The Src Family Kinase Hck Interacts with Bcr-Abl by a Kinase-independent Mechanism and Phosphorylates the Grb2-binding Site of Bcr*

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bcr-abl, the oncogene causing chronic myeloid leukemia, encodes a fusion protein with constitutively active tyrosine kinase and transforming capacity in hematopoietic cells. Various intracellular signaling intermediates become activated and/or associate by with Bcr-Abl, including the Src family kinase Hck. To elucidate some of the structural requirements and functional consequences of the association of Bcr-Abl with Hck, their interaction was investigated in transiently transfected COS7 cells. Neither the complex formation of Hck kinase with Bcr-Abl nor the activation of Hck by Bcr-Abl was dependent on the Abl kinase activity. Both inactivating point mutations of Hck and dephosphorylation of Hck enhanced its complex formation with Bcr-Abl, indicating that their physical interaction was negatively regulated by Hck (auto)phosphorylation. Finally, experiments with a series of kinase negative Bcr-Ab mutants showed that Hck phosphorylated Bcr-Abl and induced the binding of Grb2 to Tyr177 of Bcr-Abl. Taken together, our results suggest that Bcr-Abl preferentially binds inactive forms of Hck by an Abl kinase-independent mechanism. This physical interaction stimulates the Hck tyrosine kinase, which may then phosphorylate the Grb2-binding site in Bcr-Abl.

Bcr-Abl (p210bcr-abl), the transforming agent in chronic myeloid leukemia, is the gene product of the bcr-abl hybrid gene, which results from the Philadelphia translocation t(9;22) by fusing parts of the c-abl gene, normally located on chromosome 9, to the bcr gene on chromosome 22 (1, 2). Previous studies have demonstrated that Bcr-Abl is a constitutively active tyrosine kinase (3) that has transforming capacity in fibroblasts and hematopoietic cells (4, 5).

Bcr-Abl-induced transformation seems to require the activation of the Ras signaling pathway (6, 7), involving at least two different signaling intermediates, Grb2 and Shc (8–10). In addition, the association of Bcr-Abl with the SH3-SH2 adaptor protein CRKL (11), the activation of the Jak-STAT pathway (12, 13) and of the PI3-Kinase pathway (14–16), the phosphorylation of a variety of cytoskeletal proteins (17–19) and the interaction with cytokine and growth factor receptors (20, 21) may also play pivotal roles in the pathogenesis of chronic myeloid leukemia. However, the precise mechanisms of transformation by p210bcr-abl are unknown, and some characteristics of chronic myeloid leukemia, like induction of blast crisis after chronic phase or prolonged viability of chronic myeloid leukemia cells under serum starvation, are still unexplained.

Some critical domains of Bcr-Abl that are necessary for transformation and induction of leukemia have been identified. The coiled-coil oligomerization domain, localized at the N terminus of Bcr-Abl, seems to induce tetramerization of Bcr-Abl, which is in turn necessary for the constitutive activation of the tyrosine kinase of Bcr-Abl, as well as for the complex formation with other Src homology (SH)2-containing proteins (22, 23). Further important residues or domains within Bcr are the tyrosine at position 177, which is a binding site for the Ras adaptor protein Grb2 (24), and a SH2-binding motif, alternatively named A-Box and B-Box (25, 26), that binds SH2 domains by a phosphotyrosine-independent mechanism. The N-terminal portion of Abl mainly consists of molecular modules with homology to corresponding domains of the tyrosine kinase c-Src, therefore called SH domains 3 and 2 (27). The putative function of these domains is to direct the subcellular localization of Bcr-Abl to compartments where it interacts with specific proteins via specific binding motifs (28). The optimal binding motif for SH3 domains is polyproline (PXXP) (29), whereas SH2 domains predominantly bind to phosphorylated tyrosine residues in a specific amino acid context (30). In addition, the SH3 and SH2 domains of Bcr-Abl seem to regulate the tyrosine kinase activity as well as the transforming capacity of Abl proteins in vitro (26, 31, 32). As in Src family kinases, a kinase domain (SH1 domain) is located next to the SH2 domain. The C-terminal part includes proline-rich motifs that are the molecular anchor for the adaptor protein CRKL (33, 34), a nuclear localization sequence, which is “inactivated” in Abl fusion genes (35), a DNA-binding domain (36), and an actin binding site allowing interaction with the cytoskeleton (37).

We have recently described the activation and association of two members of the Src family of tyrosine kinases, p53/56lyn and p59hck, with Bcr-Abl (38). Src kinases are composed of a N-terminal unique domain, a PXXP-binding SH3 domain, a phosphotyrosine-binding SH2 domain, a tyrosine kinase domain, and a C-terminal tail, which is closely involved in negative regulation of the kinase activity (39). One of the common features of Src family kinases seems to be their mechanism of autoregulation. Two cooperative mechanisms negatively regulate the activity of Src family kinases (40): the interaction of the
SH3 domain with a polypolyine type II helix located between the SH2 domain and the kinase domain and an interaction of the tyrosine phosphorylated C-terminal tail (Tyr290 in Hck) with the SH2 domain. On the contrary, phosphorylation of a conserved autophosphorylation site within the activation loop of the kinase domain (Tyr390 in Hck) positively regulates the kinase activity (41). The resulting full-length hck fragment was cloned into pUC19 and sequenced subsequently. The mutations K269R and Y390F were accomplished using a two-fragment PCR strategy; wt hck was cloned into a pUC vector that had been modified by deleting one PvuI site. The resulting vector, pUCΔNdel/Bcr-Abl was used as a template for two PCR reactions producing PCR fragments overlapping at the remaining PvuI site within the amphiclin resistance (AmbI) gene of pUC and meeting within the hck insert near the triplet to be mutated. Thus, the primers binding in hck allowed us to obtain PCR fragments that could be blunt end-ligated without initiating a deletion; one of these primers contained a mutagenic triplet. Finally, the PCR fragments were digested and ligated. For the following primers were used: 5′-gtggtggcctggatacgtaacgagccgg-3′ and 5′-cttcgctgctgtttgaggg-3′. Sequences for primers used for the mutation of Tyr290 were 5′-gacagatttgagttgaggg-3′ and 5′-ctcttcgctgctgtttgaggg-3′. The sequences for the primers binding in pUC were 5′-ccagcaggttagggaggg-3′ and 5′-ctcttcgctgctgtttgaggg-3′. All hck constructs as well as a wt cDNA were cloned into the EcoRI site of the expression vector pAuro (vector and cDNA provided by Dr. Seth Corey, Children's Hospital of Pittsburgh, PA).

Cell Culture and Transfection—32D, 32D k.n. bcr-ABL (K1172R) and 32D wt bcr-ABL (32Dp210) cells were obtained from Dr. Brian J. Druker (Oregon Health Sciences University, Portland, OR). 32D cells were transfected by electroporation as described (44). 32D and 32D bcr-ABL k.n. cells were grown in RPMI 1640 medium (Boehringer Bioproducts, Ingelheim, Germany) supplemented with 10% fetal calf serum (Boehringer Bioproducts) and 10% WEHI-3B conditioned medium to provide murine interleukin-3. 32Dp210 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum. COS7 cells were routinely grown in Dulbecco's modified Eagle's medium containing 4.5 g/liter glucose. For transient transfection, cells of one confluent 175-cm2 flask were diluted 2.5 to 3 times and replated into 175-cm2 tissue culture flasks. 18–24 h thereafter, cells grown to 95% confluence were transfected with lipofectamine using DOPAT (Boehringer, Mannheim, Germany) according to the guidelines of the manufacturer. Briefly, 50 μg of bcr-ABL cDNA and/or 25 μg of hck cDNA were diluted to concentrations of 0.1 μg/ml and preincubated for 15 min with a 5-fold excess (in μg) of DOPAT. For transfection, Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Boehringer Bioproducts) and 1% antibiotics was used. 24 h after transfection, cells were washed twice in ice-cold phosphate-buffered saline (Life Technologies, Inc., Eggersheim, Germany) and serum-deprived by incubation in Dulbecco's modified Eagle's medium containing 1.0 g/liter glucose and 0.5% fetal calf serum. Transfected cells were normally harvested 48 h after transfection by trypsinization. To protect cells from forming irresponsively aggregating cells, 10 μg/ml aprotinin was added to the cells immediately after trypsinization.

Cell Lysis—32D cells were lysed in lysis buffer containing 1% Brij97 as described previously (38). For lysis, COS7 cells were washed twice in ice-cold phosphate-buffered saline to remove remaining serum. Thereafter, cells were lysed in lysis buffer containing 1% Nonidet P-40, 20 mm Tris (pH 8.0), 50 mm NaCl, and 10 μM EDTA as well as 1 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μM/ml leupeptin, and in most cases 2 mm sodium orthovanadate. In general, pelleted cells from one 175-cm2 tissue culture flask (about 5×10⁷ cells) were resuspended in 500 μl of lysis buffer solution and incubated on ice for 25 min. Thereafter unsoluble material was removed by centrifugation at 15,000 ×g. Afterward lysates were checked for protein concentrations using a Betageta kit.

1 Immuno precipitation and Immune Complex Kinase Assay—For immunoprecipitation (IP), 150 μl of COS cell lysate was diluted by the addition of 450 μl of incubation buffer containing 20 mm Tris (pH 8.0), 50 mm NaCl, and 10 μM EDTA, 1 μM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 mm sodium orthovanadate to inhibit phosphatase activity where desired. Lyn, Hck, and Bcr-Abl were
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FIG. 1. Complex formation of Hck kinase with Bcr-Abl in COS7 cells. A, COS7 cells were transfected by lipofection with control vector (pcDNA3) and two different vectors based on pcDNA3 that contained the full-length cDNAs of wt bcr-ABL and hck (see "Materials and Methods"). 48 h post-transfection, expressed proteins were detected by Western blotting using the indicated antibodies. B, the blot of A was stripped and rebotted with an anti-phosphotyrosine antibody (a-P-Tyr, PY20). Expression of the Hck and Bcr-Abl kinases resulted in a strong increase of the phosphotyrosine content of cellular proteins. The arrows indicate the apparent molecular masses of Bcr-Abl and Hck kinases. C, lysates of COS7 cells (from A, lane 4) coexpressing Bcr-Abl and Hck were subjected to immunoprecipitation with a polyclonal anti-Abl antibody, K-12, and subsequent SDS gel electrophoresis. Hck and Bcr-Abl were detected by immunoblotting with the indicated antibodies.

RESULTS

Complex Formation of Hck Kinase with Bcr-Abl in COS7 Cells—We have recently described the association and activation of two kinases of the Src family, p53/56hck and p59hck, with or by Bcr-Abl (38). The mechanism and function of this interaction is unknown. This led us to investigate the structural and functional requirements for the complex formation of Bcr-Abl with Hck kinase, a Src family member preferentially expressed in hematopoietic cells. To establish an expression system that would allow the rapid screening of Bcr-Abl and Hck mutants, wt full-length cDNAs of bcr-ABL and hck were cloned into appropriate mammalian expression vectors (pcDNA3 or pApuro) and prepared for transient transfection into COS7 cells. cDNAs were introduced into these cells using lipofection (see "Materials and Methods"). Fig. 1A demonstrates that considerable amounts of Bcr-Abl (lane 2) and Hck (lane 3) were expressed 48 h post-transfection when compared with lysates from cells transfected with control vector (lane 1). Cotransfection of bcr-ABL and hck cDNAs (lane 4) lead to expression of Bcr-Abl and Hck similar to that of single cDNA transfections. To demonstrate that the expressed kinases were active in vivo, the blot was stripped and rebotted with an anti-phosphotyrosine Ab, PY20. Fig. 1B shows that both kinases were highly (auto)phosphorylated and that expression of these kinases resulted in an increased overall phosphotyrosine content in cellular proteins. Because both kinases, Bcr-Abl and Hck (lanes 2 and 3), seemed maximally activated, no synergism was detectable by anti-tyrosine Ab K-12. Subsequent immunoblotting with the anti-Hck Ab N-30 demonstrated that Hck (Fig. 1C, lane 1) formed a complex with Bcr-Abl. Coprecipitation was completely blocked by the addition of corresponding blocking peptide, indicating that the coprecipitation of Hck with Bcr-Abl was not caused by unspecific binding (lane 2). Similar results were obtained when precipitated by adding 5 μg of the appropriate Abs, i.e. anti-Lyn 44 for precipitation of Lyn, anti-Hck N-30 for precipitation of Hck, and either anti-Bcr 7C6 or anti-Abl K-12 for precipitation of Bcr-Abl. IP reactions were incubated overnight at 4 °C on a rotating plate. After 18 h of incubation, 125 μl of Sepharose A beads (Pharmacia Biotech Inc., Freiburg, Germany) diluted 1:1 in IP buffer (0, 1% Nonidet P-40, 20 mM Tris (pH 8.0), 50 mM NaCl, and 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml antiprotein, 10 μg/ml leupeptin, and 2 mM sodium orthovanadate) were added to each sample. Following an additional 2 h of incubation at 4 °C, the precipitates were washed three times with IP buffer and subsequently boiled in 2× sample buffer before loading on SDS gels. Peptide blocking experiments were performed as described previously (38).

For immune complex kinase assays of Src kinases precipitated from 32D cells, cell lysis, and the IP protocol were slightly modified; IP incubation periods were reduced to 3 h, and three times of washing with IP buffer were followed by washing the precipitates one time with kinase buffer (50 mM Tris (pH 7.4), 10 mM MnCl2). Kinase reaction and analysis of autophosphorylation was performed as described (38).

Gel Electrophoresis and Immunoblotting—Gel electrophoresis and immunoblotting were performed using standard methods. Proteins were either transferred to polyvinyldene difluoride membranes (Millipore, Eschborn, Germany) or nitrocellulose (Schleicher & Schuell, Dassel, Germany). Immunoblots with PY20 were developed by using alkaline phosphatase-conjugated secondary Abs at a dilution of 1:2000 in Tris-buffered saline containing 5% bovine serum albumin when polyvinyliden difluoride membranes were probed. For detection of phosphorylated proteins transferred to nitrocellulose or other membranes, secondary horseradish peroxidase-conjugated Abs were used. The ECL detection system was used according to the guidelines of the manufacturer (Amersham, Braunschweig, Germany).

Protein Dephosphorylation—Dephosphorylation was achieved by omitting orthovanadate, a potent phosphatase inhibitor, from the lysis buffer, followed by preincubation of cleared lysate at 4 °C for 24 h prior to IP.

RESULTS

Complex Formation of Hck Kinase with Bcr-Abl in COS7 Cells—We have recently described the association and activation of two kinases of the Src family, p53/56hck and p59hck, with or by Bcr-Abl (38). The mechanism and function of this interaction is unknown. This led us to investigate the structural and functional requirements for the complex formation of Bcr-Abl with Hck kinase, a Src family member preferentially expressed in hematopoietic cells. To establish an expression system that would allow the rapid screening of Bcr-Abl and Hck mutants, wt full-length cDNAs of bcr-ABL and hck were cloned into appropriate mammalian expression vectors (pcDNA3 or pApuro) and prepared for transient transfection into COS7 cells. cDNAs were introduced into these cells using lipofection (see “Materials and Methods”). Fig. 1A demonstrates that considerable amounts of Bcr-Abl (lane 2) and Hck (lane 3) were expressed 48 h post-transfection when compared with lysates from cells transfected with control vector (lane 1). Cotransfection of bcr-ABL and hck cDNAs (lane 4) lead to expression of Bcr-Abl and Hck similar to that of single cDNA transfections. To demonstrate that the expressed kinases were active in vivo, the blot was stripped and rebotted with an anti-phosphotyrosine Ab, PY20. Fig. 1B shows that both kinases were highly (auto)phosphorylated and that expression of these kinases resulted in an increased overall phosphotyrosine content in cellular proteins. Because both kinases, Bcr-Abl and Hck (lanes 2 and 3), seemed maximally activated, no synergism was detectable by anti-phosphotyrosine blotting when Bcr-Abl and Hck were coexpressed.

We then wished to demonstrate that Bcr-Abl and Src kinases were found in a complex in COS7 cells, similar to our previous findings in myeloid cells (38). For this purpose, lysates of transiently transfected COS7 cells coexpressing Bcr-Abl and Hck were subjected to immunoprecipitation with the polyclonal anti-Abl antibody, K-12, and subsequent SDS gel electrophoresis. Hck and Bcr-Abl were detected by immunoblotting with the indicated antibodies.

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Protein Dephosphorylation—Dephosphorylation was achieved by omitting orthovanadate, a potent phosphatase inhibitor, from the lysis buffer, followed by preincubation of cleared lysate at 4 °C for 24 h prior to IP.
Bcr-Abl was precipitated using the polyclonal anti-Bcr Ab 7C6 (not shown). Moreover, we could also coprecipitate Bcr-Abl in anti-Hck IPs (not shown). Finally, similar results were obtained in cotransfection and coprecipitation experiments with Lyn and Bcr-Abl in COS7 cells (not shown).

Mutations of Several Known Binding Motifs of Bcr-Abl Do Not Disrupt Complex Formation with Hck Kinase—Several domains have been described as functionally relevant protein interaction modules of Bcr-Abl (Fig. 2A) (8, 45, 46). To investigate which of these regions were necessary for the interaction of Bcr-Abl with Src kinases, several mutations were introduced into bcr-abl cDNAs (Fig. 2A). Surprisingly, none of these mutations introduced into bcr-abl alone or in combination was able to disrupt or diminish the formation of Bcr-Abl-Hck complexes in COS7 cells (Fig. 2D). Fig. 2B shows a typical example of such an experiment. The hck gene was cotransfected into COS7 cells either in combination with a control vector (lane 1) or with various single, double, or triple bcr-abl mutants containing amino acid substitutions recently shown to be critical for Ras activation by Bcr-Abl (8): Tyr177, a binding site for the Grb2 adaptor protein; Arg1053, an amino acid embedded in the conserved FLVRESE motif of the Abl SH2 domain that is
Dephosphorylation Induces Complex Formation between Bcr-Abl and Hck—To investigate whether Bcr-Abl-Hck complex formation was due to binding of the SH2 domain of Hck to phosphorylated tyrosine residues in Bcr-Abl (similar to the binding mechanism of Grb2 to Tyr\textsuperscript{1254} or vice versa), immunoprecipitations of Bcr-Abl from COS7 cell lysates coexpressing Bcr-Abl and Hck were performed under conditions allowing dephosphorylation of cellular proteins. Dephosphorylation of cell lysates was achieved by omitting orthovanadate, a phosphatase inhibitor, from the lysis buffer, followed by preincubation of the cleared lysate at 4 °C for 24 h prior to IP. After immunoprecipitation and SDS-polyacrylamide gel electrophoresis, the precipitates were assayed by immunoblotting for coprecipitating Hck and Grb2. Dephosphorylation of the lysate resulted in nearly complete abrogation of the binding of Grb2 to Bcr-Abl (Fig. 3A, right panels, anti-Grb2 blot, lane 2). In marked contrast to Grb2, Hck was still found in a complex with Bcr-Abl, and the amount of coprecipitated Hck was even enhanced by dephosphorylation (Fig. 3A, right panels, anti-Hck blot, lanes 1 and 2). Similar results were obtained with dephosphorylation of Hck by the addition of potato acid phosphatase (not shown). Anti-Abl blotting of the precipitates indicated that similar amounts of Bcr-Abl were precipitated from both lysates (Fig. 3A, upper panels). In addition, the deletion of the coiled-coil oligomerization domain (amino acids 1–223), the complete deletion of an SH2-binding motif in bcr (A-/B-Box), and the truncation of the noncatalytical C terminus, which included binding sites for the SH2-SH3 adaptor protein CRKL and an actin-binding region, did not interfere with Bcr-Abl-src binding (Fig. 2D). In conclusion, these results suggest that the interaction of Bcr-Abl and Src kinases is not mediated by any of the known protein interaction modules of Bcr-Abl. Moreover, the co-precipitation of Hck kinase with Bcr-Abl Δ1–223 lacking the oligomerization site necessary for the constitutive activation of the Bcr-Abl tyrosine kinase (Fig. 2D), as well as the complex formation of Hck kinase with endogenous c-Abl that normally has only low kinase activity in COS7 cells, suggested that the tyrosine kinase activity of Bcr-Abl might not be indispensable for this interaction.

Dephosphorylation Enhances the Complex Formation between Bcr-Abl and Hck—To identify the different proteins at their known apparent molecular masses. Left panels, anti-Bcr-Abl, anti-Hck, and anti-Grb2 immunoblots of whole cell lysates. B, Bcr-Abl was precipitated from the same lysates used in the experiment shown in A. IPs were analyzed for precipitated Bcr-Abl (left panel). Thereafter, blots were stripped and reblotted with anti-phosphotyrosine antibodies (PY20) (right panel). C, Hck was precipitated from the same lysates used in the experiment shown in A. IPs were analyzed for precipitated Hck (left panel). Thereafter, blots were stripped and reblotted with anti-phosphotyrosine antibodies (PY20) (right panel).
blotting showed that about equal amounts of Bcr-Abl, Hck, and Grb2 were detectable in both lysates. Control experiments (Figs. 3, B and C) were performed to demonstrate that dephosphorylation of the signaling proteins involved was nearly complete. Bcr-Abl (Fig. 3B) and Hck (Fig. 3C) were precipitated from the lysates used in the experiment of Fig. 3A. Bcr-Abl and Hck blots (left panels) demonstrated that similar amounts of each protein were precipitated from the lysates using the indicated Abs. Stripping and reblotting with anti-phosphotyrosine Abs revealed that the content of phosphorylated tyrosine residues in Bcr-Abl and in Hck was substantially reduced in the dephosphorylated lysates. Similar observations were made in the bcr-abl positive human K562 cell line, where dephosphorylation enhanced the binding of Bcr-Abl to the Src family kinase Lyn (not shown). In conclusion, the results suggested that the complex formation of Src kinases and Bcr-Abl was strongly enhanced by dephosphorylation. Moreover, the interaction of Bcr-Abl and Src kinases was probably not mediated by binding of a SH2 domain to phosphorytoryne residues.

Enhanced Binding of Kinase Inactive Mutants of Hck to Bcr-Abl—The regulation of the activity of Src kinases critically depends on the phosphorylation of at least two distinct tyrosine residues (41). In Hck, C-terminal phosphorylation of Tyr501 induces a "closed" kinase inactive conformation, whereas phosphorylation of Tyr390 is necessary for full activation of the catalytic domain. Therefore, we hypothesized that the enhanced binding of Bcr-Abl to dephosphorylated Hck was due to dephosphorylation of one of these two important tyrosine residues.

To test these hypotheses, several activating (Y510F) and inactivating (K269R and Y390F) point mutations were introduced into Hck (Fig. 4A): K269R to delete the ATP-binding site of the kinase; Y390F to delete the positive regulatory autophosphorylation site of the catalytic domain; and Y501F to delete the C-terminal negative regulatory phosphorylation site. Finally, the Y501F mutation was combined with the K269R mutation. All four mutants were coexpressed with wt Bcr-Abl in COS7 cells (Fig. 4B). Bcr-Abl was precipitated from the lysates using anti-Bcr antisera (7C6). Anti-Bcr blotting showed that similar amounts of Bcr-Abl were precipitated (Fig. 4B, upper panel, lanes 1–6). Again, wild type Hck was found to co-precipitate with Bcr-Abl (Fig. 4B, lower panel, lanes 1 and 4). Co-precipitation was not enhanced with the Hck Tyr501 mutant, suggesting that "dephosphorylation" of this negative regulatory tyrosine residue was not important for the binding to Bcr-Abl. In marked contrast, co-precipitation of all three inactivating Hck mutants (K269R, Y390F, and K269R/Y501F) with Bcr-Abl was significantly increased (Fig. 4B, lanes 2, 3, and 6). Control blots of cell lysates with Hck antisera showed that similar amounts of each mutant were expressed (Fig. 4C).
These results strongly suggested that Bcr-Abl preferentially bound inactive forms of Hck kinase.

The Abl Kinase Is Not Required for Bcr-Abl-Hck Complex Formation in COS7 Cells—We asked next whether the Abl kinase was required for the interaction of Bcr-Abl with Hck. Therefore, we expressed wt Bcr-Abl or a k.n. mutant of Bcr-Abl (K1172R) with either wt or k.n. (K269R) Hck in COS7 cells. Immunoprecipitations with anti-Abl Abs showed that similar levels of wt Hck were found in complex with wt and k.n. (K1172R) with either wt or k.n. Bcr-Abl in COS7 cells. Therefore, we expressed wt Bcr-Abl or a k.n. mutant of Bcr-Abl kinase was required for the interaction of Bcr-Abl with Hck.

We asked next whether the Abl kinase bound inactive forms of Hck kinase. These results strongly suggested that Bcr-Abl preferentially might phosphorylate Tyr177 in k.n. Bcr-Abl, thus creating a phosphorylation site for Grb2. To demonstrate that Hck induced phosphorylation of k.n. Bcr-Abl, the blot from a previous experiment (Fig. 4B), co-immunoprecipitation of both wt and k.n. Bcr-Abl was stronger with k.n. Hck than with wt Hck (Fig. 5A, lower panel). Peptide controls for all precipitations demonstrated the specificity of the precipitating antibodies used (Fig. 5A, lower panel, lanes 1 and 3). As observed above (Fig. 4B), co-immunoprecipitation of both wt and k.n. Bcr-Abl was stronger with k.n. Hck than with wt Hck (Fig. 5A, lower panel). These results indicated that the complex formation of Hck with Bcr-Abl was enhanced by inactivating the Hck kinase but not the Abl kinase. Furthermore, the kinase activity of Bcr-Abl was not necessary for the complex formation with Hck kinase.

Bcr-Abl Induces Activation of Src Kinases Lyn and Hck in 32D Cells by a Kinase-independent Mechanism—Experiments on the activation of Src kinases Hck and Lyn by Bcr-Abl were difficult in COS7 cells, because both kinases showed a relatively high constitutive activation level in these cells. To address this question, we therefore had to use the murine, interleukin-3-dependent hematopoietic cell line, 32D, which shows a lower activation of Src kinases Hck and Lyn than COS7 cells. In these experiments Src kinases Lyn and Hck were immunoprecipitated from 32D cells transfected with wt or k.n. bcr-abl (K1172R). Fig. 6A shows a representative immune complex kinase assay for the Lyn kinase. Compared with control cells, both wt and k.n. Bcr-Abl induced a substantial increase of autophosphorylation of Lyn (Fig. 6A). Blots of aliquots of the IP reactions and of lysates indicated that equal amounts of precipitated Lyn were used for the assay (Fig. 6B) and that comparable amounts of Lyn were expressed in the different 32D sublines (Fig. 6C). Immune complex kinase assays with precipitated Bcr-Abl from the same cell lysates demonstrated that the k.n. Bcr-Abl had a dramatically reduced kinase activity (Fig. 6D). Again, anti-Abl immunoblotting of IP aliquots used for the assay showed that equal amounts were precipitated (Fig. 6E). Comparable results were obtained with Hck, although Hck expression was slightly lower than Lyn expression in 32D cells (not shown). In conclusion, these results further support an Abl kinase-independent mechanism for the activation of Src kinases (Hck and Lyn).

Hck Induced Association of Bcr-Abl with Grb2—Preliminary results indicated that substantial amounts of Grb2 were found in complex with Bcr-Abl, even in 32D cells expressing k.n. Bcr-Abl (K1172R), despite the fact that the docking site for Grb2, Tyr177 in Bcr, is thought to be (auto)phosphorylated by the Abl kinase. Because we had observed that k.n. Bcr-Abl activated Src kinases (Fig. 6), we hypothesized that Src kinases might phosphorylate Tyr177 in k.n. Bcr-Abl, thus creating a binding site for Grb2. To demonstrate that Hck induced phosphorylation of k.n. Bcr-Abl, the blot from a previous experiment (Fig. 5) was reprobed using the anti-phosphotyrosine Ab PY20. Fig. 7A shows that the content of phosphorylated tyrosine was dramatically reduced in k.n. Bcr-Abl (K1172R) as compared with wt Bcr-Abl (compare lanes 1 and 3 with lanes 5 and 7). However, k.n. Bcr-Abl showed a slightly increased phosphotyrosine staining, when wt Hck (but not k.n. Hck) was coexpressed, suggesting that Hck was able to induce some phosphorylation of k.n. Bcr-Abl. The same blot then was reanalyzed by anti-Grb2 blotting. As shown in the lower panel of Fig. 7A, coexpression of wt Hck induced a severalfold increase of the amount of Grb2 bound to k.n. Bcr-Abl. A larger amount of Grb2 co-precipitated with wt Bcr-Abl, indicating that Hck did not completely substitute for the Abl tyrosine kinase activity. In control blots, equal amounts of Hck and Grb2 were detected in the COS7 cell lysates used (Fig. 7B).

The SH2-binding Motif of Bcr Is Not Required for the Effects of Hck Kinase on Tyr177 Phosphorylation and Grb2 Binding—To study the effects of Hck on Tyr177 phosphorylation in k.n. Bcr-Abl (in 32D cells), the K1172R mutation was combined with the Y177F point mutation or the deletion of the SH2-binding motif (A-/B-Box). With these mutants, we wished to determine whether any of these regions was important for Tyr177 phosphorylation by Hck kinase (Fig. 8A). Previous studies indicated that isolated SH2 domains of Src kinases are able to bind to Bcr via this SH2-binding motif (A-/B-Box) in vitro (25). We therefore speculated that the binding of the Hck SH2...
domain to the A-/B-Box motif could bring the Hck kinase domain into proximity with Tyr 177, thus allowing its transphosphorylation. Fig. 8 shows an immunoblot of COS7 cells transiently transfected with these mutants. Anti-Bcr blotting of precipitated k.n. Bcr-Abl showed that similar amounts of Bcr-Abl were purified from each lysate (Fig. 8B, top panel). Only little Grb2 was detected by anti-Grb2 blotting when wt Hck was not co-expressed (Fig. 8B, bottom panel, lane 1). The expression of wt Hck induced a severalfold increase of the amount of Grb2 coprecipitating with Bcr-Abl (Fig. 8B, bottom panel, lane 2). The association of Grb2 with Bcr-Abl was completely abolished in the Y177F mutant (Fig. 8B, bottom panel, lane 3).

In marked contrast, the deletion of the A-/B-Box motif had no influence on the amount of Grb2 bound to Bcr-Abl, indicating that this region was not essential for the phosphorylation of Tyr177 by Hck (Fig. 8B, bottom panel, lane 4). In contrast to Grb2, Hck was found to coprecipitate with all three Bcr-Abl constructs (Fig. 8B, middle panel), suggesting that Tyr177 acted as a substrate but not as binding site for Hck kinase. Finally, the blot was reprobed with anti-phosphotyrosine Ab to investigate the influence of the different Bcr-Abl mutants on the tyrosine phosphorylation by Hck (Fig. 8C). Again, a slight
**Fig. 8.** The SH2-binding region of Bcr-Abl is not necessary for Hck-induced Grb2 binding. A, schematic representation of the different Bcr-Abl constructs used for the experiments. B, immunoblot analysis of IP reaction with anti-Bcr antisem (7C6) from lysates of COS7 cells transfected with the mutants shown in A. C, the blot from B (upper panel) was reprobed with anti-phosphotyrosine antibody. D, immunoblots of whole cell lysates of the same cells.
increase in tyrosine phosphorylation of k.n. Bcr-Abl was detectable when Hck was coexpressed (compare lanes 1 and 2). However, we did not find any decrease in Hck-induced phosphorylation in the two double mutants, suggesting that Tyr177 may not be the only tyrosine residue to be phosphorylated by Hck. Fig. 5D demonstrates that equal amounts of Bcr-Abl and Grb2 were expressed in all four cell lines and that no Hck was expressed in COS7 cells not transfected with the hck cDNA.

**DISCUSSION**

We have recently reported the interaction of Bcr-Abl with two Src family kinases, p53/56f and p59f in myeloid cells (38). In this manuscript, structural requirements and functional consequences of the complex formation of Bcr-Abl with Hck and Lyn kinase were investigated. Our results suggest that Hck (and Lyn) kinase might cooperate in phosphorylating a binding site for Grb2, Tyr177, in Bcr. Although we have not yet been able to identify the domain(s) necessary for the interaction of Bcr-Abl with Src kinases, experiments with dephosphorylated proteins (Fig. 3) and inactivating mutants of Hck (Figs. 4–6) gave some clues concerning potential mechanisms for this association. Src kinases are thought to be regulated by phosphorylation at distinct tyrosine residues (47). Phosphorylation of Tyr503 is necessary for full activation of Hck, whereas phosphorylation of Tyr511 is thought to negatively regulate the kinase activity by intramolecularly stabilizing an inactive conformation (39, 40). In this regard, the demonstration that dephosphorylation of cellular proteins prior to IP increased Bcr-Abl-Hck complex formation seemed particularly interesting. This observation led us to ask whether inactive Hck kinase could interact with Bcr-Abl. Therefore, we performed additional experiments using two mutations of the Hck kinase, Y390F, inactivating a critical regulatory autophosphorylation site in the kinase domain, and K269R, inactivating the catalytic ATP-binding site. Both mutants showed enhanced binding to Bcr-Abl when compared with wt Hck, confirming that Bcr-Abl preferentially bound to inactive forms of Hck. In addition, both binding and activation of Src kinases Hck and Lyn by Bcr-Abl was shown to be Abl kinase-independent, suggesting that the complex formation with Bcr-Abl alone was sufficient for activation of Src kinases. A similar mechanism has previously been reported for the activation of Lck following the complex formation with Syk in T cells (48).

The comparison of the crystallographic analysis of inactive and active forms of Src kinases has revealed that major structural differences are solely found within the kinase domain of different Src kinases (40, 49, 50). This suggests that the affinity of Src kinases for Bcr-Abl may be regulated by activation state-dependent conformational differences in this domain and that at least one interaction site in Src kinases may be the kinase domain itself, similar to the mechanism proposed for binding of Src kinases to Polyomavirus middle T antigen (51). Binding of Bcr-Abl to the kinase domain of Src kinases may then induce an alteration of the orientation of certain amino acid residues necessary for catalytic activity, finally leading to increased activity and enhanced autophosphorylation of Src kinases. A similar mechanism has previously been proposed for the induction of kinase activity of Cdk2 by Cyclin A (52, 53).

One might ask whether Src kinases are also activated by endogenous c-Abl, because the Abl tyrosine kinase activity does not seem to be necessary for Src kinase activation. Regarding this, it is important to recall that Bcr does not only alter the kinase activity of Abl but also its subcellular distribution. Although c-Abl is normally found in both the nucleus and the cytoplasm, the fusion to Bcr seems to favor its cytosolic localization (54), thus bringing it in closer proximity to Src kinases. The transfer of the (Bcr)Abl kinase to the cytoplasm is supposed to induce new signaling events, including the activation of (membrane-associated) Src kinases.

In addition to the evidence for a physical interaction of Bcr-Abl and Src kinases, our results indicate that these kinases may also functionally cooperate with each other. Co-expression of Hck with a k.n. mutant of Bcr-Abl resulted in increased tyrosine phosphorylation of Bcr-Abl. Further analysis of this Hck-induced phosphorylation of Bcr-Abl demonstrated that one tyrosine to be phosphorylated by Hck was Tyr177, resulting in increased binding of Grb2 to Abl kinase negative Bcr-Abl. A similar effect has been reported for the coexpression of Bcr and Fps/Fes tyrosine kinases in Sf9 insect cells by Maru et al. (55). In some contrast to the findings of Maru and colleagues, our kinase negative double mutants containing either a point mutation of Tyr177 or a deletion of amino acids 190–412 (comprising a total of 8 tyrosine residues) barely showed any decrease in tyrosine phosphorylation, strongly suggesting that multiple tyrosine residues within Bcr-Abl might be substrates for Src kinases. Although it is unclear whether Src kinases are necessary for the phosphorylation of tyrosine residues in wt Bcr-Abl in vivo, the phosphorylation of k.n. Bcr-Abl by activated Src kinases may explain some so far unexplained findings in 32D cells expressing high levels of k.n. Bcr-Abl. In these cells, we found substantial amounts of Grb2 in complex with k.n. Bcr-Abl, an observation that contrasts the current opinion that Tyr177 is an autophosphorylation site of the Abl tyrosine kinase. Because Src kinases were activated in these cells by an Abl kinase-independent mechanism, they were likely to substitute for the Abl kinase activity in phosphorylating Tyr177. Whether Src kinases cooperate with Bcr-Abl in activating the Ras signaling pathway remains to be determined.

Taken together, the results imply that Bcr-Abl preferentially binds inactive Hck molecules. One might hypothesize that this interaction leads to the activation of Hck by altering the three-dimensional structure of Hck. Once activated, Hck may lose its affinity for its “activation site” in Bcr-Abl and become oriented toward its substrates, one of which is located in Bcr-Abl itself. Further experiments will now have to elucidate the precise mechanism of activation of Src kinases by Bcr-Abl and the pathophysiological role of this interaction in Philadelphia chromosome positive leukemias.

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REFERENCES

1. Ben-Neriah, Y., Daley, G. Q., Mes-Masson, A. M., Witte, O. N., and Baltimore, D. (1986) Science 233, 212–214
2. Groffen, J., Stephenson, J. R., Heisterkamp, N., de Klein, A., Bartram, C. R., and Grosveld, G. (1984) Cell 36, 93–99
3. Konopka, J. B., Watanabe, S. M., and Witte, O. N. (1984) Cell 37, 1035–1042
4. Lugo, T. G., and Witte, O. N. (1989) Mol. Cell. Biol. 9, 1263–1270
5. Daley, G. Q., and Baltimore, D. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 9312–9316
6. Sawyers, C. L., McLaughlin, J., and Witte, O. N. (1995) J. Exp. Med. 181, 307–313
7. Skorski, T., Nieborowskaskorska, M., Szczylik, C., Kanakaraj, P., Perotti, D., Zan, G., Gewirtz, A., Perussia, B., and Calabretta, B. (1995) Cancer Res. 55, 2275–2279
8. Goga, A., McLaughlin, J., Apar, D. H. E., Salffran, D. C., and Witte, O. N. (1995) Cell 82, 981–988
9. Cortez, D., Kedl, L., and Pendergast, A. M. (1995) Mol. Cell. Biol. 15, 5631–5641
10. Matsuguchi, T., Salgia, R., Hallek, M., Eder, M., Druker, B., Ernst, T. J., and Griffin, J. D. (1994) J. Biol. Chem. 269, 5016–5021
11. ten Heeke, J., Arlinghaus, R., Guo, J., Heisterkamp, N., and Groffen, J. (1994) Blood 84, 1731–1736
12. Ilaria, R. L., Jr., and Van Etten, R. (1996) J. Biol. Chem. 271, 31704–31710
13. Shahi, K., Halpern, J., ten Heeke, J., Rao, X., and Sawyers, C. L. (1996) Oncogene 13, 247–254
14. Jain, S., Susa, M., Keeler, M., Carlesso, N., Druker, B., and Varticovskij, L. (1996) Blood 88, 1542–1550
15. Stattler, M., Salgia, R., Okuda, K., Uemura, N., Durrsten, M., Piasik, E. R., Xu, G., Li, J., Priaps, K., and Griffin, J. (1996) Oncogene 12, 839–846
16. Skorski, T., Kanakaraj, P., Nieborowskaskorska, M., Rajczak, M., Wen, S., Zan, G., Gewirtz, A., Perussia, B., and Calabretta, B. (1995) Blood 86,
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17. Gotoh, A., Miyazawa, K., Ohyashiki, K., Tauchi, T., Boswell, H. S., Broxmeyer, H. E., and Toyama, K. (1995) Exp. Hematol. 23, 1153–1159
18. Salgia, R., Miyaoka, K., Ernst, T., Tantravahi, R., Chen, L. B., and Griffin, J. D. (1995) J. Biol. Chem. 270, 5039–5047
19. Salgia, R., Brunkhorst, B., Pisick, E., Li, J., Lo, S., Chen, L., and Griffin, J. (1995) Oncogene 11, 1149–1155
20. Wilson-Rawls, J., Xie, S., Li, J.-L., Lo, S. H., Brunkhorst, B., Kansas, G. S., Sobhany, E. S., Sun, Y., Pisick, E., Hallek, M., Druker, B. J., Emmerich, B., and Hallek, M. (1996) Cancer Res. 56, 3589–3596
21. Hallek, M., Danhauser-Riedl, S., Herbst, R., Warmuth, M., Winkler, A., Kolb, H. J., Druker, B. J., Emmerich, B., Griffin, J. D., and Ullrich, A. (1996) Br. J. Hematol. 94, 5–16
22. Danhauser-Riedl, S., Warmuth, M., Druker, B. J., Emmerich, B., Griffin, J. D., and Ullrich, A. (1996) Br. J. Hematol. 94, 5–16
23. McWhirter, J. R., Galasso, D. L., and Wang, J. Y. J. (1993) Mol. Cell. Biol. 13, 7587–7595
24. Pendergast, A. M., Quilliam, L. A., Cripe, L. D., Bassing, C. H., Dai, Z., Li, N., Batzer, A., Rabun, K. M., Der, C. J., Schlessinger, J., and Gishizky, M. L. (1993) Cell 75, 175–185
25. Muller, A. J., Pendergast, A. M., Havlik, M. H., Puil, L., Pawson, T., and Witte, O. N. (1992) Mol. Cell. Biol. 12, 5087–5093
26. Pendergast, A. M., Muller, A. J., Havlik, M. H., Maru, Y., and Witte, O. N. (1991) Cell 66, 161–171
27. Ramakrishnan, L., and Rosenberg, N. (1989) Biochim. Biophys. Acta 989, 208–224
28. Pawson, T. (1995) Nature 373, 573–580
29. Ren, R., Mayer, B. J., Ciccotti, P., and Baltimore, D. (1993) Science 259, 1157–1161
30. Seogyeong, Z., and Cantley, L. C. (1995) Trends Biochem. Sci. 20, 470–475
31. Mayer, B., and Baltimore, D. (1994) Mol. Cell. Biol. 14, 2883–2894
32. Walkenhorst, J., Goga, A., Witte, O., and Superti-Furga, G. (1996) Oncogene 12, 1513–1520
33. Hec, T., Miyazawa, K., Feng, G.-S., Broxmeyer, H. E., and Toyama, K. (1997) J. Biol. Chem. 272, 1389–1394
34. McWhirter, J. R., Galasso, D. L., and Wang, J. Y. J. (1993) Mol. Cell. Biol. 13, 7587–7595
35. McWhirter, J. R., and Wang, J. Y. J. (1993) EMBO J. 12, 1533–1546
36. Danhauser-Riedl, S., Warmuth, M., Druker, B. J., Emmerich, B., and Hallek, M. (1996) Cancer Res. 56, 3589–3596
37. Brown, M., and Cooper, J. (1996) Biochim. Biophys. Acta 1287, 121–149
38. Ramakrishnan, L., and Rosenberg, N. (1989) Biochim. Biophys. Acta 989, 209–224
39. Pawson, T. (1995) Nature 373, 573–580
40. Sicheri, F., Moarefi, I., and Kuriyan, J. (1995) Nature 385, 602–609
41. Cooper, J., and MacAuley, A. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4232–4236
42. Boerner, R., Kassel, D., Barker, S., Ellis, B., DeLacy, P., and Knight, W. (1996) Biochemistry 35, 8519–8525
43. Daley, G. Q., Van Etten, R. A., and Baltimore, D. (1990) Science 247, 824–830
44. Druker, B. J., Okuda, K., Matulonis, U., Salgia, R., Roberts, T., and Griffin, J. D. (1992) Blood 79, 2215–2220
45. Feller, S. M., Ren, R., Hanafusa, H., and Baltimore, D. (1994) Trends Biochem. Sci. 19, 453–458
46. Cohen, G. B., Ren, R., and Baltimore, D. (1995) Cell 80, 257–248
47. Cooper, J. A., and Howell, B. W. (1993) Cell 73, 1051–1054
48. Couture, C., Baier, G., Oetken, C., Williams, S., Telford, D., Marie-Caridine, A., Baier-Bitterlich, G., Fischer, F., Burn, P., Altman, A., and Mustelin, T. (1994) Mol. Cell. Biol. 14, 5249–5258
49. Yamaguchi, H., and Hendrickson, W. A. (1996) Nature 384, 484–489
50. Moarefi, I., LaFevre-Bernt, M., Sicheri, F., Huse, M., Lee, C., Kuriyan, J., and Miller, W. (1997) Nature 385, 650–653
51. Dunant, N., Senften, M., and Ballmer-Hofer, K. (1996) J. Virol. 70, 1323–1330
52. Jeffrey, P., Russe, A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J., and Porlech, N. (1995) Nature 376, 313–320
53. Johnson, L. N., Noble, M. E. M., and Owen, D. J. (1996) Cell 85, 149–158
54. Wetzler, M., Tann, M., Van Etten, R., Hirsh-Ginsberg, C., Beran, M., and Kurzrock, R. (1993) J. Clin. Invest. 93, 1925–1939
55. Maru, Y., Peters, K. L., Afar, D. E. H., Shibuya, M., Witte, O. N., and Smithgall, T. E. (1995) Mol. Cell. Biol. 15, 835–842