The CRKL Adaptor Protein Transforms Fibroblasts and Functions in Transformation by the BCR-ABL Oncogene*

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The CRKL adaptor protein was recently identified as a substrate for the BCR-ABL tyrosine kinase in patients with chronic myelogenous leukemia, but its function is unknown. Here we report that CRKL is phosphorylated when overexpressed, activates RAS and JUN kinase signaling pathways, and transforms fibroblasts in a RAS-dependent fashion. We examined the potential role of CRKL in BCR-ABL function by deleting the CRKL binding site in BCR-ABL. This mutant BCR-ABL protein shows a 50% reduction in fibroblast transforming activity. The GRB2 adaptor protein has previously been implicated in this pathway, presumably linking BCR-ABL to RAS. To address the relative roles of CRKL and GRB2 in this system, we compared BCR-ABL mutants with defects in binding to one or both adaptors. Where each single mutant showed a 2-3-fold loss in transforming activity, the double mutant showed a 15-fold reduction, suggesting that GRB2 and CRKL both contribute to BCR-ABL transformation. These results demonstrate the oncogenic potential of CRKL and provide functional evidence that CRKL plays a role in fibroblast transformation by BCR-ABL in conjunction with other adaptor proteins.

Receptor tyrosine kinases (RTKs) function in part by activation of Ras. This activation is mediated by adaptor molecules such as GRB2 and SHC, which physically link the RTK to guanine nucleotide exchange factors that activate Ras (1, 2). Adaptor molecules have no catalytic activity but contain SH2 and SH3 (Src homology) domains, that mediate protein-protein interactions with phosphotyrosine (3) and proline (4) residues, respectively. Genetic studies have confirmed a critical role for these adaptors in RTK signal transduction (5, 6). Loss of function mutations in the Drosophila and Caenorhabditis elegans homologues of GRB2 disrupt signaling by the Sevenless and Let-23 RTKs, respectively, and the mutant phenotypes are specifically rescued by expression of wild-type GRB2 (6–9).

The CRK genes, originally identified as cellular homologues of the avian retroviral oncogene for v-CRK (10, 11), also encode adaptor molecules. CRK-I and CRK-II are alternative RNA splicing products of the c-CRK gene and contain one SH2 domain and either one (CRK-I) or two (CRK-II) SH3 domains. v-CRK contains retroviral GAG sequences fused to the SH2 and SH3 domains of CRK-I. Oncogenic transformation by v-CRK is associated with elevated levels of phosphotyrosine in cells, presumably due to assembly of activated signaling complexes containing a cellular tyrosine kinase (10, 12). The normal function of the CRK proteins is unclear. Binding studies have shown that the N-terminal SH3 domain of c-CRK binds to the guanine nucleotide exchange factors SOS and C3G (13), providing a potential link to Ras activation. The same domain also binds the c-ABL tyrosine kinase, and c-CRK is phosphorylated by c-ABL (14, 15). Structural studies suggest that c-CRK is regulated by an intramolecular interaction between its SH2 domain and tyrosine 221, which is specifically phosphorylated by c-ABL (16). The SH2 domain of v-CRK binds to specific tyrosine-phosphorylated proteins such as p130 CAS, CBL, and Paxillin (17–19). The functions of CAS and CBL are unknown, but Paxillin is localized to focal adhesion plaques (20, 21), perhaps linking CRK to adhesion molecules or the cytoskeleton (22). A new CRK family member, CRKL, was recently isolated as a phosphotyrosine substrate in leukemia cells from patients expressing the BCR-ABL fusion protein (23–25). Similar to CRK-II, CRKL contains one SH2 domain and two SH3 domains and appears to share the same range of binding properties, including interaction with SOS, C3G, c-ABL (13), and BCR-ABL (25).

BCR-ABL encodes a constitutively active cytoplasmic tyrosine kinase created by the Philadelphia chromosome translocation in patients with chronic myelogenous leukemia (CML) (26). BCR-ABL transforms fibroblasts (27) and hematopoietic cells (28) in culture and causes leukemias in mice (29–32). Transformation is kinase-dependent (33) and results from the activation of multiple signaling pathways (34), including those involving RAS (35–37), MYC (38), and JUN (39). As with RTKs, it is believed that adaptor proteins mediate the connection between BCR-ABL and Ras. Evidence for this model includes observations that (i) GRB2 and SHC are associated with BCR-ABL in transformed cells (35, 40, 41), (ii) the GRB2 binding site in BCR-ABL is required for full activity in fibroblast transformation assays (35), and (iii) overexpression of SHC restores transformation to this GRB2 binding mutant (42).

Similar to GRB2 and SHC, CRKL binds to BCR-ABL (23, 25). CRKL is a substrate for BCR-ABL in CML cells, and BCR-ABL is the only stimulus known to activate CRKL phosphorylation (24). However, the function of CRKL and its role in BCR-ABL transformation are unknown. In this report we use a well characterized fibroblast model to examine the biological activity of CRKL and its role in BCR-ABL transformation. We show that CRKL becomes phosphorylated when overexpressed, activates Ras-dependent and JNK pathways, and transforms fibroblasts, thereby demonstrating its oncogenic potential. We also show that CRKL plays a critical role as a BCR-ABL substrate.
because a BCR-ABL mutant that no longer binds CRKL has attenuated transforming activity in fibroblasts. CRKL and GRB2 both function in BCR-ABL transformation in a non-overlapping manner because a double mutant BCR-ABL incapable of binding either adaptor protein shows a greater deficit in transformation assays than either single mutant. These findings show that CRKL is a functionally relevant tyrosine kinase substrate and suggest that multiple adaptors contribute to transformation in a combinatorial manner.

MATERIALS AND METHODS

Plasmids—A full-length cDNA of the coding region of CRKL was isolated from poly(A) RNA from K562 cells by PCR using primers derived from the published sequence (43). The resulting cDNA was confirmed by sequencing and was subcloned into the pSRaMSVtkNeo retrovirus vector (44). pSRaMSVtkNeo p185BCR-ABLwt (44) and pSRaMSVtkNeo Asn-17 e-Ha-RAS (37) have been described previously. To construct a p185BCR-ABL mutant that lacks the ability to bind CRKL, an in-frame deletion of amino acids 925–975, spanning the previously identified c-CRK binding site in c-ABL (15), was made by PCR. p185BCR-ABL Y177F was kindly provided by A. M. Pendergast (Duke University). The double mutant p185BCR-ABL Y177F/925–975 was made by swapping a BsrGI to HindIII restriction fragment from BCR-ABL Y177F/925–975 with BCR-ABL Y177F. The p4XCAT plasmid containing the RAS-responsive ETS/AP-1 promoter element (45) (provided by B. Wasylk, INSERM) was used for transcription activation studies as described previously (35).

Protein Analysis—For labeling studies, cells were incubated in medium containing 1 μCi/ml orthophosphate and lysed in 10 mM sodium phosphate, pH 7.0, 1% Triton X-100, 150 mM NaCl, 100 μM/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 mM NaVO4, 50 mM NaF. CRKL protein was immunoprecipitated with 10 μl of CRKL antiserum (Santa Cruz Biotechnology) and separated by SDS-PAGE and visualized by ECL (Amersham) or subjected to autoradiography. The same filter was also analyzed by immunoblot with CRKL antisera to identify the position of unphosphorylated CRKL (data not shown).

PAGE assays and can be distinguished by anti-CRKL immunoblot analysis due to a band shift (24, 25). We obtained similar results in 293T cells transfected with a BCR-ABL expression plasmid (Fig. 1A, lane 3) compared to a NEO control (Fig. 1A, lane 1). When cells were transfected with the CRKL expression vector, we consistently observed three bands immunoreactive with antisera specific for CRKL (Fig. 1A, lane 2). These included (i) one band (lowest) which comigrates with the most prominent endogenous CRKL band observed in NEO-transfected cells (Fig. 1A, lane 1), (ii) a second, more prominent band (middle), which comigrates with the upper band in cells transfected with BCR-ABL, and (iii) a third band (top) of even slower mobility. These same three bands were also seen in cells transfected with CRKL and BCR-ABL; however, the intensity of the upper band was greatly enhanced (Fig. 1A, lane 4).

Next, we asked if these slower mobility bands represent phosphorylated forms of CRKL by performing orthophosphate labeling experiments. As expected, CRKL immunoprecipitated from cells transfected with BCR-ABL showed a large increase in phosphate content (Fig. 1B, lane 3) when compared to the NEO-transfected control (Fig. 1B, lane 1). Anti-CRKL immunoblot analysis performed using the same filter confirmed that the prominent, phosphate-labeled band seen in cells transfected with BCR-ABL is the slower mobility CRKL band, as previously reported (24, 25). In cells overexpressing CRKL, two phosphate-labeled bands were seen (Fig. 1B, lane 2), one of similar mobility to that observed in cells transfected with BCR-ABL and a second slower mobility band. This upper band was labeled even more prominently in cells co-transfected with both CRKL and BCR-ABL (Fig. 1B, lane 4). Phosphotyrosine immunoblot analysis confirmed that the slower migrating bands contained phosphotyrosine (data not shown). Based on these results, we conclude that CRKL becomes phosphorylated when overexpressed, similar to the effects induced by BCR-ABL. Differences in the mobility of phosphorylated forms of CRKL induced by BCR-ABL versus CRKL overexpression may result from differences in the stoichiometry and sites of phosphorylation and will require further study.

CRKL Activates Intracellular Signaling Pathways Involving RAS and JUN Kinase—Because overexpression of CRKL was...
sufficient to activate its phosphorylation, we next asked if CRKL overexpression mimics the effects of BCR-ABL on intracellular signaling pathways. BCR-ABL is known to activate pathways involving RAS (34, 36, 47) and JNK (39). We tested the ability of CRKL to activate these pathways in Rat-1 fibroblasts, which have been extensively utilized for studies of BCR-ABL signal transduction (27, 34). Stable cell lines expressing either CRKL (Rat-1/CRKL) or the G418 (NEO) drug resistance marker (Rat-1/NEO) were generated by retrovirus infection and drug selection for 2 weeks. Overexpression of CRKL was confirmed by immunoblot (Fig. 2B, bottom). Previous studies have shown that the plasmid pB4XCAT, which contains a Ras-responsive enhancer element adjacent to a minimal promoter driving expression of the CAT reporter gene (45), is activated by BCR-ABL in a RAS-dependent manner (35). Therefore, we measured the effect of CRKL on the activity of this RAS-dependent reporter gene. After transfection with pB4XCAT, cells expressing CRKL consistently showed 10-fold higher levels of CAT activity when compared to the Rat-1/NEO control (Fig. 2B, bottom). Studies have shown that the plasmid pB4XCAT, which contains a Ras-responsive enhancer element adjacent to a minimal promoter driving expression of the CAT reporter gene (45), is activated by BCR-ABL in a RAS-dependent manner (35). Therefore, we measured the effect of CRKL on the activity of this RAS-dependent reporter gene. After transfection with pB4XCAT, cells expressing CRKL consistently showed 10-fold higher levels of CAT activity when compared to the Rat-1/NEO control (Fig. 2A). The magnitude of this response is comparable to that previously reported for BCR-ABL (35). Next we examined the effect of CRKL on JNK. We have previously shown that JNK is activated 4–5-fold by BCR-ABL (39). Endogenous JNK was immunoprecipitated from Rat-1/NEO and Rat-1/CRKL cells, and its kinase activity was measured in vitro using GST-JUN as a substrate. JNK was activated in Rat-1/CRKL cells when compared to the Rat-1/NEO control (Fig. 2B, top). Similar activation was seen with transient retroviral infection, followed by JNK kinase assay (data not shown). Thus, overexpression of CRKL is sufficient to activate intracellular signaling pathways similar to those activated by BCR-ABL.

CRKL Transforms Fibroblasts in a RAS-dependent Manner—Next we asked if CRKL overexpression leads to cellular transformation using a single-step soft agar colony assay, which measures anchorage-independent growth. Rat-1 fibroblasts were infected with retrovirus stocks as indicated in the figure. Lysates were analyzed by SDS-PAGE (12.5%) followed by anti-CRKL immunoblot. B, infected Rat-1 fibroblasts were plated into soft agar and colonies counted after 2 weeks. Error bars represent the average of two experiments (± standard deviation). C, photographs of colonies are shown after 2 weeks.

Because CRKL activates the RAS-dependent pB4XCAT construct, we asked if RAS plays a role in transformation by CRKL. For these experiments Rat-1 fibroblasts were simultaneously infected with retroviruses expressing either CRKL and NEO or CRKL and Asn-17 RAS, a previously described dominant negative RAS mutant (48). Asn-17 RAS cannot be converted to an active GTP-bound state and is believed to function as a dominant negative mutation by titration of guanine nucleotide exchange factors (48). In two independent experiments, Asn-17 RAS completely blocked transformation by CRKL (Fig. 4A). This result is comparable to our previous
studies of Asn-17 RAS and BCR-ABL (37). Immunoblot analysis confirmed expression of CRKL and Asn-17 RAS in the appropriate cell populations (Fig. 4B). These findings show that overexpression of CRKL transforms fibroblasts cells by activation of a Ras-dependent pathway.

**CRKL** **Binds to a Proline-rich Region of BCR-ABL**—The fact that overexpression of CRKL is oncogenic provides functional evidence that it may be a physiologically relevant substrate. To directly examine the role of CRKL as an adaptor protein in signal transduction by a tyrosine kinase, we designed a strategy to disrupt the signal from BCR-ABL to CRKL. Previous studies of the GRB2 adaptor molecule showed that mutation of the GRB2 binding site in BCR-ABL disrupts complex formation between BCR-ABL and GRB2. This mutant has impaired transforming activity in fibroblasts (35), thereby implicating GRB2 in the BCR-ABL transformation pathway. We adapted this approach to address the role of CRKL in BCR-ABL transformation. First, we had to define the CRKL binding site in BCR-ABL. A prior observation from studies of c-ABL and c-CRK showed that the N-terminal SH3 domain of c-CRK can bind two adjacent P-X-P motifs in c-ABL (15). We created a deletion in BCR-ABL that encompassed both of these P-X-P motifs (BCR-ABL925–975) (Fig. 5A) and tested the effect of this deletion on binding to CRKL in two cell types commonly used for studies of BCR-ABL function: 293T cells and Rat-1 fibroblasts. Cells were transfected (293T) or infected (Rat-1/CRKL) with retrovirus constructs expressing NEO, wild-type BCR-ABL, or BCR-ABL925–975. Immunoprecipitations with CRKL antisera were performed on lysates from each cell type, then analyzed for co-precipitation of BCR-ABL by immunoblot using ABL antisera. As expected, wild-type BCR-ABL was present in anti-CRKL immunoprecipitates from both cell types (Fig. 5B, top panel, lanes 2 and 5), indicating these two proteins form a complex. However, the BCR-ABL925–975 mutant was not present in anti-CRKL immunoprecipitates from 293T cells or Rat-1/CRKL cells (Fig. 5B, top panel, lanes 3 and 6), despite high levels of expression of BCR-ABL925–975 (Fig. 5B, bottom panel, lanes 3 and 6). This result defines the CRKL binding site in BCR-ABL and shows that deletion of this site disrupts complex formation between BCR-ABL and CRKL in these two cell types.

**Deletion of the CRKL Binding Site in BCR-ABL Impairs Fibroblast Transformation**—Next we examined the consequences of lack of CRKL binding on the biological activity of BCR-ABL. Because the CRKL binding site in BCR-ABL is adjacent to the tyrosine kinase domain, we first compared the kinase activity of wild-type BCR-ABL to the BCR-ABL925–975 mutant using autophosphorylation on tyrosine in vivo as an end point. Whole cell lysates from 293T cells transfected with neo, wild-type BCR-ABL, or BCR-ABL925–975 were analyzed by immunoblot for expression of BCR-ABL and auto-phosphorylation activity. Wild-type BCR-ABL and BCR-ABL925–975 were expressed at similar levels and were phosphorylated at comparable levels on tyrosine (Fig. 6A). This result suggests that deleting the CRKL binding site does not alter the kinase activity of BCR-ABL. Next we measured the effect of this deletion on the transforming activity of BCR-ABL in Rat-1 fibroblasts. After infection with retrovirus stocks expressing NEO, wild-type BCR-ABL, or BCR-ABL925–975, Rat-1 cells were plated into soft agar to assess anchorage-independent growth as a measure of transformation. In two independent experiments plated in duplicate, cells infected with wild-type BCR-ABL retrovirus (Fig. 6B) Immunoblot analysis of whole cell lysates using ABL antisera confirmed that equivalent levels of expression of the wild-type and BCR-ABL925–975 proteins were seen following retrovirus infec-
The conditions of this assay, suggesting a potential role for ABLY177F impaired transforming activity. However, it is important to note that this mutant does retain 30% activity under the conditions of this assay, suggesting a potential role for other adaptor proteins. A single mutation of the CRKL binding mutant (BCR-ABLΔ925–975) showed a 50% decrease in colony formation, consistent with the experiments reported in Fig. 6. When combined within the same protein, the effect of each single mutation was enhanced 5–10-fold. The double mutant incapable of binding either GRB2 or CRKL (BCR-ABLΔ177F-Δ925–975) showed a 15-fold reduction in transforming activity compared to wild-type BCR-ABL. This loss of function was not due to a change in kinase activity because the BCR-ABLΔ177FΔ925–975 double mutant showed levels of auto-phosphorylation equivalent to the wild-type protein as measured by phosphotyrosine immunoblot (data not shown). These results show that fibroblast transformation by BCR-ABL requires interaction with both GRB2 and CRKL, suggesting that multiple adaptors mediate the biological activity of this tyrosine kinase.

**DISCUSSION**

One approach toward defining mechanisms of transformation by tyrosine kinases is to identify relevant cellular substrates. CRKL was identified (49) and isolated (23, 24) from CML cell lines and patient samples in a search for substrates for the BCR-ABL tyrosine kinase. CRKL is of particular interest because it is an adaptor protein that can bind a number of molecules which might affect signal transduction pathways. These include GDP/GTP exchange factors for RAS such as SOS and C3G and the tyrosine-phosphorylated proteins paxillin, CBL, and CAS (13, 25). Despite these compelling biochemical associations, no functional studies of the biological activity of CRKL have been reported. In this work we have examined the biological effects of CRKL by overexpression in fibroblasts. We find that overexpression of CRKL activates cellular signaling pathways and leads to fibroblast transformation, thereby implicating CRKL as an oncogene. We have also examined the role of CRKL as an adaptor protein in RTK signaling using fibroblast transformation by BCR-ABL as a model system. Deletion of the CRKL binding site in BCR-ABL disrupts complex formation between the two proteins and causes a partial reduction in transforming activity. Because BCR-ABL also binds the GRB2 adaptor protein, we addressed the relative roles of CRKL and GRB2 in BCR-ABL function by preparing mutants that fail to bind to either one or both proteins. The results suggest that both GRB2 and CRKL contribute to transformation. Taken together, these findings provide evidence that CRKL is a physiologically relevant BCR-ABL substrate and functions in linking BCR-ABL to transformation pathways.

To date, BCR-ABL is the only stimulus known to activate human CRKL phosphorylation, despite an exhaustive look at cytokines and other mitogenic stimuli (24). Recent studies of murine CRKL have shown ubiquitous expression of the protein with highest levels in hematopoietic cells. Tyrosine-phosphorylated CRKL was found only during early embryogenesis and in lung tissue (50). For these reasons, we were surprised by our initial studies showing that overexpression of CRKL in fibroblasts was sufficient to lead to its phosphorylation. This result implies that a balance between cellular tyrosine kinases and phosphatases maintains CRKL in a mostly unphosphorylated state. Overexpression of CRKL might perturb this balance in favor of its tyrosine phosphorylation, perhaps by overcoming a regulatory phosphatase. Alternatively, high levels of CRKL might allow unhindered access to a cellular tyrosine kinase. The consequence is activation of cellular signal transduction pathways and transformation. Although similar findings have been reported for other adaptor proteins such as v-CRK (10, 11), CRK-I (51), NCK (52), and SHC (53), the closest homologue of CRKL, CRK-II, does not transform fibroblasts (51). Our
results suggest that the C-terminal SH3 domain of CRKL, which is postulated to negatively affect transforming activity in CRK-II (51), may be regulated differently in the context of CRKL. The mechanism of transformation by CRK proteins is unknown. Recent studies of v-Crk implicate the Src kinases because v-Crk can interfere with the normal regulation of Src by Crk (54, 55). RAS pathway activation also appears to be of functional importance in mediating the effects of v-Crk in PC12 cells (56–58), which is in agreement with our finding of RAS-dependent transformation by CRKL.

What role does CRKL play in the transforming activity of BCR-ABL? In addition to interacting with BCR-ABL, CRKL binds two guanine nucleotide exchange factors, SOS and C3G, which activate RAS (13). Our finding that CRKL transformation is RAS-dependent suggests that one function of CRKL might be to connect BCR-ABL to the RAS pathway. Consistent with this hypothesis, the CRKL binding mutant of BCR-ABL shows reduced activation of the RAS-responsive pB4XCAT reporter, similar to results reported for the GRB2 binding mutant (35). Two other adaptor molecules, GRB2 (35) and SHC (42), have also been implicated in BCR-ABL transformation, presumably by activating RAS through the formation of signaling complexes. GRB2 links BCR-ABL to SOS by binding directly to both proteins through its SH2 and SH3 domains, respectively (35, 40). SHC may function as an intermediate between BCR-ABL and GRB2, since both proteins are found in SHC immunoprecipitates (41), but the biochemical details of the SHC/BCR-ABL interaction are not yet clear. Because CRKL also binds BCR-ABL and SOS, it is possible that it not be expected to bind two proteins simultaneously, it is unlikely that CRKL functions simply as a bridge between BCR-ABL and SOS. Alternatively, one consequence of direct binding between BCR-ABL and CRKL might be the efficient phosphorylation of CRKL by BCR-ABL, as has recently been reported for c-ABL and c-Crk (60). Once phosphorylated, CRKL might bind a novel set of proteins that play a role in signal transduction by BCR-ABL. In addition to RAS pathway activation through SOS and C3G, it is likely that phosphorylated CRKL affects other signaling pathways through interaction with phosphorytrosine containing proteins through its SH2 domain. CBL (18, 61) and paxillin (22) are two such CRKL-binding proteins. Paxillin is of particular interest, since it forms a complex with CRKL and BCR-ABL and becomes phosphorylated in CML cells (22, 62). Paxillin is also a substrate for focal adhesion kinase and is localized to focal adhesion plaques (21, 63, 64). It is likely that interaction with these proteins may contribute to the transformation phenotype. These observations may also provide insight into the observation that CML cells have defects in cellular adhesion (65).

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CRKL and BCR-ABL

| Gene       | Neo | Colony Number1 | Colony Size2 | Acidification3 |
|------------|-----|----------------|--------------|---------------|
| BCR CRKL Binding | WT Bcr-Abl | 334 (+/- 38) | Large | +++ |
|            | Bcr-Abl/Y177F | 136 (+/- 27) | Small | + |
|            | Bcr-Abl Δ925-975 | 226 (+/- 14) | Large | ++ |
|            | Bcr-Abl/Y177F Δ925-975 | 21 (+/- 19) | Small | - |

1 Results from three independent experiments plated in duplicate in 20% fetal calf serum
2 Large = > 0.5 mm; Small = < 0.5 mm
3 Degree of acidity as measured by phenol red; = basic; +++,+++ represent degrees of acidity

FIG. 7. GRB2 and CRKL contribute independently to fibroblast transformation by BCR-ABL.

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