

The interaction of the Bcr-Abl tyrosine kinase with the Src kinase Hck is mediated by multiple binding domains

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Bcr-Abl is found in more than 95% of cases with CML. The mechanism of Bcr-Abl-induced transformation is not fully understood. Bcr-Abl is a constitutively active tyrosine kinase with transforming capacity for hematopoietic cells. We demonstrated recently that the Src kinase Hck interacts directly with Bcr-Abl by a kinase-independent mechanism. Moreover, the inhibition of the Hck kinase seems to block some of the transforming effects of Bcr-Abl. To identify the binding domains mediating this interaction of Hck with Bcr-Abl, we co-expressed different plasmid and baculovirus vectors containing mutants or single domains of Bcr-Abl and/or Hck in COS7 and Sf9 cells. At least four independent binding regions for Hck were identified in Bcr-Abl, one in Bcr, one in the region comprising the SH3 and SH2 domain of Abl, one in the SH1 domain of Abl, and one in the C-terminal domain of Abl. In the Hck kinase, deletion of the SH2 and/or the SH3 region abolished binding to Bcr-Abl. In contrast, deletion of the Hck SH1 domain enhanced binding of Hck to Abl and Bcr-Abl. In conclusion, the results indicate that the interaction of Bcr-Abl with Hck is mediated by a novel, complex mechanism that involves multiple domains of Bcr-Abl and the SH2 and SH3 domains of Hck.

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Introduction

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder of primitive hematopoietic progenitor cells. The CML is characterized by the Philadelphia translocation t(9;22) that fuses parts of the long arm of chromosome 9 to chromosome 22 creating a hybrid gene, *bcr-abl*.¹ The protein product of this fusion, Bcr-Abl, is found in more than 95% of CML patients and represents a major causative factor of the disease.^{2–5} In contrast to its cellular counterpart, c-Abl, Bcr-Abl has an increased tyrosine kinase activity.⁶ It is preferentially located in the cytoplasm,⁷ where it is found in a complex with cytoskeletal and focal adhesion proteins like paxillin or actin.^{8,9} Overexpression of Bcr-Abl in myeloid cells activates different cellular signalling cascades, like the Ras pathway,¹⁰ the MAPK pathway,^{5,11} the Jak-STAT pathway^{12,13} and the PI3K pathway.¹⁴

We could recently show that the Src kinases Hck and Lyn are also target proteins of Bcr-Abl. Bcr-Abl binds to and activates Hck and Lyn.^{15–17} The Hck–Bcr-Abl complex formation revealed that neither the kinase activity of Bcr-Abl nor of Hck was necessary for complex formation. Moreover, the Bcr-Abl kinase activity was not required for the activation of Hck by Bcr-Abl.¹⁵

Src kinases comprise a family of nine highly homologous members which are expressed in a tissue-specific manner. They are involved in the signalling of receptor tyrosine kinases, cytokine receptors, G-protein coupled receptors or integrin receptors.^{18,19}

To characterize the mechanism of interaction of Hck with Bcr-Abl in more detail, we expressed deletion mutants of both proteins in COS7 cells and Sf9 cells using baculovirus vectors. The studies revealed a highly complex mode of interaction of Bcr-Abl with Hck involving multiple binding domains²⁰ as recently also shown by Lionberger *et al.*²¹ Moreover, dominant-negative Hck seems to block – at least in part – the transforming activity of Bcr-Abl.²¹ In addition to the results of Lionberger and colleagues, we provide here a much more detailed analysis of the interaction of Hck with Bcr-Abl by demonstrating that (1) not only Abl but also Bcr are involved in the interaction with Hck; (2) the deletion of the Hck SH1 domain leads to an enhanced binding of Hck to Abl and Bcr-Abl; and (3) the SH3 domain of Hck interacts with Bcr and Abl, while the SH1 and SH2 domains are not necessary.

Materials and methods

Antibodies and reagents

Reagents for cell lysis were purchased from Sigma Chemicals (Deisenhofen, Germany). SDS-polyacrylamide gel electrophoresis was performed with chemicals provided by Bio-Rad (München, Germany). Acrylamide/bisacrylamide was purchased from Boehringer Bioproducts (Ingelheim, Germany). Polyclonal antibodies against Hck (N-30), Bcr (N-20, 7C6), Abl (K12) and GST (Z-5) and the corresponding blocking peptides were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The monoclonal anti-Abl antibodies Ab-2 and Ab-3 were purchased from Oncogene Science (Uniondale, NY, USA). Secondary antibodies (coupled with horseradish peroxidase, ECL detection system) were purchased from Amersham (Freiburg, Germany). Primary antibodies were used at 1:1000 dilutions; except for Ab-2 and Ab-3 which were used in a 1:500 dilution; secondary antibodies were used in dilution from 1:2500 to 1:5000.

Cloning of Bcr-Abl and Hck mutants

All mutants were cloned by PCR mutagenesis. The following primers were used: 5'-GCCAATTCATATGGGGTGCATG AAG-3' (Hck-SH3 sense), 5'-CTGAATTCCTACTACTAATAGT TGCTGGGATGTAGCCCTC-3' (Hck-SH3 antisense), 5'-CCGAATTCATATGGTCCGCCGCGTTGAC-3' (Hck-SH2 sense), 5'-GAGAATTCTCAGGAAGACATGCAGG-3' (Hck-SH2 antisense), 5'-CTGAATTCATATGAAGCCCCAGAAGCCT TGGGAGAAA-3', (Hck-SH1 sense) 5'-CTGAATTCATCAT

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CATGGCTGCTGTTGGTACTGG-3' (Hck-SH1 antisense). Hck-SH23 was cloned using the Hck-SH3 sense and the Hck-SH2 antisense primers. Hck- Δ SH3 was cloned using the Hck-SH2 sense and the Hck-SH1 antisense. Hck- Δ SH2 was cloned by a two-time PCR with overlapping primers (Hck Δ SH2-OP1, -OP2) at the transition from the SH3 to the SH1 domain, 5'-CTACATCCCAAGCAACTAT/AAGCCCCAGAAGCCTTGG-3' (Hck Δ SH2-OP1), 5'-CCAAGGCTTCTGGGGCTTATAGTTGCTTGGGATGTAC-3' (Hck Δ SH2-OP2).

5'-TGGAATTCATATGGCCCTTCAGCGGCCAGTAG-3' (Abl sense), 5'-GCGAATTCTACTACCTCTGCACTATGTC-3' (Abl antisense), 5'-TAGAATTCATATGGCCCCAGAGCTGCC ACC-3' (Abl-CT sense), 5'-GCGAATTCTACTAGGGGGACAC ACCATAGAC-3' (Abl-SH23 antisense), 5'-TGGAATTCATATG AACTAGACAAGTGGGAG-3' (Abl-SH1 sense), 5'-GGGAA TTCTACTACTGCAGCAAGGTACTCACAG-3' (Abl-SH1 antisense). Abl-CT was cloned using the Abl-CT sense and the Abl antisense primer. Abl-SH23 was cloned using the Abl sense and the Abl-SH23 antisense primer.

All PCR reactions were done with the Vent polymerase (New England Biolabs, Beverly, MA, USA). All PCR fragments were completely sequenced and cloned into a pCDNA3 vector (Invitrogen, Leek, The Netherlands) for expression in COS7 cells or in the pCLV1393 and pAcG2T transfer vectors (Pharmingen, San Diego, CA, USA) for expression in the baculovirus/Sf9 system.

Cell culture

COS7 cells were grown in Dulbecco's modified Eagle's medium containing 4.5 g/l glucose and 10% fetal calf serum (Gibco, Karlsruhe, Germany). Cells were transiently transfected by lipofection using DOTAP as described previously.¹⁵ For binding studies COS7 cells were cotransfected with two different plasmids coding for the proteins investigated.

Sf9 cells were grown in TC100 medium (Gibco) supplemented with 10% pretested fetal calf serum (Gibco). Recombinant baculovirus was obtained by using the BaculoGold system (Pharmingen, San Diego, CA, USA) as described.²² Briefly, 2 μ g of plasmid DNA were cotransfected with 0.25 μ g BaculoGold DNA into Sf9 cells. After incubation at 27°C for 5 days the supernatant was collected and used for another round of infection. A dilution series of this supernatant was made and added to freshly seeded Sf9 cells for plaque assay. Appropriate plaques were picked and eluted overnight in TC100 medium. The medium was used for virus amplification. Proteins were isolated from plaques and assayed by SDS-PAGE.

For protein expression, Sf9 cells were infected with a high titer recombinant virus at a MOI of 5–10 depending on the virus stock in 75 cm² tissue culture flasks. After 3 days of incubation cells from one culture flask were lysed in 100 μ l of lysis buffer.

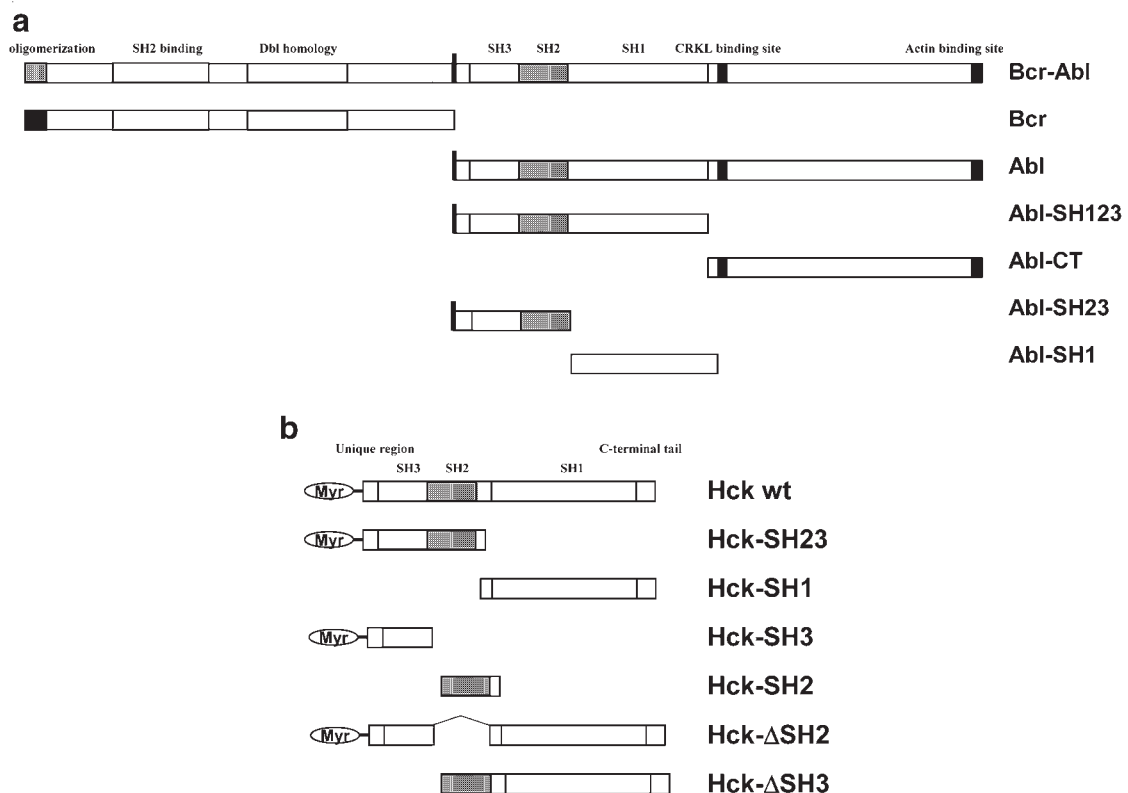


Figure 1 Structure and mutation of known functional domains of Bcr-Abl and Hck. (a) Schematic presentation of deletions introduced in Bcr-Abl. Bcr and Abl denote the corresponding parts of the Bcr-Abl protein and not the cellular counterparts. (b) Schematic presentation of deletions introduced in Hck. All cDNAs were cloned using *Eco*RI restriction sites in pCDNA3 vectors for expression in the COS7 cell system and in pVL1393 transfer vectors for expression of untagged proteins in the Baculovirus/SF9 system and in pAcG2T transfer vector for expression of GST-fusion proteins, respectively.

Co-precipitation assays and immunoblotting

Co-immunoprecipitation experiments were performed as described elsewhere,¹⁵ with the exception that only half of the amounts were used. For co-precipitation assays with GST-tagged proteins, Sf9 lysates expressing the recombinant proteins of interest were mixed (protein amounts were estimated by Western blotting and the same amounts as for the immunoprecipitation experiments were used), diluted with PBS (supplemented with 1 mmol/l phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 2 mmol/l sodium orthovanadate) to a final volume of 275 µl incubated overnight at 4°C. After 18–20 h, 100 µl of GST-sepharose beads diluted 1:1 in PBS (supplemented with 1 mmol/l phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 2 mmol/l sodium orthovanadate) were added. After an additional incubation for 2 h, the precipitates were washed three times with PBS supplemented as above. To elute specifically bound proteins precipitates were incubated with 30 µl of reduced glutathione for 1 min at room temperature and eluted proteins were separated by centrifugation (5 min, 3000 r.p.m., RT). The elution step was repeated once and eluted protein fractions were pooled.

Protein extracts were mixed with 5 × gel loading buffer, denaturated by heating for 5 min at 95°C and loaded on 10–12% SDS-PAGE gels. Gel electrophoresis and immunoblotting were performed using standard methods. After electrophoresis, proteins were transferred to a PVDF (Millipore, Eschborn, Germany) or a nitro-cellulose (Schleicher and Schüll, Dassel, Germany) membrane. After transfer membranes were blocked with 5% skim milk, incubated with a primary antibody for at least 1 h, washed and incubated for 40 min with

a horseradish peroxidase-conjugated secondary antibody. Proteins were visualized using the ECL detection system (Amersham, Freiburg, Germany) according to the instructions of the manufacturer.

Results

Hck kinase binds to Bcr as well as to the SH1 domain, the combined SH2/3 domain and the C-terminus of Abl

We recently showed that the Src kinases Hck and Lyn interact with Bcr-Abl.¹⁷ In order to elucidate the mode of interaction between Bcr-Abl and Hck, we introduced several point mutations in functional domains of the Bcr-Abl protein, like the Grb-binding site (Y177F), the SH2 domain (R1053L) or the kinase domain (Y1291F). Surprisingly, these mutations did not abolish Hck binding¹⁵ (data not shown). We therefore sought to determine the domains responsible for interaction of both proteins by expressing their functional domains or deletion mutants thereof.

First, we coexpressed the mutants of Bcr-Abl together with Hck in COS7 cells (Figure 1). Co-immunoprecipitation with an anti Hck N-30 antibody demonstrated that not only Bcr-Abl but also Bcr and Abl alone interacted with Hck (Figure 2). The specificity of the binding was demonstrated by using blocking peptides for the Hck antibody, which completely abolished Hck binding. Co-immunoprecipitation with proteins comprising the Abl SH1, 2 and 3 domains (AblSH123) or the Abl C-terminal domain (Abl CT) revealed that both domains of Abl interacted with Hck. These results indicate

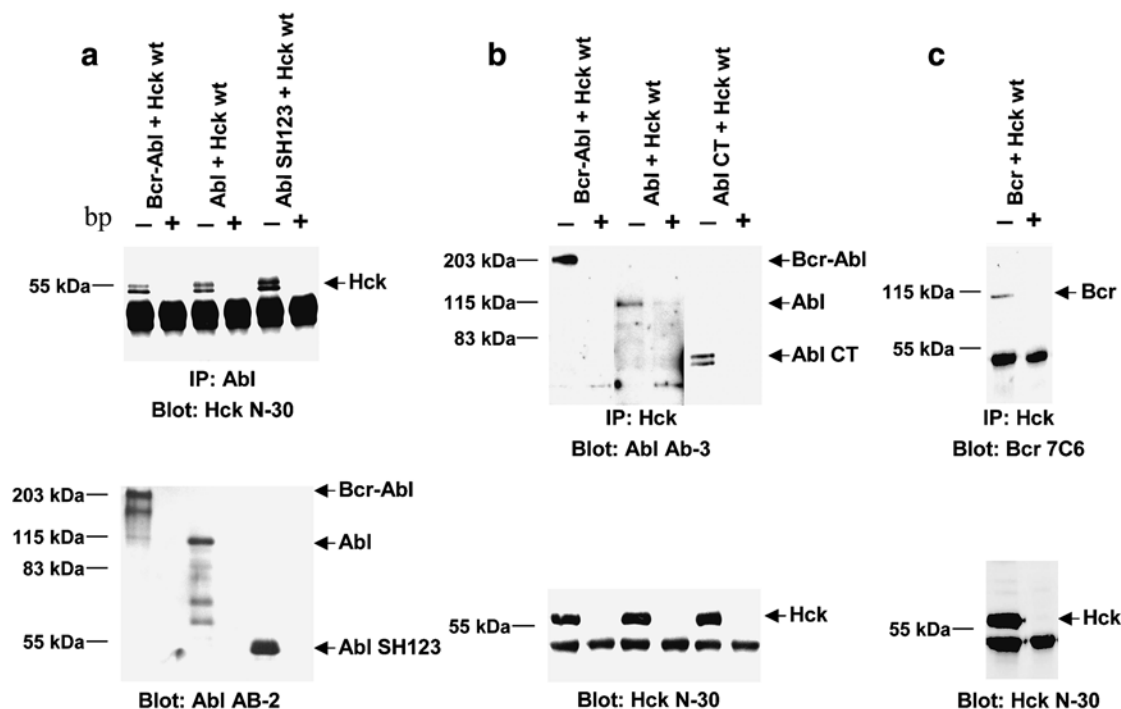


Figure 2 Interaction of Bcr-Abl and Hck in the COS7 cell system. Hck was transiently transfected in Cos7 cells with the Bcr-Abl constructs as indicated. (a) Cell lysates were used for immunoprecipitation with an anti-Abl antibody and subsequent Western blot analysis with anti-Hck (upper panel) and anti-Abl (lower panel) antibodies. (b) Cell lysates were used for immunoprecipitation with an anti-Hck antibody and subsequent Western blot analysis with anti-Abl (upper panel) and anti-Hck (lower panel) antibodies. (c) Cell lysates were used for immunoprecipitation with an anti-Hck antibody and subsequent Western blot analysis with anti-Bcr (upper panel) and anti-Hck (lower panel) antibodies. Specific blocking peptides (bp) against the antibodies used for immunoprecipitation were added to test for the specificity of the complex formation.

SPOTLIGHT

that at least three regions of Bcr-Abl were able to interact with Hck, namely the Abl SH123 region, the Abl C-terminus²¹ and Bcr.

We then used the baculovirus/Sf9 expression system for further examination of the binding mode. Abl, Abl-SH123 and Abl-CT cDNAs were cloned in the pAcG2T transfer vector, which allowed the expression of fusion proteins with an N-terminal GST-tag. The Hck cDNA was cloned in the pVL1393 transfer vector for expression of untagged Hck protein. In contrast to COS7 cells, proteins were not co-expressed in Sf9 cells, but cell lysates of single transfectants were used for further studies, because Hck expression was not consistent in double transfectants despite the use of equal concentrations (MOI) of recombinant baculovirus. Conditions for binding studies were optimized to obtain similar protein concentrations as with COS7 lysates. Glutathione bead-mediated precipitation of preincubated GST-tagged Abl mutant proteins and untagged wt Hck confirmed that wt Hck was able to form complexes with Abl, Abl-SH123 and Abl-CT (Figure 3). Anti-GST immunoprecipitation with the same proteins revealed similar results (data not shown). Further examination of the Abl-SH123 region using smaller fragments revealed that an Abl-SH23 fragment and the Abl kinase domain formed complexes with wt Hck (Figure 3). In addition, Bcr also interacted with Hck kinase. Taken together, these results indicate that at least four regions of Bcr-Abl carry binding motifs for Hck, suggesting that a new, very complex mechanism mediates the interaction of both proteins. A comparable, complex interaction was recently described for Bcr-Abl and RIN1 (Ras

interaction/interference), a protein interfering with Ras activity in yeast.²³ RIN1 interacts with the SH3, SH2 and kinase domain of Bcr-Abl. In contrast to the Bcr-Abl Hck interaction, however, the physical interaction of RIN1 with Bcr-Abl was blocked by a deletion of the Abl SH3 domain.

The SH3 domain but not the SH2 or SH1 domains of Hck kinase are required for the binding to Bcr or Abl

We then wanted to determine which Hck domain(s) bound to Bcr-Abl. For this purpose, the combined Hck SH2/3 domains and the SH1 domain were expressed as GST fusion proteins (Figure 4a). Co-precipitation with wt Bcr-Abl, Bcr and Abl indicated that none of them interacted with the Hck kinase (Hck SH1) domain. Surprisingly, both Bcr-Abl and Abl alone showed an enhanced binding with the Hck-SH2/3 deletion mutant, confirming earlier observations of our group using Hck point mutants.¹⁵ The two kinase-deficient Hck mutants, K269R and Y390F, showed a significantly increased binding to Bcr-Abl. The deletion of the entire Hck kinase domain also showed an enhanced binding to Bcr-Abl. This effect seemed to be mediated by Abl, because Bcr alone did not show an increased binding to Hck-SH2/3 (Figure 4a). Stripping and reblotting the membranes with a GST antibody showed that similar amounts of the GST fusion proteins were precipitated (Figure 4a).

Next, we wanted to explore whether the enhanced binding was mediated by the Hck SH2 or Hck SH3 domain. We there-

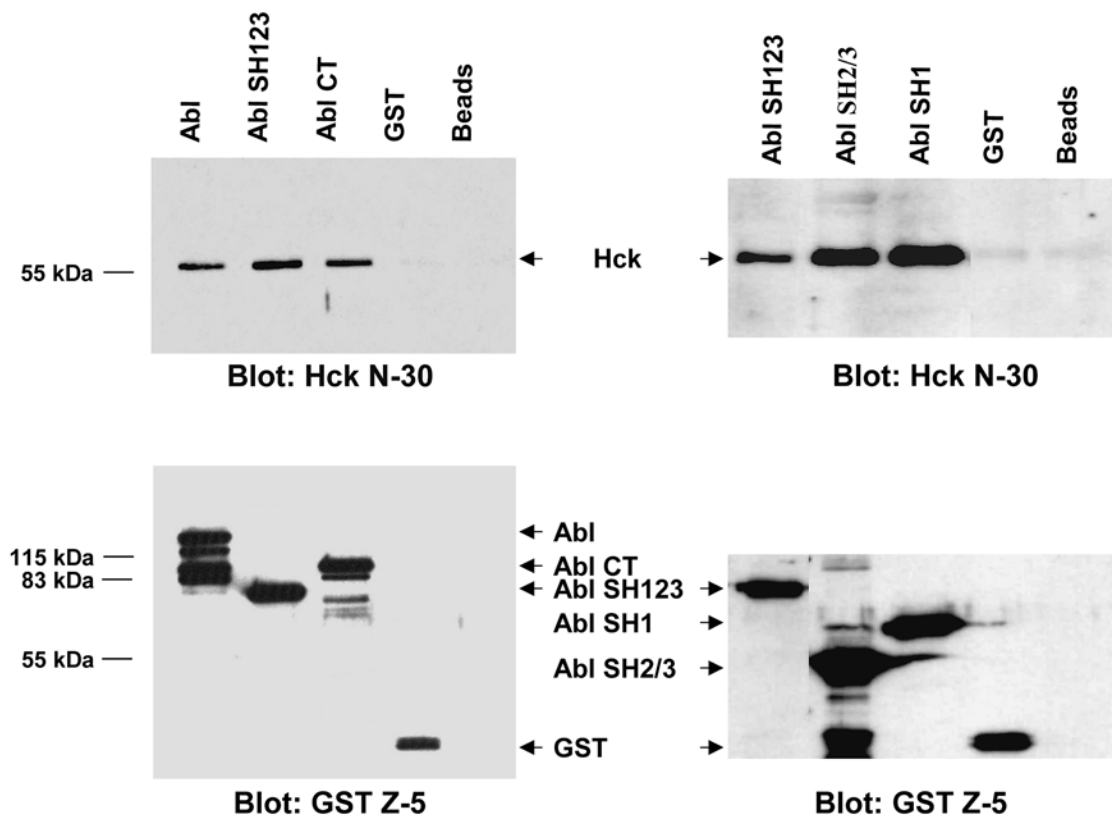


Figure 3 Interaction of Abl and Hck in the baculovirus/Sf9 system. Lysates of Sf9 cells expressing GST-tagged Abl mutants were mixed with Sf9 lysates expressing untagged Hck. Proteins were precipitated with glutathione beads and bound proteins were eluted with reduced glutathione and detected by Western blotting. Left panels: Hck is precipitated by Abl-SH123 and Abl-CT as detected with an anti-Hck antibody (upper panel). The amount of precipitated fusion proteins was controlled with an anti-GST antibody (lower panel). Right panels: Both Abl-SH23 and Abl-SH1 can precipitate Hck (upper panel), control with an anti-GST antibody (lower panel)

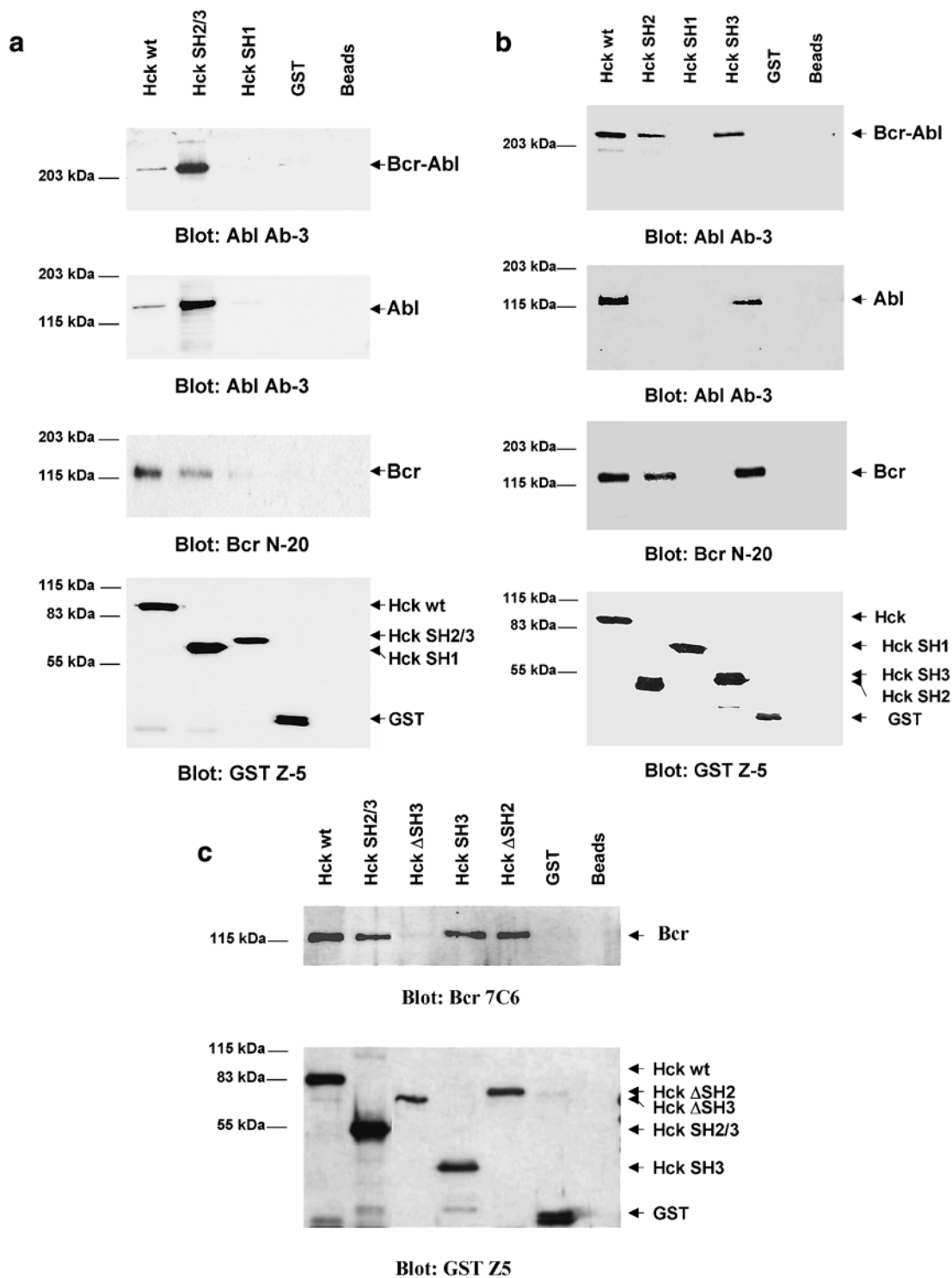


Figure 4 Interaction of Bcr-Abl and Hck in the baculovirus/Sf9 system. Lysates of Sf9 cells expressing GST-tagged Hck mutants were mixed with Sf9 lysates expressing untagged Bcr-Abl, Abl or Bcr. The amount of precipitated fusion proteins was controlled with an anti-GST antibody. In (a) and (b) only one control is shown, as all three controls were comparable. (a) The Hck-SH2/3 domain showed an enhanced binding to Bcr-Abl and Abl but not to Bcr. (b) The Hck-SH3 domain binds to Bcr-Abl, Abl and Bcr while the Hck-SH1 domain does not bind any of these proteins. (c) Deletion of the Hck-SH2 domain does not influence binding to Bcr but deletion of the Hck-SH3 dramatically reduces binding to Bcr.

fore expressed both domains as GST fusion proteins in Sf9 cells and tested the complex formation with Bcr-Abl, Abl and Bcr (Figure 4b). Both individual Hck domains interacted with Bcr-Abl. The SH2 domain bound to Bcr only and not to Abl, while the Hck SH3 domain interacted with both Bcr and Bcr.

This indicates that the Hck SH3 domain plays a major role in binding of Hck to Bcr-Abl.

In order to analyze the role of the Hck-SH2 domain in more detail, an Hck mutant containing a deletion of the SH2 domain was generated and co-precipitated with Bcr by GST-

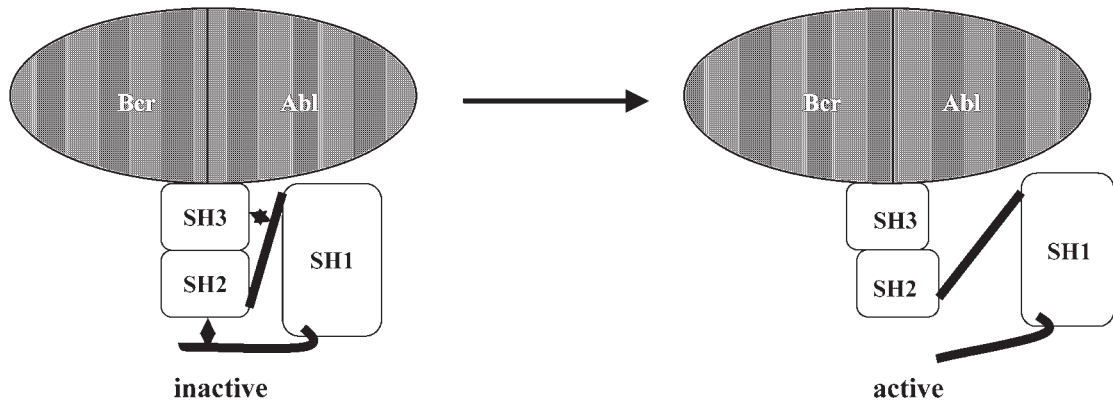


Figure 5 Bcr-Abl mediates activation of Hck. Binding of Bcr-Abl to the Hck-SH3 domain destabilizes the inactivating interaction of the Hck-SH3 domain with the SH2-SH1 linker and between the SH2-domain and a phosphotyrosine residue located in the C-terminal tail. This leads to an open conformation and activation of Hck.

pull down assays (Figure 4c). This Hck mutant showed binding to Bcr comparable to the Hck SH3 domain and Hck SH2/3 domain (Figure 4c). The deletion of the Hck SH3 domain drastically reduced binding to Bcr. Longer exposure of the ECL film revealed that small amounts of Bcr could be precipitated with the Hck-SH21 mutant (data not shown). These results suggested that the Hck SH2 domain was not involved in the interaction of Bcr with Hck kinase. In comparison with the combined Hck SH2 and SH3 domains, binding of the single Hck domains, SH2 and SH3, was reduced at least two-fold (Figure 4b). It is possible that the combined SH2 and SH3 domains adopt a conformation, which supports Bcr-Abl binding more than the separated SH2 or SH3 domains of Hck. The results with regard to the SH2 domain may appear contradictory, because the isolated SH2 domain of Hck seems to interact with Bcr, while the deletion of the same SH2 domain in Hck does not negatively affect binding of Hck to Bcr. However, it is possible that the isolated SH2 domain exposes regions that are normally not accessible in wt Hck, creating new *in vitro* binding sites for Bcr. Taken together, the results show that the SH3 but not the SH2 domain of Hck is necessary for the interaction with Bcr and Abl.

Discussion

The findings presented in our study show that binding of Hck to Bcr-Abl occurs in a close inactive conformation of Hck. It is mediated by the Hck-SH3 domain, which makes multiple contacts with Bcr-Abl. This physical interaction might destabilize the intramolecular interaction in Hck, which is designed to stabilize a closed, inactive conformation, supporting the interactions of the Hck-SH3 domain with the SH2-SH1 linker and of the SH2 domain with the C-terminal tail.²⁴ After this destabilization, Hck adopts an open conformation leading to the activation of its catalytic domain (Figure 5) and to the phosphorylation of potential Hck substrates, one of which is Bcr-Abl. The activated Hck kinase displays a lower affinity to Bcr-Abl than inactive Hck. This might lead to rapid dissociation of the complex. Activation of Hck is mediated by the physical contact with Bcr-Abl, but not by the Bcr-Abl kinase activity. Similarly, Moarefi *et al.*²⁵ have shown that binding of HIV-1 protein Nef to the Hck SH3 domain leads to full activation of Hck.

Work presented here also confirms some results of Lion-

berger *et al.*²¹ who showed that Bcr-Abl and Hck interact by a novel and highly complex mechanism so far not described for the interaction of Bcr-Abl with other signalling molecules. Due to the complexity of this binding mechanism, it will be difficult to explore the function of this interaction by any mutagenesis approach, because the disruption of multiple binding sites will not allow conservation of the structure of Bcr-Abl. Therefore, alternative strategies are necessary to study the biological relevance of the Bcr-Abl-Hck interaction for the pathogenesis of CML. One such strategy is the use of dominant-negative mutants.²¹ Another strategy is the use of chemical inhibitors. In recent experiments using the Src kinase specific inhibitor, PP1, we observed that inhibition of Src kinases shortened the survival of Bcr-Abl-positive myeloid cells, but not Bcr-Abl-negative myeloid cells.²⁶ We also showed that PP1 co-operated with the Abl-kinase inhibitor STI 571 to induce apoptosis in these cells. This suggests that the combined inhibition of Src and Abl kinases may be superior to the inhibition of Abl alone for the treatment of Ph-positive leukemias.

Acknowledgements

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