The Adapter Protein Crkl Links Cbl to C3G after Integrin Ligation and Enhances Cell Migration*

Naoki Uemura and James D. Griffin‡§

From the ‡Department of Adult Oncology, Dana-Farber Cancer Institute, and the Departments of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts 02115

Crkl, an SH2-SH3-SH3 adapter protein, is one of the major tyrosine phosphoproteins detected in cells from patients with chronic myelogenous leukemia. Crkl binds to BCR/ABL through its N-terminal SH3 domain and is known to interact with several signaling proteins that have been implicated in integrin signaling, including Cbl, Cas, Hef-1, and paxillin. We have previously shown that overexpression of Crkl enhances adhesion to extracellular matrix proteins through β1 integrins. In this study, the effects of Crkl on spontaneous and chemokine-directed migration of the hematopoietic cell line Ba/F3 were examined. Full-length, SH2-, and SH3(N)-domain deletion mutants of Crkl were expressed transiently as fusion proteins with green fluorescent protein. Successfully transfected cells were isolated by fluorescence-activated cell sorting. The ability of these cells to migrate across a fibronectin-coated membrane, either spontaneously or in response to the chemokine stromal-derived factor-1, was determined. Cells expressing green fluorescent protein alone were not distinguishable from untransfected or mock transfected Ba/F3 cells. However, Ba/F3 cells overexpressing full-length Crkl were found to have an increase in spontaneous migration of 2.8 ± 0.6-fold in seven independent assays. The enhancement of migration required both the SH2 domain and the N-terminal SH3 domain. Migration in response to stromal-derived factor-1a was not significantly enhanced by overexpression of Crkl. Overexpression of Crkii also augmented spontaneous migration but to a lesser degree than did Crkl. Because the SH2 domain was required for enhanced migration, we looked for changes in phosphotyrosine containing proteins coprecipitating with Crkl, but not CrklΔSH2, after integrin cross-linking. Full-length Crkl, but not CrklΔSH2, coprecipitated with a single major tyrosine phosphoprotein with an Mr of approximately 120 kDa, identified as Cbl. The major Crkl SH3-binding protein in these cells was found to be the guanine nucleotide exchange factor, C3G. Interestingly, overexpression of C3G also enhanced migration, suggesting that a Cbl-Crkl-C3G complex may be involved in migration signaling in Ba/F3 cells. These data suggest that Crkl is involved in signaling pathways that regulate migration, possibly through a complex with Cbl and C3G.

MATERIALS AND METHODS

Plasmids—A human Crkl cDNA was obtained from Dr. John Groffen (Children’s Hospital, Los Angeles, CA). In-frame deletions of amino acids 14–64, 131–179, 238–290, and 131–290, respectively, were made by polymerase chain reactions to create an SH2-, an N-terminal SH3-, a C-terminal SH3-, and both SH3-domain(s) deletion mutants (hereafter termed CrklΔSH2, CrklΔSH3(N), CrklΔSH3(C), and CrklΔSH3(N+C), respectively) (29). Crkl and Crk deletion mutants were cloned into mammalian expression vectors, pEBB and pEGFP-C (CLONTECH, Palo Alto, CA), resulting in the expression of green fluorescent protein (GFP)—Crk fusion proteins. A human Grb2 cDNA was obtained from Dr. Akio Yakamawaki (Dana-Farber Cancer Institute), and was also cloned in frame in the pEGFP-C vector. A murine c-ABL cDNA was obtained from Dr. Richard A. Van Etten (Center for Blood Research, Harvard Medical School, Boston, MA). A human C3G cDNA and a human DOCK180 cDNA were obtained from Dr. Michiyuki Matsuda (International Medical Center of Japan, Tokyo, Japan) and cloned into the pEBB vector. A hemagglutinin-tagged human Cbl cDNA in the pALTER-MAX expression vector was obtained from Dr. Hamid Band (Harvard Medical School).

* This work was supported by National Institutes of Health Grants CA36167 and DK560654 (to J. D. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dept. of Adult Oncology, Dana-Farber Cancer Inst., 44 Binney St., Boston, MA 02115. Tel.: 617-632-3360; Fax: 617-632-4388.

The abbreviations used are: GFP, green fluorescent protein; BSA, bovine serum albumin; FACS, fluorescence-activated cell sorting; SDF-1α, stromal-derived factor-1α; Ab, antibody.
Transient Transfection of Ba/F3 Cells—The murine hematopoietic cell line, Ba/F3, was cultured in RPMI 1640 containing 10% fetal calf serum and 15% WEHI3B cell conditioned medium as a source of murine interleukin-3. Ba/F3 cells were transfected with plasmids by electroporation using a Gene Pulser apparatus (Bio-Rad). For migration assays, GFP-expressing cells were purified by fluorescence-activated cell sorting 18 h after transfection using an EPICS Elite-EPS cytometer (Coulter Corp., Hialeah, FL).

Migration Assays—Migration assays were performed using Transwell plates (0.33 cm² insert growth area, 8 µm pores; Corning Coster Corp. Cambridge, MA). Both sides of the porous polycarbonate membrane were coated with human fibronectin (5 µg/ml). The lower chamber contained 600 µl of RPMI 1640 medium containing 1% bovine serum albumin (BSA). Fluorescence-activated cell sorting (FACS)-purified cells were starved in RPMI 1640 containing 1% BSA for 6 h, placed into the upper chamber (1 × 10⁵ cells/100 µl of RPMI 1640 containing 1% BSA), and allowed to migrate into the lower chamber for 6–8 h. In some experiments, recombinant human SDF-1α (R & D Systems, Minneapolis, MN) (31) was added to the lower chamber.

Integrin Cross-linking—18 h after transfection cells were removed from interlin-3 containing medium and placed in RPMI 1640 containing 1% BSA for 6 h. To induce integrin signaling, cells were suspended in phosphate-buffered saline (1 × 10⁶/ml), incubated for 10 min on ice with antibody against murine β1 integrin (HA2/5, Pharmingen, San Diego, CA) or hamster IgM as a control, washed in phosphate-buffered saline, and stimulated by cross-linking using anti-hamster IgM at 37 °C for 5 min.

Immunoblotting and Immunoprecipitation—Cells were lysed in buffer containing 50 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol, 1% IgdM at 37 °C for 5 min.

RESULTS

Overexpression of Crkl and Crk, but Not Grb2, Increases Random Cell Migration on Fibronectin-coated Surfaces—The hematopoieticU cell line Ba/F3 was selected for these studies because it is capable of migration on two-dimensional surfaces and across three-dimensional membranes, and because it expresses appropriate receptors for extracellular matrix proteins, hematopoietic growth factors, and chemokinones such as SDF-1α (the CXCR4 receptor).2 Migration assays were performed using modified Boyden chambers (Transwell membranes) coated with fibronectin on both surfaces. Crkl, mutants of Crkl, Crk, Grb2, or other test genes were expressed transiently along with appropriate controls in all cell migration experiments. To obtain cell populations in which the transgenes were overexpressed in more than 90% of cells, plasmids encoding GFP fusion proteins were transfected by electroporation, and positive cells were isolated by FACS. The protein expression of GFP fusion proteins was confirmed by immunoblotting using specific antibodies and an anti-GFP antibody. This technique had the advantage of allowing for purification of cell populations that expressed approximately equivalent amounts of each transgene, because cells were isolated that expressed approximately equivalent amounts of fluorescence by FACS.

Migration of GFP-Crkl-overexpressing cells was significantly increased (2.8-fold) compared with GFP-expressing cells (Fig. 1A). Overexpression of GFP alone had no significant effect on migration compared with untransfected cells or sham transfected cells (data not shown). To ensure that the observed effects were because of Crkl and unique to the fusion protein, Crkl and GFP were individually expressed in the same cells from different vectors, and migration experiments were repeated. Again, Crkl overexpression enhanced spontaneous migration, suggesting that the presence of the GFP tag did not alter Crkl function in this assay (data not shown). The Crkl-related adapter protein, Crkii, also increased migration but was reproducibly less effective than Crkl in these hematopoietic cells. The enhanced migration was likely to involve β1 integrins, because there was no significant migration if the Transwell membranes were not coated with fibronectin (data not shown). Also, the effects of Crkl on Transwell migration were not a general property of adapter proteins, because overexpression of Grb2 did not have any significant effects on cell migration in this system (Fig. 1B).

Both SH2 and N-terminal SH3 Domains of Crkl Are Necessary to Increase Migration—Overexpression of full-length Crkl was compared with deletion mutants in which the SH2 or SH3 domains had been deleted (Fig. 1, A and C). In contrast to full-length Crkl, overexpression of GFP-CrklΔSH2 did not alter cell migration, and overexpression of GFP-CrklΔSH3(N) significantly inhibited migration of Ba/F3 cells (Fig. 1C). Overexpression of GFP-CrklΔSH3(C) increased migration, although to a lesser degree than that of GFP-Crkl. Overexpression of GFP-CrklΔSH3(N+C) reduced migration. These results indicate that the SH2 and the N-terminal, but not the C-terminal, SH3 domains are required to enhance random migration of Ba/F3 cells. Further, because overexpression of GFP-CrklΔSH3(N) inhibited migration below the base-line value exhibited by control cells, this mutant may function as dominant-negative in a pathway required for regulating migration.

SDF-1α Overrides the Modulation of Migration by Overexpression of Crkl—The above findings support the hypothesis that Crkl may play an important role in overall random migration of hematopoietic cells on fibronectin-coated surfaces. This raised the question whether overexpression of Crkl also affects cell migration induced by chemottractant gradients. We have previously shown that Ba/F3 cells express the receptor for SDF-1α, CXCR4, and migrate avidly along an SDF-1α gradient in vitro and in vivo.3 The effect of overexpression of Crkl on SDF-1α-directed cell migration was evaluated. Adding SDF-1α into the lower chamber increased migration of all transfected cells tested. Overexpression of GFP-Crkl or GFP-CrklΔSH3(N) did not alter migration of transfected Ba/F3 cells in response to SDF-1α (data not shown).

Identification of Cbl as the Major Crkl-SH2-binding Protein after β1-integrin Ligation—The above results suggest that Crkl is likely to enhance spontaneous migration by linking to other proteins using its SH2 domain and the N-terminal SH3 domain. To look for proteins interacting with the Crkl-SH2 domain, β1 integrins were cross-linked using an anti-β1 integrin antibody, and cell lysates were subjected to anti-Crkl immunoprecipitation followed by immunoblotting with the anti-phosphotyrosine monoclonal antibody 4G10 (Fig. 2). Cross-linking β1 integrins in mock transfected cells resulted in a substantial increase in the coprecipitation of a 120-kDa phosphoprotein with Crkl. After overexpression of wild type Crkl, the associa-

2 N. Uemura, R. Salgia, M. Sattler, and J. D. Griffin, unpublished data.

3 R. Salgia, E. Quackenbush, J. Lin, N. Souchkova, M. Sattler, D. Ewanuik, K. M. Kulcher, G. Q. Daley, S. K. Kraeft, U. H. von Andrian, L. B. Chen, J.-C. Gutierrez-Ramos, A.-M. Pendergast, and J. D. Griffin, submitted.
tion of Crkl with pp120 was increased before integrin activation. Several other proteins were detected in Crkl-overexpressing cells that were not observed in mock transfected cells, including bands at approximately 140, 95, 60, and 40 kDa. The interaction of each of these proteins with Crkl, and the enhanced binding to pp120, required the SH2 domain, because cells overexpressing Crkl\textsubscript{SH2} were indistinguishable from mock transfected cells (Fig. 2).

The pp120 protein(s) coprecipitating with Crkl was found to be composed predominantly of Cbl (Fig. 3). Cbl coprecipitated with GFP-Crkl after the cross-linking of \(\beta\)-integrins (Fig. 3A). This was not affected by mutation of tyrosine 207 to phenylalanine, but Cbl did not coprecipitate with GFP-Crkl\textsubscript{SH2}, consistent with this interaction being mediated by the SH2 domain of Crkl (Fig. 3A). In contrast to the SH2 deletion, the integrin-induced association of Cbl with Crkl was only slightly reduced by deleting either SH3 domain (Fig. 3B). To determine whether Cbl was the major 120-kDa phosphoprotein induced to coprecipitate with Crkl after integrin ligation, immunodepletion using an anti-Cbl antibody was performed. Cbl immunodepletion significantly reduced the amount of pp120 coprecipitating with Crkl, suggesting that the Cbl is the major component of this band (Fig. 3C). An anti-Cas antibody was also used to determine whether Crkl coprecipitated with Cas. However, coprecipitation of Cas with Crkl in Crkl-overexpressing cells was at the lower limit of detection. Further, in these cells, cross-linking of \(\beta\) integrins induced only marginal tyrosine phosphorylation of Cas (data not shown). Thus, integrin signaling in Ba/F3 cells induces Crkl-SH2 to bind primarily to Cbl.

In Crkl-overexpressing cells, a small amount of Cbl copre-
Precipitated with Crkl even in resting, non-adherent, cells. This finding led us to examine whether overexpression of Crkl prolongs tyrosine phosphorylation of Cbl by blocking access of phosphatases to phosphotyrosine residues. Cbl was rapidly tyrosine-phosphorylated after integrin ligation, followed by nearly complete dephosphorylation within 60 min in either mock or Crkl-transfected cells (Fig. 3D). This result suggests that Crkl overexpression does not affect the kinetics of tyrosine phosphorylation or dephosphorylation of Cbl after integrin ligation. The increased association of Cbl with Crkl in resting, Crkl-overexpressing cells may be due to the fact that Cbl is relatively abundant in the cell and that the formation of a Cbl-Crkl complex is limited by the available Crkl. Interestingly, CrklΔSH3(N) competed with wild type endogenous Crkl for binding to phospho-Cbl (Fig. 3E). Anti-Crkl monoclonal antibody 2–2, which recognizes an epitope in the N-terminal SH3 domain, was used to specifically detect binding of endogenous, full-length Crkl to Cbl. Overexpression of CrklΔSH3(N) reduced the integrin-induced association of Cbl with endogenous Crkl as compared with mock transfected cells (Fig. 3E). One explanation for these results is that both the level of Crkl and the level of Cbl tyrosine phosphorylation are important to migration signaling. The ability of CrklΔSH3(N) to compete with full-length Crkl for binding to phospho-Cbl...
may contribute to the dominant-negative effects this Crkl mutant had on spontaneous cell migration.

Because Cbl was the major protein interacting with the CrklΔSH2 domain in Ba/F3 cells after integrin cross-linking, the effect of overexpression of Cbl (approximately 3-fold) on cell migration was examined. Overexpression of Cbl with either GFP or GFP-Crkl did not have any additional significant effect on cell migration in multiple experiments (data not shown). It is possible that Cbl is not involved in regulating migration despite interacting directly with Crkl, that the amount of endogenous Cbl is not rate-limiting for migration, or that overexpressing Cbl does not increase the amount of phospho-Cbl available for binding to Crkl.

Some of the other tyrosine phosphoproteins that are coprecipitated with Crkl after integrin cross-linking were identified. The protein at 140 kDa was identified as SHIP (33) (data not shown). The protein at 40 kDa was tyrosine-phosphorylated Crkl (data not shown). In a longer exposure, a 70-kDa protein detected in Crkl immune complexes after integrin cross-linking in Crkl-overexpressing cells was identified as the SH2 containing tyrosine phosphatase SHP-2 (34) (data not shown). It is also possible that one or more of these proteins play a role in the regulation of migration, and further studies are underway to address this point.

C3G Cooperates with Crkl to Increase Cell Migration—Because overexpression of Crkl enhanced migration and overexpression of CrklΔSH3(N) inhibited migration, it is likely that one or more CrklΔSH3 domain-binding proteins also contributes to the regulation of migration in Ba/F3 cells. Several molecules have been reported to bind to SH3 domains of Crk family proteins including c-ABL, SOS, C3G, EPS15, and DOCK180 (19, 20, 28, 35–37). Among these molecules, several have previously been linked to cytoskeletal regulation. The guanine nucleotide exchange factor C3G has been found to be abundant in Crkl complexes and is reported to play an important role in the signaling through Crk family proteins (2, 18, 20, 27, 35, 36, 38). The kinase activity and localization of c-ABL have been reported to be regulated by integrin activation (39). DOCK180 has been found to regulate cytoskeletal function by cooperating with the Crk-Cas complex (40, 41). However, DOCK180 is not expressed in hematopoietic cells, although a homologue may be.

To examine the role of some of these proteins, we overexpressed C3G, c-ABL, or DOCK180 and determined if they cooperated with Crkl to increase cell migration. As expected, overexpression of C3G, c-ABL, or DOCK180 with Crkl led to the detection of increased complexes of Crkl with C3G, ABL, or DOCK180, respectively (Fig. 4A). Cotransfection of C3G and GFP-Crkl further increased cell migration significantly as compared with transfection of GFP-Crkl alone, whereas cotransfection of c-ABL or DOCK180 did not have any further effect on migration of Crkl-overexpressing cells (Fig. 4B). Interestingly, overexpression of C3G alone did not have any significant effects on cell migration. However, C3G cooperated with Crkl to enhance cell migration (Fig. 4C). These results indicate that the Crkl-C3G complex may play an important role in increasing cell migration and that Crkl, but not C3G, is rate-limiting for this effect.

The association of C3G with Crkl in cells overexpressing deletion mutants of Crkl was also examined. Overexpression of wild type Crkl, CrklΔSH2, and CrklΔSH3(C), but not CrklΔSH3(N), led to increased formation of Crkl-C3G complexes, consistent with the hypothesis that C3G binds to Crkl through the N-terminal SH3 domain, further suggesting that this complex is important in migration (Fig. 5A). Because we found that Cbl was the most abundant Crkl-SH2-binding part-

![Fig. 4. C3G cooperates with Crkl to increase cell migration. A, Ba/F3 cells were co-transfected with pEBB-Crkl (10 μg) and either pEBB-mock, pEBB-c-ABL, pEBB-C3G, or pEBB-DOCK180 (20 μg each). Cell lysates were prepared 18 h after transfection and were immunoprecipitated with anti-Crkl Ab 5–6 or preimmune mouse serum as a control and then immunoblotted with anti-ABL Ab, anti-C3G Ab, or anti-DOCK180 Ab. Immunoprecipitation and immunoblotting with anti-Crkl Ab (data not shown) detected a comparable amount of Crkl in each transfaction. B, the migration ratio was determined in cells transfected with pEBB-Crkl and cotransfected with mock, c-ABL, C3G, or DOCK180 vectors as indicated. C, Ba/F3 cells were co-transfected with pEGFP-control or pEGFP-C-Crkl (20 μg each) plus either pEBB or pEBB-C3G (20 μg each). For B and C, GFP-expressing cells were enriched to >90% purity by FACS 18 h after transfection, cultured in serum- and growth factor-free medium for 6 h, and then tested for migration as described above. Shown are results from two independent experiments (mean ± S.D.). Each experiment was performed using triplicate wells.](image-url)
Crkl Involvement in Cell Migration

FIG. 5. C3G-Crkl complexes interact with Cbl after β1, integrin cross-linking. Ba/F3 cells were transfected with pEBB, pEBB-Crkl, pEBB-CrklΔSH2, pEBB-CrklΔSH3(N), or pEBB-CrklΔSH3(C) as indicated. Starvation and β1 integrin cross-linking were performed as described above. A, lysates from transfected cells were immunoprecipitated with anti-CrkI Ab 5–6 and immunoblotted with anti-C3G Ab. B, lysates from transfected cells were immunoprecipitated with anti-Cbl Ab and immunoblotted with anti-C3G Ab or anti-Cbl Ab.

DISCUSSION

Cell migration is a fundamentally important process that is not well understood at the level of signal transduction. During development, many precursor cells migrate in response to specific stimuli such as a gradient of chemoattractant molecules. In addition, numerous cells of the adult organism are also capable of extensive migration, particularly cells involved in wound healing and those of the hematopoietic and immune systems. Migration involves a carefully orchestrated series of events that allows cells to extend a cellular protrusion, attach to extracellular matrix proteins through integrins or other adhesive molecules, move in the direction of attachment, and simultaneously detach at the trailing edge (42). Whereas much is known about the events regulating changes in actin structure during cell movement, little is known about the signals from integrins or other receptors that initiate migration.

Members of the Crk family have previously been shown to participate in signaling pathways activated by integrins and have been further linked to the control of adhesion and migration (2, 18, 29, 30, 41, 43). For example, after integrin cross-linking in hematopoietic cells, Crkl is rapidly tyrosine-phosphorylated and binds through its SH2 domain to other cellular tyrosine phosphoproteins (29). The SH3 domains of Crk and Crkl bind constitutively to an overlapping set of cellular proteins that have also been linked in some cases to integrin signaling, including c-ABL, DOCK180, and C3G (17). Overexpression of either CrkII or Crkl has also been shown to increase short-term integrin-mediated adhesion of 32D and Ba/F3 hematopoietic cells to fibronectin-coated surfaces (2, 29). CrkII, but not Crkl, has also been linked to migration (44). Klemke et al. (44) showed that CrkII-Cas complexes correlated with enhanced migration of the FG-M carcinoma cell line and that dominant-negative mutants of either CrkII or Cas inhibited spontaneous migration of this cell line. Thus, it is likely that both Crkl and CrkII participate in signal transduction initiated by β integrins and, as adapter proteins, may serve to link or transport critical signaling molecules that regulate adhesion and possibly migration.

The goal of the current studies was to develop a model system in which the effects of specific signaling proteins activated by β integrins could be studied in hematopoietic cells. Compared with more commonly used fibroblastic or epithelial cell lines, hematopoietic cells display higher levels of motility and migration using in vitro assays. The Ba/F3 cell line was selected for this study because its expression of integrin receptors and other adhesion molecules is well characterized.2 Cross-linking of β integrins on Ba/F3 cells was found to cause rapid tyrosine phosphorylation of Crkl and association of Crkl with other tyrosine phosphoproteins, notably Cbl. Although CrkII is also expressed at high levels in Ba/F3 cells, compared with Crkl, CrkII was only minimally tyrosine-phosphorylated after integrin cross-linking and was less involved in forming new protein-protein complexes. Transient overexpression of intact Crkl enhanced spontaneous migration on fibronectin-coated surfaces but not on uncoated surfaces. The role of the SH2 and SH3 domains of Crkl were further explored by creating specific deletion mutants and transiently overexpressing these mutants as GFP fusion proteins in Ba/F3 cells. Interestingly, deletion of the SH2 domain resulted in loss of the migration-enhancing properties of intact Crkl but did not, at least at the levels of expression achieved in the present studies in Ba/F3 cells, generate a dominant-negative mutant. In contrast, deletion of the N-terminal, but not the C-terminal, SH3 domain both resulted in loss of the migration-enhancing effects of Crkl and reduced spontaneous migration below control levels. However, the effects of overexpressing Crkl were limited to spontaneous migration, because after the addition of a strong chemoattractant, the chemokine SDF-1α, overexpression of Crkl or CrkII mutants had no additional effects.

The results presented here suggest that Crkl plays a similar role in Ba/F3 cells to that of CrkII in epithelial carcinoma cells (44). As shown here, overexpression of CrkII in Ba/F3 cells also enhanced migration, but to a substantially less degree than did Crkl. These results suggest that Crkl may be more significantly involved in integrin signaling and migration in hematopoietic cells, whereas CrkII is more important in non-hematopoietic cells. Interestingly, overexpression of Crkl, but not CrkII, has been reported to transform fibroblasts (45, 46). Overall, our results significantly extend the understanding of the functions of Crkl and provide additional evidence that Crkl is involved in signaling pathways that involve integrins.

Potential mechanisms to explain the migration-enhancing effects of Crkl were explored by identifying the major cellular proteins that associate with the CrklΔSH2 domain after integrin ligation and by overexpression studies of some of the known proteins that constitutively bind to the Crkl SH3 domains. The major Crkl SH2-binding phosphoprotein was identified as Cbl, a proto-oncogene with multiple YYXP phosphorylation sites that serve as CrklΔSH2-binding sites (47). v-Cbl is the transforming gene of the mouse NS-1 virus, which causes lymphomas in mice (48). Cbl is the mammalian homologue of the Sli-1 gene in Caenorhabditis elegans that is involved in negative regulation of the epidermal growth factor receptor (49). Targeted disruption of the Cbl locus is the dominant-negative mutant of either CrkII or Cas inhibited spontaneous migration of this cell line. Thus, it is likely that both Crkl and CrkII participate in signal transduction initiated by β integrins and, as adapter proteins, may serve to link or transport critical signaling molecules that regulate adhesion and possibly migration.

The goal of the current studies was to develop a model system in which the effects of specific signaling proteins activated by β integrins could be studied in hematopoietic cells. Compared with more commonly used fibroblastic or epithelial cell lines, hematopoietic cells display higher levels of motility and migration using in vitro assays. The Ba/F3 cell line was selected for this study because its expression of integrin receptors and other adhesion molecules is well characterized.2 Cross-linking of β integrins on Ba/F3 cells was found to cause rapid tyrosine phosphorylation of Crkl and association of Crkl with other tyrosine phosphoproteins, notably Cbl. Although CrkII is also expressed at high levels in Ba/F3 cells, compared with Crkl, CrkII was only minimally tyrosine-phosphorylated after integrin cross-linking and was less involved in forming new protein-protein complexes. Transient overexpression of intact Crkl enhanced spontaneous migration on fibronectin-coated surfaces but not on uncoated surfaces. The role of the SH2 and SH3 domains of Crkl were further explored by creating specific deletion mutants and transiently overexpressing these mutants as GFP fusion proteins in Ba/F3 cells. Interestingly, deletion of the SH2 domain resulted in loss of the migration-enhancing properties of intact Crkl but did not, at least at the levels of expression achieved in the present studies in Ba/F3 cells, generate a dominant-negative mutant. In contrast, deletion of the N-terminal, but not the C-terminal, SH3 domain both resulted in loss of the migration-enhancing effects of Crkl and reduced spontaneous migration below control levels. However, the effects of overexpressing Crkl were limited to spontaneous migration, because after the addition of a strong chemoattractant, the chemokine SDF-1α, overexpression of Crkl or CrkII mutants had no additional effects.

The results presented here suggest that Crkl plays a similar role in Ba/F3 cells to that of CrkII in epithelial carcinoma cells (44). As shown here, overexpression of CrkII in Ba/F3 cells also enhanced migration, but to a substantially less degree than did Crkl. These results suggest that Crkl may be more significantly involved in integrin signaling and migration in hematopoietic cells, whereas CrkII is more important in non-hematopoietic cells. Interestingly, overexpression of Crkl, but not CrkII, has been reported to transform fibroblasts (45, 46). Overall, our results significantly extend the understanding of the functions of Crkl and provide additional evidence that Crkl is involved in signaling pathways that involve integrins.

Potential mechanisms to explain the migration-enhancing effects of Crkl were explored by identifying the major cellular proteins that associate with the CrklΔSH2 domain after integrin ligation and by overexpression studies of some of the known proteins that constitutively bind to the Crkl SH3 domains. The major Crkl SH2-binding phosphoprotein was identified as Cbl, a proto-oncogene with multiple YYXP phosphorylation sites that serve as CrklΔSH2-binding sites (47). v-Cbl is the transforming gene of the mouse NS-1 virus, which causes lymphomas in mice (48). Cbl is the mammalian homologue of the Sli-1 gene in Caenorhabditis elegans that is involved in negative regulation of the epidermal growth factor receptor (49). Targeted disruption of the Cbl locus is the dominant-negative mutant of either CrkII or Cas inhibited spontaneous migration of this cell line. Thus, it is likely that both Crkl and CrkII participate in signal transduction initiated by β integrins and, as adapter proteins, may serve to link or transport critical signaling molecules that regulate adhesion and possibly migration.
Cbl has also been associated with integrin signaling and the cytoskeleton. Cbl is prominently tyrosine-phosphorylated after integrin-mediated adhesion in many cell types (27, 52). It is localized to the cytoplasm and has been shown to interact directly with several cytoskeletal proteins, although the significance of these interactions is unclear (53). Oncogenic forms of Cbl abrogate anchorage dependence of fibroblasts for proliferation (52). After integrin ligation, Cbl forms complexes with other proteins involved in cytoskeletal functional regulation, including Src and PI3K (54). However, the role of Cbl in regulating migration is unclear. In our study, overexpression of Cbl in Ba/F3 cells did alter short-term adhesion or spontaneous migration, although Cbl is already a relatively abundant protein in these cells. The association and activation of PI3K by Cbl may provide a pathway that links Crkl to adhesion and migration, and further studies are warranted to explore this possibility.

A second well known target of the Crkl SH2 domain is Cas, which is likely to be involved in integrin signaling and regulation in some cell types. Cas was discovered as a Crk-binding protein, and has multiple YXXP motifs for binding Crk SH2 domains. Like Cbl, Cas functions as a docking protein after integrin ligation, interacting with 14–3–3 proteins (53, 55). In transient expression assays, Crk-Cas complexes have been shown to activate c-Jun kinase through activation of p21^{ras} (56). In these cells, activation of Rac was believed to occur through DOCK180, which was brought to Cas by Crkii (41). Homologues of DOCK180 in both Drosophila melanogaster (Myoblast Cell) and C. elegans (ced-5) have also been linked to the formation of cell extensions, migration, or both (57). Activation of Rac is frequently associated with enhanced migration and adhesion and would explain the enhanced migration of cells transiently overexpressing Crk (41). However, DOCK180 is not expressed at detectable levels in Ba/F3 and many other hematopoietic cell lines, suggesting that either a related protein exists or that there are other pathways involved. Of note, v-Crk does not activate Rac in PC12 cells but does activate Rho, thereby inducing cell spreading and focal adhesion formation (58).

Overexpression of another Crkl SH3-binding protein, C3G, did enhance spontaneous migration but only when expressed with Crkl. C3G is a guanine nucleotide exchange factor for the small G protein Rap1, also known as smg p21 or Krev-1, originally identified as an anti-oncogenic protein capable of reversing transformation in some cell types. Cas was discovered as a Crk-binding protein in hematopoietic cells transiently overexpressing Crkl (41). However, DOCK180 activation of Rac is frequently associated with enhanced migration or membrane (44). In hematopoietic cells, Crkl-Crkii complexes predominate, but downstream signals are unclear. C3G is proposed as one pathway that can induce migration through this complex. Crkl is likely to link C3G to Cbl after integrin cross-linking, because Cbl is the predominant target of the Crkl SH2 domain in hematopoietic cells. In cells transformed by tyrosine kinase oncoproteins such as p210BCR-ABL, Crkl and Cbl are constitutively tyrosine-phosphorylated and form a permanent complex independent of integrin activation (17, 25). This complex is likely to contribute to the abnormal adhesion and migration exhibited by BCR/ABL transformed hematopoietic cells.

REFERENCES

1. ten Hoeve, J., Morris, C., Heisterkamp, N., and Groffen, J. (1993) Oncogene 8, 2469–2474
2. Arai, A., Nosaka, Y., Kohsaka, H., Miyasaka, N., and Miura, O. (1999) Blood 93, 3713–3722
3. Barber, D. L., Mason, J. M., Fukazawa, T., Reedquist, K. A., Druker, B. J., Band, H., and D’Andrea, A. D. (1997) Blood 89, 3166–3174
4. Bonnafous, V. A., Freeman, G. J., Berezovskaya, A., Barber, D. L., and Nadler, S. E. (1997) Science 277, 1314–1317
5. Butler, A. A., Yakar, S., Gewell, I. H., Karas, M., Okubo, Y., and LeRoith, D. (1998) Comp Biochem Physiol 121, 19–26
6. Fozzato, S. M., Posern, G., Westwood, J., Kardinal, C., Sakkah, D., Zheng, J., and Knudsen, S. B. (1998) J Cell Physiol 177, 535–552
7. Geshert, F., Garbay, C., and Bertolgi, J. (1998) J Biol Chem 273, 3886–3993
8. Reedquist, K. A., Fukazawa, T., Panchamoorthy, G., Langdon, W. Y., Shoessin, E., Druker, B. J., and Band, H. (1996) J Biol Chem 271, 8435–8442
9. de Jong, R., van Wijck, A., Haataja, L., Heisterkamp, N., and Groffen, J. (1997) J Biol Chem 272, 32649–32655
10. Bhat, A., Johnson, K. J., Oda, T., Corbin, A. S., and Druker, B. J. (1998) J Biol Chem 273, 32360–32368
11. Heaney, C., Koliabka, K., Bhat, A., Oda, T., Ohno, S., Fanning, S., and Druker, B. J. (1997) Blood 89, 318–340
12. Koliabka, K. S., Bhat, A., Heaney, C., Oda, T., and Druker, B. J. (1999) Leuk Lymphoma 33, 119–126
13. Nichols, G. L., Raines, M. A., Vera, J. C., Lacomis, L., Tempst, P., and Golde, D. W. (1994) Blood 84, 2912–2919
14. Oda, T., Heaney, C., Hagepan, J. R., Okuda, K., Griffin, J. D., and Druker, B. J. (1994) J Biol Chem 269, 22925–22929
15. Salgia, R., Uemura, N., Okuda, K., Li, J. L., Fissik, E., Sattler, M., de Jong, R., Druker, B., Heisterkamp, N., Chen, L. B., and Griffin, J. D. (1995) J Biol Chem 270, 29145–29150
16. ten Hoeve, J., Arlinghaus, R. B., Guo, J. Q., Heisterkamp, N., and Groffen, J. (1994) Blood 84, 1731–1740
17. Uemura, N., Salgia, R., Li, J. L., Fissik, E., Sattler, M., and Griffin, J. D. (1997) Leukemia (Baltimore) 11, 376–385
18. Azorin, A., Manie, S. N., Law, P. S., Canty, T., Hagaybhi, N., Druker, B. J., Salgia, R., Golemis, E. A., and Freedman, A. S. (1997) Leuk & Lymphoma 28, 65–72
19. Feller, S. M., Knudsen, B., and Hanafusa, H. (1995) Oncogene 10, 1465–1473
20. Kolligs, F. T., de Jong, R., Park, R. K., Liu, Y., Heisterkamp, N., Groffen, J., and Durden, D. L. (1998) J Immunol 161, 5555–5563
21. Koval, A. P., Karas, M., Zick, Y., and LeRoith, D. (1998) J Biol Chem 273, 14780–14787
22. Ling, P., Yao, Z., Meyer, C. F., Wang, X. S., Oehrli, W., Feller, S. M., and Tan, T. H. (1999) Mol Cell Biol 19, 1359–1368
23. Manie, S. N., Beck, A. R. P., Azorin, A., Law, P. S., Canty, T., Hirai, H., Druker, B. J., Avraham, H., Hagaybhi, N., Sattler, M., Salgia, R., Griffin, J. D., Golemis, E. A., and Freedman, A. S. (1997) J Biol Chem 272, 4230–4236
24. Ota, J., Kimura, F., Sato, K., Wakimoto, N., Nakamura, Y., Nagata, S., Suzu, S., Yamada, M., Shimamura, N., and Motoyoshi, K. (1998) Biochem Biophys Res Commun 252, 779–786
25. Sattler, M., Salgia, R., Okuda, K., Uemura, N., Durstln, M. A., Fissik, E., Xu, G., Asano, J. L., Prasad, K. V., and Griffin, J. D. (1996) Oncogene 12, 839–846
26. Salgia, R., Fissik, E., Sattler, M., Li, J. L., Uemura, N., Wong, W.-K., Burky, S. A., Hirai, H., Chen, L. B., and Griffin, J. D. (1996) J Biol Chem 271, 25198–25203
27. Sattler, M., Salgia, R., Shrikhande, G., Verma, S., Uemura, N., Law, S. F., Golemis, E. A., and Druker, J. J. (1997) J Biol Chem 272, 4320–4326
28. Ota, J., Kimura, F., Sato, K., Wakimoto, N., Nakamura, Y., Nagata, S., Suzu, S., Yamada, M., Shimamura, N., and Motoyoshi, K. (1998) Biochem Biophys Res Commun 252, 779–786
29. Sattler, M., Salgia, R., Okuda, K., Uemura, N., Durstln, M. A., Fissik, E., Xu, G., Asano, J. L., Prasad, K. V., and Griffin, J. D. (1996) Oncogene 12, 839–846
30. Salgia, R., Posern, G., Weh, C. K., Rapp, U. R., and Feller, S. M. (1998) J Biol Chem 273, 24297–24300
31. Ware, M. D., Rostem, P., Damen, J. E., Liu, L., Humphries, R. K., and Krystal, G. (1996) Blood 88, 2833–2840
34. Sattler, M., Salgia, R., Shrikhande, G., Verma, S., Choi, J. L., Rohrschneider, L. R., and Griffin, J. D. (1997) *Oncogene* **15**, 2379–2384
35. de Jong, R., van Wijk, A., Heisterkamp, N., and Groffen, J. (1998) *Oncogene* **17**, 2805–2810
36. Ichiba, T., Kuraishi, Y., Sakai, O., Nagata, S., Groffen, J., Kurata, T., Hattori, S., and Matsuda, M. (1997) *J. Biol. Chem.* **272**, 22215–22220
37. Posern, G., Zheng, J., Kruisbeek, A. M., Muller, K. B., Moll, J., Shishido, T., Cowburn, D., Cheng, G., Wang, B., Kruh, G. D., Burrell, S. K., Jacobson, C. A., Lenz, D. M., Zamore, D. J., Adermann, K., Hanafusa, H., and Feller, S. M. (1998) *Oncogene* **16**, 1903–1912
38. Gotoh, T., Hattori, S., Nakamura, S., Kitayama, H., Noda, M., Takai, Y., Kaibuchi, K., Matsu, H., Hatase, O., Takahashi, H. (1995) *Mol. Cell. Biol.* **15**, 6746–6753
39. Lewis, J. M., Baskaran, R., Taagepera, S., Schwartz, M. A., and Wang, J. Y. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 15174–15179
40. Kiyokawa, E., Hashimoto, Y., Kurata, T., Sugimura, H., and Matsuda, M. (1998) *J. Biol. Chem.* **273**, 24479–24484
41. Kiyokawa, E., Hashimoto, Y., Kobayashi, S., Sugimura, H., Kurata, T., and Matsuda, M. (1998) *Genes Dev.* **12**, 3331–3336
42. Lauffenburger, D. A., and Horwitz, A. F. (1996) *Cell* **84**, 359–369
43. Polte, T. R., and Hanks, S. K. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 10678–10682
44. Klemke, R. L., Leng, J., Molander, R., Brooks, P. C., Vuori, K., and Cheresh, D. A. (1998) *J. Cell Biol.* **140**, 961–972
45. Senechal, K., Heaney, C., Drucker, B., and Sawyer, C. L. (1998) *Mol. Cell. Biol.* **18**, 5082–5090
46. Senechal, K., Halpern, J., and Sawyer, C. L. (1996) *J. Biol. Chem.* **271**, 23255–23261
47. Andoniou, C. E., Thien, C. B., and Langdon, W. Y. (1996) *Oncogene* **12**, 1981–1989
48. Langdon, W. Y., Hartley, J. W., Klinken, S. P., Rucet, S. K., and Morse, H. C. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 1168–1172
49. Yoon, C. H., Lee, J., Jongeward, G. D., and Sternberg, P. W. (1995) *Science* **269**, 1102–1105
50. Murphy, M. A., Schnall, R. G., Venter, D. J., Barnett, L., Berenson, I., Thien, C. B., Langdon, W. Y., and Bowtell, D. D. (1998) *Mol. Cell. Biol.* **18**, 4872–4882
51. Levkovitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguin, L., Geiger, B., and Yarden, Y. (1998) *Genes Dev.* **12**, 3663–3674
52. Ojaniemi, M., Langdon, W. Y., and Vuori, K. (1998) *Oncogene* **16**, 3159–3167
53. Robertson, H., Langdon, W. Y., Thien, C. B., and Bowtell, D. D. (1997) *Biochem. Biophys. Res. Commun.* **240**, 46–50
54. Ojaniemi, M., Martin, S. S., Dolfi, P., Olefsky, J. M., and Vuori, K. (1997) *J. Biol. Chem.* **272**, 3780–3787
55. Liu, Y., Liu, Y. C., Meller, N., Giampa, L., Elly, C., Doyle, M., and Altman, A. (1999) *J. Immunol.* **162**, 7095–7101
56. Dolfi, F., Garcia-Guzman, M., Ojaniemi, M., Nakamura, H., Matsuda, M., and Vuori, K. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 15394–15399
57. Wu, Y. C., and Horvitz, H. R. (1998) *Nature* **392**, 501–504
58. Altun-Gultekin, Z. F., Chandriani, S., Bougeret, C., Ishizaki, T., Narumiya, S., de Graaf, P., Van Bergen en Henegouwen, P., Hanafusa, H., Wagner, J. A., and Birge, R. B. (1998) *Mol. Cell. Biol.* **18**, 3044–3058
59. Kitayama, H., Sugimoto, Y., Matsuzaki, T., Ikawa, Y., and Noda, M. (1989) *Cell* **56**, 77–84
60. Kirsh, K. H., Georgescu, M. M., and Hanafusa, H. (1998) *J. Biol. Chem.* **273**, 25673–25679