xba1 digested pcDNA/HisMaxB vector (Invitrogen, San Diego, CA). An expression plasmid for murine ERK2, pcDNA-ERK2, was constructed by subcloning the PmnI/Xba1 insert of pRmErk2, obtained through the RIKEN Gene Bank (Ibaraki, Japan), into the pcDNA2 vector (Invitrogen). An expression plasmid used for constitutively activated Ha-Ras, pcDNA-Ha-Ras61L was constructed by subcloning cDNA coding for Ha-Ras61L (Upstate Biotechnology) into the pcDNA3 vector (Invitrogen). Expression plasmids for HA-tagged ERK2 (SHH-ERK2) and dominant negative R-Ras (pcDNA-R-Ras4N) were kindly provided by Dr. Michael Karin (University of California at San Diego) and by Dr. Erik Ruoslabhi (La Jolla Cancer Research Center, La Jolla, CA), respectively.

Expression plasmids for dominant negative and constitutively activated mutants of Rap1/K-Rev1 (29) were tagged with the T7 epitope, pSso-T7-Rap1A-17N and pSso-T7-Rap1A-12V, respectively, kindly provided by Drs. Makoto Noda and Masakazu Hattori (Kyoto University, Kyoto, Japan).

Transfection—For transient expression in 32D/Epo-R-Wt cells, 1 × 106 cells were electroporated at 960 microfarads and 300 V with indicated plasmids using a Gene Pulser apparatus (Bio-Rad). After overnight starvation from Epo, cells were sub-

ected to luciferase reporter assays or immunoprecipitation followed by immunoblotting.

Transfection of expression plasmids into COS7 cells was carried out using the Lipofectamin reagent (Life Technologies, Inc.), as described previously (31). Two days after transfection, cells were harvested for analysis with immunoprecipitation and immunoblotting.

Luciferase Reporter Assays—Luciferase reporter assays of transiently transfected cells were performed essentially as described previously (30). In brief, 32D/Epo-R-Wt cells were electroporated with indicated plasmids and starved overnight in medium without Epo. Cells were then incubated for 5 h in medium with or without cytokines, as indicated, and harvested for the luciferase assay using Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. 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. Except for dose dependence experiments, all the luciferase assays in Results were performed in duplicate, unless otherwise described, and repeated at least three times with reproducible results.

Immunoprecipitation and Immunoblotting—Cells were lysed in a lysis buffer containing 1% Triton X-100, 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml each of aprotinin and leupeptin. Cell lysates were subjected to immunoprecipitation as described previously (32). For immunoprecipitation of endogenous ERK1 and ERK2, 1 × 106 cells were lysed in 100 µl of boiling lysis buffer containing 1% SDS and 10 mM Tris-HCl (pH 7.4) and boiled for another 5 min, as described previously (4). The lysate was then diluted 10-fold with the Triton X-100 lysis buffer, and denatured ERK1 and ERK2 were immunoprecipitated with anti-ERK1/2. For immunoblot analysis of total cell lysates, an aliquot of the clarified supernatant was directly mixed with equal volumes of 2 × Laemmli’s sample buffer and heated at 100 °C for 5 min. Samples were separated by SDS-polyacrylamide gel electrophoresis and electrotransferred to Immobilon-P membranes (Millipore, Bedford, MA). The membranes were probed with an indicated antibody followed by detection using enhanced chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). For reprobing of the membranes, they were treated with stripping buffer composed of 100 mM ethanol 2-mercaptoethanol, 2% SDS, and 62.5 mM/liter Tris-HCl (pH 6.7) at 50 °C for 30 min and subsequently probed with a different antibody.

JNK Activation Assay—The activity of JNK was assayed by using SAPK/JNK Assay Kit (New England Biolabs) according to the manufacturer’s instructions. In brief, JNK was pulled down from cell lysates by using a GST-Jun fusion protein and allowed to phosphorylate the fusion protein in vitro. The in vitro kinase reaction products were then analyzed by Western blotting with an antibody specifically reactive with c-Jun phosphorylated on Ser-63.

Ras Activation Assay—Activation of Ras was examined essentially as described by de Rooji and Bos (33). In brief, the portion of Raf-1 cDNA coding for the minimal Ras-binding domain (RBD; amino acids 51–131) was subcloned into the BamHI-EcoRI site of pGEX-4T-3 to prepare the glutathione S-transferase (GST)-Raf-RBD fusion protein, as described previously (33). Cells were lysed in radioimmunoprecipitation buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and 10 µg/ml each of aprotinin and leupeptin) and incubated with GST-Raf-RBD purified on glutathione-agarose beads for 1 h at 4 °C. Affinity-purified Ras-GTP was then detected by Western blot analysis using anti-pan-Ras monoclonal antibody.

RESULTS

Overexpression of CrkL Enhances Epo- or IL-3-induced Activation of the c-fos Gene Promoter and Elk-1—Previously, we have shown that Epo or IL-3 induces the transcriptional activation of the c-fos gene in 32D/Epo-R-Wt cells, an IL-3-dependent 32D clone expressing the EpoR (25). To explore the involvement of CrkL in cytokine receptor signaling in hematopoietic cells, we first examined the effect of CrkL overexpression on Epo- or IL-3-induced activation of the c-fos gene promoter. As shown in Fig. 1A, CrkL significantly augmented the Epo- or IL-3-induced activation of the c-fos gene promoter when transiently overexpressed in 32D/Epo-R-Wt, although the basal transcriptional level of the c-fos gene promoter was also elevated slightly by overexpression of CrkL. Because the growth
factor-induced activation of the c-fos gene has been shown to be mediated through Elk-1, a transcription factor activated mainly by the Raf/ERK signaling pathway, we next examined the effect of CrkL overexpression on the Elk-1 activation by using the PathDetect in vivo reporting system, as described under "Materials and Methods." In accordance with the effect of CrkL overexpression on the c-fos gene promoter activation, overexpression of CrkL augmented the Epo- or IL-3-induced activation of Elk-1, as shown in Fig. 1B. These data suggest that CrkL is involved in activation of a signaling pathway from the receptors for Epo and IL-3 to up-regulate the c-fos gene transcription through the activation of Elk-1.

The Raf/MEK/ERK Signaling Pathway Is Involved in the CrkL-enhanced Activation of Elk-1-To define the downstream signal transduction pathway leading to the Elk-1 activation, we next examined the possible involvement of the Raf/MEK/ERK cascade. We first examined ERK2, which directly activates Elk-1, by transiently overexpressing this serine/threonine kinase alone or in combination with CrkL in 32D/EpoR-Wt cells. As shown in Fig. 2A, ERK2 only modestly augmented the Epo-induced activation of Elk-1 when overexpressed alone. However, when ERK2 was overexpressed in combination with CrkL, the basal and Epo-induced levels of Elk-1 activity were synergistically increased to very high levels (Fig. 2A). This is compatible with the idea that ERK2 is a downstream effector of CrkL and thus further amplifies the signal from CrkL to activate Elk-1. The possible involvement of MEK, an activator of ERK2, was then examined by using a MEK inhibitor, PD98059. As shown in Fig. 2B, treatment of 32D/EpoR-Wt cells with PD98059 showed a very strong inhibitory effect on Epo-induced activation of Elk-1. PD98059 also significantly inhibited the CrkL-enhanced activation of Elk-1 (Fig. 2B). These data indicate that MEK plays a crucial role in Epo-induced activation of Elk-1 and is also involved in CrkL-enhanced activation of Elk-1. We next examined Raf-1, an activator of MEK, by transiently expressing a dominant negative mutant of Raf-1, which lacks the kinase domain, in 32D/EpoR-Wt cells. As shown in Fig. 2C, the Raf-1 mutant strongly inhibited Epo-induced Elk-1 activation in 32D/EpoR-Wt cells as well as in cells overexpressing CrkL. The dominant negative Raf-1 mutant exhibited similar inhibitory effects on Elk-1 activation in IL-3-stimulated 32D/EpoR-Wt cells (data not shown). Together, these results suggest that CrkL transduces the Elk-1 activation signal through the Raf/MEK/ERK pathway.

To confirm and further analyze the involvement of Raf/MEK/ERK pathway in CrkL-mediated signaling from the EpoR, we next examined Epo-induced activation of ERK1 and ERK2 in 32DE/Tet-CrkL, a clone of 32D/EpoR-Wt cells, which inducibly overexpress CrkL when withdrawn from tetracycline (24). In accordance with our previous results in 32D/EpoR-Wt cells (4), Epo stimulation induced activation of ERK1 and ERK2, which was detected by their phosphorylation on tyrosines, in 32DE/Tet-CrkL cells (Fig. 3). A dose-dependent experiment in Fig. 3A demonstrates that, when 32DE/Tet-CrkL cells were cultured in the absence of tetracycline, Epo-induced activation of ERK1 and ERK2 was observed at a lower Epo concentration (0.1 unit/ml) than when cultured in the presence of tetracycline. Moreover, the activation of ERK1 and ERK2 in tetracycline-deprived cells reached higher levels with increasing Epo concentrations as compared with those observed in cells cultured in the presence of tetracycline (Fig. 3A). A time course experiment in Fig. 3B further demonstrates that Epo-induced activation of ERK1 and ERK2 in 32DE/Tet-CrkL cells cultured without tetracycline was not only intensified but also prolonged as compared with that in cells cultured with tetracycline. In 32DE/TA cells, which express the tetracycline transactivator alone at a comparable level with that in 32DE/Tet-CrkL, tetracycline did not show any effect on Epo-induced activation of ERK1 and ERK2 (data not shown). These data have thus confirmed that CrkL is involved in Epo-induced activation of ERK1 and ERK2.

Overexpression of CrkL Also Augments the Epo-induced Activation of JNK in 32D Cells—In fibroblasts, CrkL has been shown to activate JNK (21, 22), which also activates Elk-1 by phosphorylation. Previous studies have also shown that Epo as well as IL-3 induces the JNK activation in hematopoietic cells (34–37). We therefore examined the effect of CrkL overexpression on the JNK activity in 32D cells. As demonstrated in Fig. 4A, Epo induced a dose-dependent activation of JNK in 32DE/Tet-CrkL cells. When overexpression of CrkL was induced by withdrawing cells from tetracycline, the Epo-induced activation of JNK as well as that induced by anisomycin was moderately enhanced. A time course experiment shown in Fig. 5B revealed that the Epo-induced activation of JNK peaks at 20 min after stimulation and significantly declines at 60 min. Overexpression of CrkL did not significantly change the time course of Epo-induced JNK activation, although the activation level at the peak was moderately enhanced (Fig. 5B). These results raise the possibility that CrkL may play a role in Epo-induced activation of JNK and suggest that the CrkL-enhanced activation of JNK may also contribute, although less significantly than that of ERK, to the augmented activation of Elk-1.

The Guanine Nucleotide Exchange Domain of C3G Is Required for CrkL-mediated Activation of Elk-1—The N-terminal SH3 domain of CrkL has been shown to bind Sos1 and C3G, guanine nucleotide exchange factors for the Ras subfamily of GTP-binding proteins. However, we have demonstrated previously that CrkL exclusively binds C3G in 32D/EpoR-Wt cells (17), as is also the case in various other hematopoietic cell lines (38–40). Thus, we examined whether C3G is involved in CrkL-mediated activation of the Raf/ERK signaling pathway. First, wild-type C3G was overexpressed alone or in combination with CrkL, and its effect on Epo-induced Elk-1 activation was examined. As shown in Fig. 5A, overexpression of C3G alone only slightly enhanced the Epo-induced Elk-1 activation. However, when overexpressed in combination with CrkL, a synergistic enhancement of Elk-1 activation was observed, thus suggesting that C3G may cooperate with CrkL to activate the signaling pathway leading to Elk-1 activation. To confirm this, we next expressed the dominant negative C3G-dSS mutant, lacking the
guanine nucleotide exchange domain, along with CrkL in 32D/EpoR-Wt cells. As shown in Fig. 5B, C3G-dSS demonstrated a dose-dependent inhibitory effect on the CrkL-enhanced activation of Elk-1. Overexpression of C3G or its dominant negative mutant exhibited the same effect on Elk-1 activation in IL-3-stimulated cells as compared with that in Epo-stimulated cells (data not shown). As shown in Fig. 5C, when transiently overexpressed in 32D/EpoR-Wt cells, CrkL associated not only with C3G but also with Sos1, a guanine-nucleotide exchange factor for Ras. It is thus possible that Sos1 may also contribute, to some extent, to the enhanced activation of the Ras/ERK pathway in CrkL-overexpressing cells. However, co-expression of C3G or C3G-dSS drastically increased or decreased, respectively, the CrkL-enhanced activation of Elk-1, whereas they similarly and only moderately decreased the amount of Sos1 associated with CrkL (Fig. 5C). It was thus concluded that C3G was directly involved in the CrkL-enhanced activation of Elk-1.

The dominant negative effect of C3G-dSS observed on the CrkL-enhanced Elk-1 activation indicates that C3G mediates the CrkL-induced Elk-1 activation rather than acts through a separate pathway. To confirm this, we constructed an expression plasmid for mutant C3G (pcDNA-C3G-dN) that lacks the CrkL-SH3 binding domain, as shown in Fig. 5D. When expressed in COS7 cells, the dN mutant was demonstrated not to coimmunoprecipitate with coexpressed CrkL, while wild-type C3G as well as the dominant negative C3G mutant (C3G-dSS) coimmunoprecipitated with CrkL (Fig. 5E). We then transiently expressed C3G-dN alone or in combination with CrkL in 32D/EpoR-Wt cells and examined the effects on Epo-induced activation of Elk-1. As shown in Fig. 5F, when expressed alone, C3G-dN modestly enhanced the Epo-induced Elk-1 activation. Unlike wild-type C3G, however, C3G-dN acted only additively.
but not synergistically with CrkL to enhance the Elk-1 activation. Thus, the complex formation with CrkL is required for C3G to synergize with CrkL to activate Elk-1, which indicates that C3G acts downstream of CrkL to mediate activation of the Elk-1-activating pathway from the cytokine receptors.

**Activation of the CrkL- and C3G-mediated Elk-1 Activation Pathway Is Dependent on Ras**—C3G is a guanine nucleotide exchange factor for the Ras subfamily of small GTP-binding proteins, which includes Ras, Rap1/K-Rev1, and R-Ras. To determine whether these small GTP-binding proteins are involved in CrkL-mediated activation of the Raf/ERK signaling pathway, dominant negative mutants of these molecules were expressed in 32D/EpoR-Wt cells to examine their effects on CrkL-enhanced Elk-1 activation. Expression of a dominant negative mutant of Ras, Ha-Ras17N, drastically inhibited Epo- or IL-3-induced Elk-1 activation when expressed alone or in combination with CrkL or with both CrkL and C3G (Fig. 5A and data not shown). On the other hand, in repeated experiments, dominant negative mutants of R-Ras and Rap1/K-Rev1, R-Ras43N and Rap1-17N, respectively, significantly enhanced the CrkL-C3G-induced Elk-1 activation, as shown in Fig. 6A, or did not show significant effects (data not shown). Furthermore, a constitutively activated mutant of Rap1/K-Rev1, Rap1-12V, showed an inhibitory effect on the Elk-1 activation (Fig. 6A). These data suggest that Ras, but not R-Ras or Rap1/K-Rev1, mediates the CrkL-C3G-induced activation of Elk-1.

We next examined the effect of Ha-Ras17N on Epo-induced ERK2 activation enhanced by CrkL and C3G. In accordance with the results of luciferase assays on Elk-1 activity shown in Fig. 5A, transient overexpression of both CrkL and C3G in 32D/EpoR-Wt cells induced a constitutive activation of cotransfected HA-ERK2 and significantly enhanced its activation induced by Epo, as detected by its tyrosine phosphorylation (Fig. 6B). However, the Epo-induced activation of HA-ERK2 became only slightly detectable when Ha-Ras17N was coexpressed with CrkL and C3G. These data thus confirm that the Epo-induced activation of the Raf/ERK pathway through CrkL and C3G is dependent on Ras.
To further confirm the involvement of Ras in the signaling pathway downstream from CrkL and C3G, we next examined Epo-induced activation of Ras in 32DE/Tet-CrkL cells. When cells were cultured in the presence of tetracycline, Epo stimulation only slightly and transiently increased the activity of Ras, as detected by anti-Ras immunoblotting of GTP-bound Ras, as shown in Fig. 6C. We next examined the functional significance of Ras/ERK signaling pathway.

Finally, we examined whether C3G physically interacts with Ras. As shown in Fig. 6D, the dominant negative Ha-Ras17N mutant was found to coimmunoprecipitate with C3G when coexpressed in COS cells. When CrkL was also coexpressed, an increase in the amount of Ha-Ras17N associated with C3G was observed. More remarkably, the C3G-dN mutant, which retains the guanine nucleotide exchange domain but lacks most of the region N-terminal to this domain (Fig. 5D), bound a much larger amount of Ha-Ras17N as compared with wild-type C3G, as shown in Fig. 6D. The activated Ha-Ras17N mutant also bound C3G-dN but to a lesser extent as compared with Ha-Ras17N. These results indicate that C3G physically interacts with Ras and that this interaction facilitates the interaction of C3G with Ha-Ras. These observations thus agree with the idea that C3G mediates the CrkL-induced activation of the Ras/ERK signaling pathway.

The SH2 and N-terminal SH3 Domains of CrkL Play Crucial Roles in Activation of the Signaling Pathway Leading to Elk-1 Activation—We next examined the functional significance of each domain of CrkL on activation of the Ras/ERK pathway by transiently transfecting various CrkL mutants previously characterized (24) and shown in Fig. 6. When transiently expressed in 32D/EpoR-Wt, these mutants were expressed at comparable levels, as shown in Fig. 7A. Under the similar conditions, the dY mutant, which lacks a 12-amino acid region containing the site of tyrosine phosphorylation (16), did not show any impairment in the ability to enhance the Epo-induced Elk-1 activation, as determined by the PathDetect Elk-1 reporter assay (Fig. 7C). The dSH2C, dSH3N, and dSH2N mutants, in which significant portions of the SH2 and N-terminal SH3 domains, respectively, are deleted, did not significantly enhance the Epo-induced activation of Elk-1 (Fig. 7C). This result indicates that both SH2 and N-terminal SH3 domains of CrkL play crucial roles in activation of the signaling pathway leading to Elk-1 activation, whereas the C-terminal SH3 domain as well as the tyrosine phosphorylation of CrkL is dispensable for this function. The critical importance of the CrkL N-terminal SH3 domain is in agreement with the involvement of C3G, which binds CrkL through this domain. Next, these CrkL mutants were transiently expressed, along with HA-tagged ERK2, in 32D/EpoR-Wt to examine their effects on Epo-induced activation of ERK2. In accordance with
the results in 32DE/Tet-CrkL cells shown in Fig. 3, transient overexpression of wild-type CrkL augmented and prolonged the Epo-induced ERK2 activation (Fig. 7D), although the augmentation was not as remarkable as when both CrkL and C3G were overexpressed as shown in Fig. 6B. The dSH3C mutant demonstrated a similar effect with wild-type CrkL (Fig. 7D), which is in agreement with their abilities to enhance the Epo-induced activation of Elk-1 (Fig. 7C). Importantly, both dSH3N and dSH2 mutants showed inhibitory effects on the Epo-induced ERK2 activation in repeated experiments (Fig. 7D and data not shown). The dominant negative effects demonstrated by these mutants not only confirm that both SH2 and N-terminal SH3 domains play critical roles in activation of the Ras/ERK pathway but also indicate that CrkL, even when not overexpressed, should partly mediate the Epo-induced activation of this pathway in 32D/EpoR-Wt cells.

DISCUSSION

The present studies have demonstrated that the adaptor protein CrkL mediates activation of the Raf/ERK signaling pathway leading to activation of Elk-1 and the c-fos promoter activity in hematopoietic cells stimulated with Epo or IL-3. The activation of this pathway by CrkL was dependent on the guanine nucleotide exchange factor C3G and the Ras GTP-binding protein, as demonstrated by the inhibitory effects of dominant negative mutants of C3G and Ha-Ras. In agreement with this, the N-terminal SH3 domain of CrkL, which mediates binding with C3G, was shown to play a crucial role in activation of this pathway. However, it should be noted that C3G has been characterized as a guanine nucleotide exchange factor that activates Ras as well as Rap1/K-Rev1 during Drosophila development (48). It is thus speculated that C3G directly activates Ras when efficiently recruited to its vicinity by CrkL in hematopoietic cells. In accordance with this idea, C3G was shown to interact with Ras when co-expressed in COS7 cells (Fig. 6D).

It should be noted that C3G has been characterized as a guanine nucleotide exchange factor that activates Ras in vitro (46) as well as in transfected COS cells (47). C3G has also been implicated in Ras activation by yeast complementation studies (10). Furthermore, a very recent study has shown that Drosophila C3G can trigger activation of Ras as well as Rap1/K-Rev1 during Drosophila development (48). It is thus speculated that C3G directly activates Ras when efficiently recruited to its vicinity by CrkL in hematopoietic cells. In accordance with this idea, C3G was shown to interact with Ras when co-expressed in COS7 cells (Fig. 6D).

Previously, the Crk adaptor proteins, including CrkL (21), have been implicated in activation of the Ras/ERK signaling pathway in fibroblastic cells (21, 41–43) and in PC12 pheochromocytoma cells (44, 45). However, unlike in hematopoietic cells, the Crk proteins have been shown to bind Sos1 as well as C3G in these cells. It is thus implied that the Crk-mediated activation of Ras in these cells is through Sos1. In fact, the association of Sos1 with CrkL was also observed in 32DE/Tet-CrkL cells when CrkL was overexpressed (Fig. 5C). It is thus likely that the enhancement of Ras activation induced by overexpression of CrkL is partly mediated through the recruitment of Sos1 by CrkL to the plasma membrane. However, because overexpression of C3G or its mutant defective in the guanine nucleotide exchange domain synergistically increased or significantly inhibited, respectively, the CrkL-mediated enhancement of Elk-1 activation pathway, C3G should also account for the increased activation of Ras observed in CrkL-overexpressing cells. Previously, C3G was shown to activate, although weakly, Ras in vitro (46) as well as in transfected COS cells (47). C3G has also been implicated in Ras activation by yeast complementation studies (10). Furthermore, a very recent study has shown that Drosophila C3G can trigger activation of Ras as well as Rap1/K-Rev1 during Drosophila development (48). It is thus speculated that C3G directly activates Ras when efficiently recruited to its vicinity by CrkL in hematopoietic cells. In accordance with this idea, C3G was shown to interact with Ras when co-expressed in COS7 cells (Fig. 6D).

It should be noted that C3G has been characterized as a guanine nucleotide exchange factor that activates Rap1/K-Rev1 most efficiently and R-Ras moderately (46). Originally isolated as a suppressor of K-Ras transformation (49), Rap1/K-Rev1 has been hypothesized to antagonize Ras signaling. In accordance with this, the activated Rap1-12V mutant was shown to inhibit Ras-dependent activation of ERKs in Rat-1 fibroblast (50), which agrees with our result that Rap1-12V inhibited the Epo-induced Elk-1 activation in CrkL-C3G overexpressing cells (Fig. 6A). However, it has been recently re-
ported that Rap1/K-Rev1 mediates, through specific activation of B-Raf, sustained activation of ERKs induced by CAMP (51) or by nerve growth factor (52) in PC12 cells, which was significantly inhibited by Rap1-17N. We therefore examined the effect of Rap1-17N on the CrkL/C3G-enhanced activation of Elk-1 in 32D/EpoR-Wt cells, although the dominant negative effect of Rap1-17N on C3G has been disputed recently (53). In contrast to the inhibitory effect observed in PC12 cells (51, 52), Rap1-17N did not show any inhibitory effect but rather augmented the Elk-1 activation in 32D/EpoR-Wt cells (Fig. 6A). In this regard, it should be noted that activation of ERKs by Rap1/K-Rev1 in PC12 cells has also been disputed very recently (54). Taken together with the inhibitory effect of Rap1-V12, it was thus concluded that the Elk-1 activation mediated by CrkL/C3G does not depend on Rap1/K-Rev1. Similarly, R-Ras43N, the dominant negative mutant of R-Ras that was previously shown to inhibit the CrkL/C3G-induced activation of integrin in 32D/EpoR-Wt cells (24), did not show any inhibitory effect on the Elk-1 activation (Fig. 6A). Therefore, it was concluded that neither Rap1/K-Rev1 nor R-Ras is responsible for the CrkL/C3G-mediated activation of Elk-1, although both of these small GTP-binding proteins might be activated by C3G in 32D/EpoR-Wt cells.

Importantly, the dSH2 and dSH3N CrkL mutants inhibited the Epo-induced ERK activation in 32D/EpoR-Wt cells (Fig. 7D). The dominant negative effects of these mutants indicate that, without overexpression, CrkL mediates the activation of ERK by the EpoR, at least partly, in these cells. However, because of the redundancy with the activation mechanism involving the Grb2-Sos1 complex, the biological significance of the CrkL-mediated activation of the Ras/ERK signaling pathway by the EpoR remains to be determined. In this regard, it should be noted that the CrkL overexpression not only augmented but also prolonged the activation of Ras as well as ERK1/2 (Figs. 3, 6C, and 7D). It is thus suggested that the CrkL/C3G complex activates the Ras/ERK pathway with a different time course as compared with the Grb2-Sos1 complex does. It has been well established that the time course of ERK activation has a physiological significance in regulation of cell growth and differentiation; in PC12 cells, whereas epidermal growth factor transiently activates ERK to induce cellular proliferation, nerve growth factor induces sustained activation of ERK, which plays a crucial role in neuronal differentiation (55). Interestingly, the Crk adaptor proteins (44, 45, 52), as well as C3G (52), have been implicated in the nerve growth factor-induced sustained ERK activation and neuronal differentiation in PC12 cells, which may (52) or may not (54) involve the activation of Rap1/K-Rev1 by the CrkL-C3G complex. In hematopoietic cells, the sustained activation of ERK by thrombopoietin has been shown to play a crucial role in megakaryocytic differentiation (56, 57), although the activation mechanism remains to be determined. It is thus tempting to speculate that, in addition to the putative roles in regulation of proliferation and apoptosis, the CrkL-mediated sustained activation of ERK may play a role in regulation of differentiation of hematopoietic cells. Further studies are in progress in our laboratory to examine the possible involvement of CrkL-mediated signaling in erythroid differentiation using cells susceptible for Epo-induced differentiation.

Previous studies in fibroblasts have shown that overexpression of the Crk proteins (21, 22, 58, 59), including CrkL (21, 22), activates JNK, which also phosphorylates and activates Elk-1. Moreover, Epo as well as IL-3 has been shown to induce the JNK activation in hematopoietic cells (34–37). Therefore, we examined the effect of CrkL on Epo-induced activation of JNK in 32D cells and found that the JNK activity stimulated by Epo was moderately augmented in cells overexpressing CrkL (Fig. 4). Because the JNK activation in hematopoietic cells has been shown to be dependent on Ras (34, 35) and to require higher levels of Ras-GTP than the activation of ERK (35), it is speculated that CrkL may enhance the JNK activation through the augmentation of Ras activation. In fibroblastic cells, however, C3G has been shown to activate JNK by a Ras-independent mechanism through a pathway involving the mixed lineage kinase family of proteins (59). Furthermore, very recent studies (60, 61) have implicated DOCK180 (62), another Crk SH3-binding protein, in Crk-mediated activation of JNK through a pathway dependent on the small GTP-binding protein Rac1. Further studies are thus required to determine the mechanisms by which CrkL may mediate the JNK activation pathway from the cytokine receptors in hematopoietic cells.

Overexpression of CrkL in hematopoietic cells activates cellular adhesion to fibronectin (23, 24). Our previous studies have further demonstrated that CrkL activates through C3G a signaling pathway leading to activation of the VLA-4 and VLA-5 integrins, which mediate the cell adhesion with fibronectin (24). This signaling pathway is most likely mediated through R-Ras or other Ras family small GTP-binding proteins, such as Rap1/K-Rev, but is independent from the Ras/ERK pathway investigated in the present study, because the dominant negative mutant of Ha-Ras or Raf-1 actually enhanced the cell adhesion (24). The opposing effects of R-Ras and Ha-Ras on cell adhesion have also been reported previously (28, 63). Thus, CrkL may activate through C3G different small GTP-binding proteins to regulate different events in hematopoietic cells.

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REFERENCES

CrkL Mediates Ras-dependent Activation of the Raf/ERK Pathway through the Guanine Nucleotide Exchange Factor C3G in Hematopoietic Cells Stimulated with Erythropoietin or Interleukin-3

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