Transformation of Myeloid Leukemia Cells to Cytokine Independence by Bcr-Abl Is Suppressed by Kinase-defective Hck*

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Bcr-Abl is the constitutively active protein-tyrosine kinase expressed as a result of the Philadelphia translocation in chronic myelogenous leukemia. Bcr-Abl is coupled to many of the same signaling pathways normally regulated by hematopoietic cytokines. Recent work shows that Hck, a member of the Src tyrosine kinase family with myeloid-restricted expression, associates with and is activated by Bcr-Abl. Here we investigated the mechanism of Hck interaction with Bcr-Abl and the requirement for Hck activation in Bcr-Abl transformation signaling. Binding studies demonstrated that the Hck SH3 and SH2 domains are sufficient for interaction with Bcr-Abl in vitro. Hck binding localizes to the Abl SH2, SH3, and kinase domains as well as the distal portion of the C-terminal tail. To address the requirement for endogenous Src family kinase activation in Bcr-Abl signaling, a kinase-defective mutant of Hck was stably expressed in the cytokine-dependent myeloid leukemia cell line DAGM. Kinase-defective Hck dramatically suppressed Bcr-Abl-induced outgrowth of these cells in the absence of cytokine compared with a control cell line expressing β-galactosidase. In contrast, kinase-defective Hck did not affect cell proliferation in response to interleukin-3, suggesting that the effect is specific for Bcr-Abl. These data show that Hck interacts with Bcr-Abl through a complex mechanism involving kinase-dependent and -independent components and that interaction with Hck or other Src family members is essential for transformation signaling by Bcr-Abl.

Several human leukemias are characterized by the presence of the Philadelphia chromosome which results from the translocation of the c-abl locus on chromosome 9 and the bcr locus chromosome 22 (reviewed in Refs. 1 and 2). This translocation results in the expression of a family of chimeric Bcr-Abl oncoproteins associated with specific leukemias (3–6). Chronic myelogenous leukemia (CML) results from the 210-kDa form of Bcr-Abl (p210), while acute lymphocytic leukemia (ALL) is associated with a 185-kDa form (p185) (7, 8). Both forms of Bcr-Abl are constitutively active protein-tyrosine kinases that have been shown to transform cells in culture and to produce CML- and ALL-like syndromes in transgenic mice, providing strong evidence that Bcr-Abl is responsible for the development of these leukemias (9–11).

Bcr-Abl has been shown to activate multiple signal transduction pathways normally associated with the growth, survival, and differentiation of hematopoietic cells. For example, tyrosine-phosphorylated Bcr-Abl can interact directly with the Grb-2/Sos guanine nucleotide exchange factor, leading to the activation of Ras (12, 13). Bcr-Abl can also activate Ras via She, an adaptor protein that couples the receptors for many growth factors and cytokines to the Grb2/Sos complex (14). Other work has identified Crk-L as a binding partner and substrate for Bcr-Abl (15, 16). Crk-L may couple Bcr-Abl to the guanine-nucleotide exchange factor C3G, providing an additional connection to Ras activation (17). In addition to the Ras pathway, Bcr-Abl is coupled to PI-3K signaling and activation of Akt/PKB kinases downstream (18). This pathway may promote cytokine-independent survival by Bcr-Abl (19, 20). Other studies have demonstrated that Bcr-Abl induces activation of Stat transcription factors, which have also been implicated in proliferative and survival signaling in a wide variety of hematopoietic cell types (21–24). All of these signaling pathways involve components with SH2 and SH3 domains and are dependent upon tyrosine phosphorylation.

Although Bcr-Abl possesses a constitutively active tyrosine kinase domain, recent work suggests that it may initiate signaling by activating other nonreceptor tyrosine kinases, including members of the Fps/Fes and Src families (25–28). Regarding the Src family, Bcr-Abl has been shown to associate directly with Lyn and Hck in 32Dcl3 cells. This interaction correlates with increased Lyn and Hck tyrosine kinase activity (25). More recently, Hck has been shown to phosphorylate p210 Bcr-Abl on Tyr 177, the site responsible for direct recruitment of Grb-2/Sos through the Grb-2 SH2 domain (26). This result suggests that Src family kinases may facilitate the coupling of Bcr-Abl to Ras and other signaling pathways related to transformation. In this study, we investigated the mechanism of Hck interaction with Bcr-Abl. We observed that the Hck SH2 and SH3 domains bind strongly to recombinant Bcr-Abl in vitro. Constitutive binding was observed with the SH3 domain, while SH2-mediated binding was found to require Bcr-Abl autophosphorylation. In reciprocal experiments, we observed that multiple regions of Abl, including the SH3, SH2, and kinase domains as well as the distal portion of the C-terminal region interact directly with Hck. We also found that a kinase-inactive mutant of Hck strongly suppressed Bcr-Abl proliferative signals in cytokine-dependent myeloid leukemia cells, suggesting that activation of Hck or other members of the Src kinase family is
required for Bcr-Abl transformation signaling. Activation of Src family kinases by Bcr-Abl may contribute to the diversity of signaling pathways activated by this transforming tyrosine kinase.

**EXPERIMENTAL PROCEDURES**

**GST-Hck Fusion Protein Binding Assay—Construction of pGEX-2T vectors and expression of the noncatastrophic regions of Hck as GST fusion proteins is described elsewhere (29). Briefly, *Escherichia coli* DH5α were transformed with pGEX-Hck constructs, and GST fusion protein expression was induced with isopropyl-β-D-thiogalactopyranoside. Following induction, recombinant fusion proteins were isolated from clarified cell extracts with glutathione-agarose beads. The concentration of each protein was determined on Coomassie-stained gels by two-dimensional laser densitometry using bovine serum albumin as a standard. For the binding reactions, wild-type and kinase-defective forms of Bcr-Abl were expressed in SF-9 insect cells using recombinant baculoviruses as described elsewhere (28). Immobilized GST fusion proteins (20 μg or GST alone as a negative control) were added to 1 ml aliquots of clarified SF-9 cell lysates and rotated at 4 °C for 2 h. Fusion proteins were pelleted by centrifugation and washed once with 1 ml Hck lysis buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM MgCl2, 1% Triton X-100) followed by three washes with RIPA buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM MgCl2, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS). Precipitated proteins were solubilized in SDS-PAGE sample buffer and associated Bcr-Abl was detected by immunoblotting.

**Co-precipitation of Ab1 Domains and Hck in SF-9 Cells**—The coding sequences for the human Ab1 SH3 and SH2 domains (Gly73–Thr224) and kinase domain (Tyr215–Ile489) were amplified by polymerase chain reaction and subcloned into the baculovirus transfer vector pVL-GST (30). The C-terminal region of Ab1 was similarly amplified as a series of four sequences encoding residues Pro460–Gly638 (CT1), Arg639–Leu813 (CT2), Ile801–Ala993 (CT3), and Gly994–Arg1130 (CT4), which were subcloned into the same vector. The resulting pVL-GST constructs were used to create recombinant baculoviruses for the expression of these GST Ab1 regions as GST fusion proteins. SF-9 cell cultures (2.5 × 10⁷) were co-infected with each of the GST-Ab1 baculoviruses (or a GST baculovirus as a negative control) and an Hck baculovirus. Forty-eight h postinfection, the cells were lysed in 1.0 ml of Hck lysis buffer, and GST fusion proteins were precipitated with glutathione-agarose beads (Sigma; 20 μl of a 50% slurry suspension). The precipitates were washed three times with 1.0 ml of RIPA buffer and bound proteins were eluted by heating in SDS-PAGE sample buffer. Proteins were resolved on duplicate SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Associated Hck was visualized by immunoblotting with Hck polyclonal antibodies (Santa Cruz). The amount of precipitated GST-Abl fusion protein present in each reaction was determined by immunoblotting with anti-GST antibodies (Santa Cruz). Equivalent expression of Hck in each culture was verified by immunoblot analysis of the clarified cell lysates. The amount of precipitated bound Hck and GST-Abl fusion protein present in each lane was determined by densitometry (Bio-Rad GS-710 Imaging Densitometer), and the amount of Hck bound was corrected to the amount of GST-Abl fusion protein present in each sample.

**Retroviral Constructs**—Subcloning of the Hck wild-type and kinase-inactive (Hck-KE) cDNAs into the retroviral expression vector pSRαMSVtkneo (31) has been described elsewhere (32). The coding sequence for bacterial β-galactosidase was subcloned from the expression vector pCMVβSPORT (Life Technologies, Inc.) into pSRαMSVtkneo. Similar pSRαMSVtkneo constructs containing the coding sequences for the p210 and p185 forms of Bcr-Abl were obtained from Dr. Owen Witte, Howard Hughes Medical Institute, UCLA. To make retroviral stocks, subconfluent 100-mm dishes of 293T cells were transfected with 30 μg of each retroviral construct and an ectopic packaging vector using the calcium phosphate method described elsewhere (33, 34). Viral supernatant was collected 48, 72, and 96 h posttransfection, pooled, filtered with 0.45-μm filters, and stored at −80 °C.

**DAGM Cell Proliferation Assays**—DAGM myeloid leukemia cells (14) were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 50 μg/ml gentamycin, and 2.5 ng/ml IL-3. Cells were infected with β-gal or Hck retroviruses and selected with G-418 at 800 μg/ml. Expression of Hck proteins in the drug-resistant cell populations was verified by immunoblotting. Hck and β-gal control retroviruses were then superinfected with Bcr-Abl or β-gal control retroviruses as follows: 10⁶ cells were resuspended in 5 ml of viral supernatant in the presence of 4 μg/ml polybrene. To enhance retroviral gene transfer, culture plates were centrifuged at 2,400 rpm for 4 h at 20 °C (35). The virus was replaced with fresh medium containing G-418 and IL-3 and the cultures incubated for 72 h. For analysis of IL-3-independent outgrowth, cells were washed free of cytokine and 4 × 10⁵ cells were plated in 100 μl of complete medium in each well of a 96-well plate. To quantitate cell outgrowth, cells in each well were combined with 25 μl of MTT reagent (5 mg/ml in H2O) and incubated for 4 h at 37 °C. The reaction was stopped by adding 100 μl of MTT lysis buffer (50% N,N-dimethylformamide in H2O containing 20% SDS, 2.5% glacial acetic acid, and 2.5% HCl, pH 1) Plates were incubated overnight at 37 °C, and the absorbance of each well was read at 570 nm. Positive controls were analyzed in a similar manner in the presence of IL-3. For all plates, a well containing only culture medium and no cells served as a background control for the absorbance readings.

**RESULTS**

The Hck SH2 and SH3 Domains Bind Directly to Bcr-Abl in Vitro—Previous studies have shown that Bcr-Abl forms a complex with endogenous Hck and Lyn in 32Dcl3 myeloid leukemia cells and that interaction correlates with activation of these Src family kinases (25). To determine the molecular basis for this interaction, we expressed a family of GST fusion proteins containing each of the noncatastrophic domains of Hck (N-terminal, SH3, and SH2 either alone or in various combinations) in E. coli and immobilized them on glutathione-agarose beads. Equal amounts of each fusion protein were incubated with lysates from SF-9 cells expressing the p210 form of Bcr-Abl (p210), a kinase-defective mutant of p210 (p210-KR), or the p185 form of Bcr-Abl (p185). Following incubation and washing, bound Bcr-Abl proteins were visualized by immunoblotting. Control blots of the SF-9 cell lysates showed Bcr-Abl bands of identical electrophoretic mobilities and comparable intensities as the pull-down result (data not shown).

FIG. 1. Association of the Hck noncatastrophic region with Bcr-Abl in vitro. The noncatastrophic domains of Hck (unique N-terminal, SH3 and SH2) were expressed either alone or in the combinations shown in A as GST fusion proteins in E. coli and immobilized on glutathione-agarose beads. B, the immobilized fusion proteins, GST alone, or the glutathione-agarose resin (GSH) were mixed with lysates from SF-9 cells expressing the p210 form of Bcr-Abl (p210), a kinase-defective mutant of p210 (p210-KR), or the p185 form of Bcr-Abl (p185). Following incubation and washing, bound Bcr-Abl proteins were visualized by immunoblotting. Control blots of the SF-9 cell lysates showed Bcr-Abl bands of identical electrophoretic mobilities and comparable intensities as the pull-down result (data not shown).
with extracts from SF-9 cells expressing the kinase-defective form of p210 Bcr-Abl. As shown in Fig. 1, this mutant was unable to bind to the isolated Hck SH2 domain, strongly suggesting that autophosphorylation of Bcr-Abl creates a specific binding site for the SH2 domain of Hck. In contrast, the kinase-defective mutant bound to the fusion proteins containing the SH3 domain to the same extent as kinase-active Bcr-Abl, as expected for an SH3-mediated interaction. These results indicate that multiple docking sites for Src family kinases exist within Bcr-Abl. Recent studies have shown that SH3 and SH2 engagement is sufficient to activate Src kinases in a variety of cellular contexts. The SH2/SH3-mediated interaction of Hck with Bcr-Abl may provide a mechanistic basis for previous reports of Hck activation by Bcr-Abl in vivo (see “Discussion”).

Multiple Regions of Abl Contribute to Interactions with Hck—To identify the regions of Bcr-Abl that interact with Hck, a series of GST-Abl fusion proteins was co-expressed with wild-type Hck in SF-9 cells. The primary structures of these GST-Abl fusion proteins are shown in Fig. 2 and include the SH3/SH2 region, the kinase domain, and the long C-terminal tail as a series of four fusion proteins. The GST-Abl proteins were precipitated from infected cell lysates with glutathione-agarose beads, and associated Hck was visualized by immunoblotting. As shown in Fig. 3, several regions of Abl bound independently to Hck, including the SH3/SH2 region, the kinase domain, and the distal portion of the C-terminal tail. Hck did not bind to the Abl C-terminal fusion proteins containing proline-rich sequences involved in SH3 binding to the Crk-L adaptor protein (16). These results indicate that Hck interacts with the Abl portion of Bcr-Abl through multiple sites that may include a novel interaction with the C-terminal actin-binding domain (see “Discussion”).

Kinase-defective Hck Inhibits Transformation of DAGM Cells to Cytokine Independence—Previous studies have demonstrated a direct interaction of Bcr-Abl with Hck and Lyn in myeloid leukemia cells, suggesting that activation of Src kinases may contribute to Bcr-Abl signaling (25). To determine whether interaction with endogenous Src kinases is required for Bcr-Abl signal transduction, we tested the effect of kinase-inactive Hck on Bcr-Abl-induced transformation of the IL-3-dependent myeloid leukemia cell line, DAGM. Introduction of Bcr-Abl into these cells with a recombinant retrovirus has been shown to result in cytokine-independent proliferation (14). DAGM cells were infected with recombinant retroviruses carrying either the kinase-defective form of Hck (Hck-KE) or β-galactosidase (β-gal) as a negative control. Cells were selected in the presence of G-418 and cytokine, and expression of Hck-KE was verified by immunoblotting (data not shown). The DAGM cell populations stably expressing Hck-KE or β-gal were then re-infected with retroviruses carrying p210 Bcr-Abl, p185 Bcr-Abl, or the β-gal control. Cytokine-independent proliferation was monitored using an MTT reduction assay, and the results are presented in Fig. 4. As expected, both p185 and p210 Bcr-Abl induced rapid cellular outgrowth in the β-gal control cell populations, consistent with previous results. However, the presence of kinase-defective Hck dramatically suppressed DAGM cell outgrowth by both forms of Bcr-Abl relative to the β-gal control cells. No outgrowth was observed with any of the cell populations following superinfection with the β-gal control virus, indicating that transformation to cytokine independence is dependent upon Bcr-Abl. These data provide the first evidence that Src kinases play an essential role in transformation signaling downstream of Bcr/Abl.

To determine whether expression of kinase-defective Hck produced a general suppression of proliferative capacity, the populations of DAGM cells expressing Hck-KE as well as the β-gal control cells were treated with IL-3 and outgrowth was measured using the MTT assay. As shown in Fig. 5, all of the cells grew rapidly in the presence of IL-3, suggesting that the actions of Hck-KE are specific for Bcr-Abl and are not the result of a general suppressive effect on cell proliferation or survival.

In a final control experiment, we examined whether overexpression of wild-type Hck affected p210 Bcr-Abl-induced conversion of DAGM cells to cytokine independence. Cell populations were produced which stably expressed β-gal, wild-type Hck, or kinase-defective Hck as described in the preceding section. These cell populations were then re-infected with retroviruses carrying p210 Bcr-Abl or the β-gal control, and cytokine-independent proliferation was monitored using the MTT reduction assay. As shown in Fig. 6, wild-type Hck partially suppressed Bcr-Abl-induced conversion of DAGM cells to cytokine independence relative to the β-gal control. In contrast, kinase-defective Hck produced a complete block in outgrowth.
protein of HIV-1 leads to Hck activation and transformation of Rat-2 fibroblasts (32, 36). This effect is due to the ability of Nef to displace the SH3 domain of Hck from an intramolecular interaction with the linker connecting the SH2 and kinase domains (37). Together with intramolecular binding of the tyrosine-phosphorylated tail region to the SH2 domain, SH3-linker interaction is a key part of negative regulation of Src family kinases (38). Binding of Hck or other Src kinases to Bcr-Abl through their SH3 and SH2 domains may result in kinase activation though a similar mechanism involving disruption of intramolecular regulatory interactions.

Three regions within the Abl portion of Bcr-Abl demonstrated strong interactions with Hck, including the SH3/SH2 region, the kinase domain, and the distal portion of the C-terminal tail. Binding of the Abl SH3/SH2 region to Hck may occur through sites of Hck tyrosine autophosphorylation and/or the polyproline linker connecting the SH2 and kinase domains. Interaction of the Abl SH3 domain with the Hck linker could contribute to Hck activation by disturbing intramolecular SH3-linker interactions as described above. Interestingly, the SH2/SH3 region of Bcr-Abl has recently been shown to play a role in the activation of Stat5, although direct interaction of Bcr-Abl with Stat5 does not occur to a significant extent (24). Our observation that the SH3/SH2 region of Bcr-Abl is involved in the recruitment of Hck suggests that Src kinases may serve as intermediates linking Bcr-Abl to Stat5 activation and downstream survival signaling.

Interaction experiments involving the Abl C-terminal region also produced unexpected results. Fusion proteins encompassing the C-terminal regions of Abl with known SH3-binding sites for other proteins such as Crk-L did not interact strongly with Hck (Figs. 2 and 3). On the other hand, the fusion protein containing the C-terminal 137 amino acids of Abl (CT4) associated strongly with Hck, despite the lack of tyrosine phosphorylation or an obvious PxxP motif. This region of Abl encompasses the F-actin binding domain and is required for Bcr-Abl transforming activity (39–41). Whether or not this region also contributes to interactions with Src-related kinases in vivo will require further study.

In addition to investigating the mechanism of Bcr-Abl interaction with Src kinases, we also present evidence that activa-
Expression of Bcr-Abl with a kinase-defective mutant of Hck strongly suppressed both p185 and p210 Bcr-Abl signals for cytokine-independent outgrowth of the DAGM myeloid leukemia cell line (Fig. 4). This result is consistent with the data shown in Fig. 1, as both forms of Bcr-Abl demonstrated equivalent binding to the SH2 and SH3 domains of Hck. In contrast, kinase-defective Hck had no effect on cytokine-induced cell outgrowth, arguing against a nonspecific effect of Hck on cellular proliferation (Fig. 5). These results suggest that the kinase-defective mutant of Hck interacts directly with Bcr-Abl and competes for association with endogenous Src family kinases, resulting in the observed suppression of DAGM cell proliferation. The partial suppressive effect of wild-type Hck in this system (Fig. 6) suggests that activation of more than one Src kinase family member may contribute to survival and proliferative signaling. Overexpression of wild-type Hck may compete for activation of other Src family members required for a complete biological signal, while maintaining the signal from Hck. Consistent with this idea is the observation that Lyn and Fyn associate with the same regions of Bcr-Abl as Hck. However, kinase-defective Hck may interfere with growth factor and cytokine signal transduction and block growth factor induced entry into the cell cycle in some cases (reviewed in Ref. 42). Whether or not Src kinases are required to couple Bcr-Abl to proliferative and survival signaling pathways during the development of CML will require further investigation.

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