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The SH2-containing adapter protein GRB10 interacts with BCR-ABL

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Bcr-Abl is an oncogenic tyrosine kinase expressed in tumor cells of CML and a subset of ALL which in its unregulated and activated state is thought to cause cell transformation and leukemia. Bcr-Abl contains several autophosphorylation sites which serve as potential docking sites for SH2-containing signaling molecules. Mutational analysis has indicated that these autophosphorylation sites play a critical role in the transforming capability of Bcr-Abl. It has been shown that the SH2-containing adapter protein Grb2 binds to the autophosphorylation site Tyr(p)177 whereby it couples Bcr-Abl to the Ras pathway. The biological consequences of this interaction, however, are presently unclear. A Tyr177-mutated Bcr-Abl which lacks the ability to interact with the Grb2-SH2 domain still transforms myeloid cells and generates tumors in nude mice. We performed a yeast two-hybrid screen to identify signaling proteins which bind to distinct Bcr-Abl autophosphorylation sites. Autophosphorylation of Bcr-Abl in yeast was accomplished by using the DNA binding protein LexA which permits dimerization and crossphosphorylation of the fused bait. Using a LexA-Bcr-Abl full length fusion protein as bait, we identified several SH2-containing proteins. Among them we confirmed molecules already shown by others to interact with Bcr-Abl, *in vivo*, including Grb2, PI-3-kinase and Crk indicating that dimerization in yeast leads to autophosphorylation of tyrosine residues crucial for Bcr-Abl signaling *in vivo*. More importantly, we identified the SH2-containing protein Grb10 as a new binding partner for Bcr-Abl. This binding occurs in a phosphotyrosine-dependent manner at Bcr sites of Bcr-Abl. Both Abl and Bcr alone, as well as a kinase-defective Bcr-Abl, failed to interact with Grb10 in yeast. Mutational analysis uncovered a new SH2 binding site in Bcr-Abl located between Bcr aa242-446, which is different from the Grb2 binding site. Binding could be demonstrated *in vitro* and also *in vivo* as shown by co-immunoprecipitation analysis in CML cells. Using a temperature sensitive Bcr-Abl stably overexpressed in hematopoetic cells, we demonstrated that complex formation of Grb10 with Bcr-Abl was kinase activation-dependent *in vivo*. Notably, a Bcr-Abl mutant protein (Bcr/1-242-Abl) which lacks the ability to interact with Grb10 partially alleviated IL-3 dependence of Ba/F3 cells, indicating that the Grb10/Bcr-Abl interaction is important for Bcr-Abl-induced IL-3 independence of Ba/F3 cells. In addition, the Bcr/1-242-Abl mutant has a reduced capacity to induce focus formation in fibroblasts.

Keywords: SH2; CML; Grb10; Bcr-Abl; yeast two-hybrid; tyrosine kinase; signal transduction

Introduction

Oncogenes transform cells by virtue of their unregulated and activated tyrosine kinase. Substrate phosphorylation and autophosphorylation leads to constitutive association of the activated tyrosine kinase with intracellular signaling molecules. This association appears to be mediated by interactions of autophosphorylated tyrosine residues with SH2-containing signaling molecules which either exhibit catalytic activity on their own or serve as adapter molecules for proteins with catalytic activity capable of initiating signaling cascades that lead to proliferation and transformation of the cell (Pawson, 1995). Chronic myelogenous leukemia (CML) and a subset of acute lymphoblastic leukemia (ALL) are clonal proliferative disorders associated with the t(9;22) translocation whereby the breakpoint cluster region (bcr) on chromosome 22 juxtaposes to the c-Abl gene on chromosome 9. The chimeric gene generates a fusion protein containing the first 927 or 902 aminoacids (aa) of Bcr in CML, while in ALL only the first 426 aa of Bcr are fused to c-Abl (Chan *et al*., 1987; de Klein *et al*., 1982; Kurzrock *et al*., 1987). Both Bcr-Abl fusion proteins exhibit an increased tyrosine kinase activity and their oncogenic potential has been demonstrated using *in vitro* cell culture systems as well as in *in vivo* mouse models (Daley and Baltimore, 1988; Gishizky and White, 1992; Lugo *et al*., 1990; Lugo and Witte, 1989; McLaughlin *et al*., 1987; Young and Witte, 1988).

SH2-containing molecules known to interact with Bcr-Abl include the adapter molecule Grb2 and SHC (Pendergast *et al*., 1993b; Puil *et al*., 1994; Tauchi *et al*., 1994a), the phosphotyrosine phosphatase Syp (Tauchi *et al*., 1994b) and the regulatory subunit (p85) of phosphatidylinositol 3′-kinase (p85PI3K) (Skorski *et al*., 1995).

It has been shown recently, that the association of Bcr-Abl with the Ras adapter molecule Grb2 correlates with activation of the Ras pathway in Bcr-Abl transformed fibroblasts (Pendergast *et al*. 1993b; Puil *et al*., 1994; Tauchi *et al*., 1994a). Mutation of the Grb2 binding site in Bcr-Abl at position tyrosine 177 (Y177) to phenylanaline (F177) is associated with reduction of the transforming activity of Bcr-Abl in

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fibroblasts (Pendergast *et al*., 1993b; Puil *et al*., 1994). Furthermore, expression of a dominant-negative Grb2 in CML cells could reverse the transformed phenotype (Gishizky *et al*., 1995). It has, therefore, been hypothesized that Bcr-Abl through binding to the Ras adapter molecule, Grb2, leads to constitutive Ras activation and, thereby, transformation of the cell. However, the significance of this Bcr-Abl/Grb2 interaction for the transforming activity of Bcr-Abl is unclear as Bcr-Abl mutants which lack the Grb2 binding site can still activate Ras leading to a mitogenic signal in some cellular systems (Cortez *et al*., 1995; Goga *et al*., 1995). Therefore, it was speculated that redundant signaling pathways must exist for Bcr-Abl to induce a mitogenic signal. It is clear in all biological model systems tested so far that the potential of Bcr-Abl to transform cells requires an upregulated and active tyrosine kinase indicating that autophosphorylation and/or substrate phosphorylation is indispensable for Bcr-Abl transformation. Since Grb2 binding to Bcr-Abl at Tyr(P)177 seems to be insufficient for transformation of hematopoietic cells, we have attempted to isolate additional SH2-containing proteins binding to Bcr-Abl at different autophosphorylation sites which may contribute to the mitogenic signal initiated by Bcr-Abl. To screen for phosphotyrosine-dependent protein–protein interactions, we performed a modified yeast two-hybrid screen with Bcr-Abl. Autophosphorylation of Bcr-Abl in this modified yeast system was accomplished by using the DNA-binding protein LexA that leads to dimerization and crossphosphorylation of the fused bait (O'Neill *et al*., 1994; Weidner *et al*., 1996). In this article, we describe the identification of Grb10, a novel SH2-containing protein, which interacts with Bcr-Abl in yeast, *in vitro* and *in vivo*. Grb10 was recently cloned by screening of an expression library with the tyrosine-phosphorylated carboxy-terminus of the epidermal growth factor receptor (EGFR) (Ooi *et al*., 1995). Three distinct splice variants of Grb10 have been described (mGrb10, hGrb-IR, hGrbsvIR) (Liu and Roth, 1995; O'Neill *et al*., 1996; Frantz *et al*., 1997). Grb10 belongs to an emerging family of SH2-containing signaling molecules including Grb7 and Grb14. These family members share a SH2 domain, a pleckstrin domain and a proline-rich region (Ooi *et al*., 1995; Daly *et al*., 1996). The precise function for these SH2-containing adapter molecules has not been fully elucidated. However, the existence of highly conserved regions known to be of potential importance for protein–protein interactions suggests a role for these adapter proteins in growth regulation. Despite its cloning in conjunction with the EGF receptor, Grb10 could not be shown to bind to the EGF receptor *in vivo* (Ooi *et al*., 1995). In contrast, recently an *in vivo* association of Grb10 with the Ret receptor, the Insulin receptor and the ELK receptor was demonstrated suggesting that Grb10 may play a role in the biology of these receptors (Hansen *et al*., 1996; Pandey *et al*., 1995; Stein *et al*., 1996; Frantz *et al*., 1997; O'Neill *et al*., 1996).

Binding of Grb10 to Bcr-Abl is kinase-dependent and occurs between the Grb10 SH2 domain and a Bcr-Abl autophosphorylation site that is different from Tyr(P)177. This interaction was confirmed *in vitro*, *in vivo*, i.e. in lymphoid cells overexpressing Bcr-Abl as well as in CML cells obtained from a patient in CML blast crisis (K562). No tyrosine phosphorylation of Grb10 was observed after complex formation with Bcr-Abl in various cells investigated. McWhirter *et al*. (1993) showed that a mutant lacking the Grb10 autophosphorylation site had a reduced capacity to transform Rat-1 fibroblasts. In line, herewith, the Grb10-Bcr-Abl complex also seems to be important for Bcr/Abl-induced interleukin-3 (IL-3) independent growth of Ba/F3 cells. These data indicate that the adapter molecule Grb10 plays a role in the Bcr-Abl mediated oncogenicity.

Results

Bcr-Abl binds to Grb10 in the yeast two-hybrid system

A yeast two-hybrid screen was performed to identify proteins which interact with Bcr-Abl in a phosphotyrosine-dependent manner. To achieve autophosphorylation of the bait in yeast, Bcr-Abl was fused to LexA, a DNA binding protein which leads to dimerization and subsequent phosphorylation of the fused bait (Weidner *et al*., 1996). Interaction of two proteins in this system allows for growth on histidine-free medium and for expression of β-galactosidase. A mouse embryo c-DNA library was screened with Bcr-AblΔSal fused to LexA (the actin-binding domain in Bcr-Abl was deleted to avoid the isolation of actin coding clones). Expression and phosphorylation of the transfected Bcr-Abl constructs in yeast was verified by Western blotting analysis (data not shown) and the clone displaying the highest autokinase activity was subsequently used. 3×10^7 colonies were screened and 62 clones were isolated which specifically interacted with Bcr-Abl in the yeast system. Among these clones were proteins already known to bind to Bcr-Abl both in a phosphotyrosine-dependent and -independent manner like the SH2 domains of Grb2 and p85PI3K, and the SH3 domain of Crk, respectively (Pendergast *et al*., 1993b; Puil *et al*., 1994; Ren *et al*., 1994). Ten of the clones obtained coded for the novel SH2-containing adapter molecule Grb10 (Ooi *et al*., 1995). Six out of these ten clones represented individual Grb10 sequences as shown in Figure 1. All the Grb10 clones isolated included the SH2 domain of Grb10 (Figure 1) suggesting that the interaction takes place between the SH2 domain of Grb10 and a Tyr(P) autophosphorylation site in Bcr-Abl. Interaction of Bcr-Abl and Grb10 in yeast was specific since none of the Grb10 clones interacted with a nonspecific control protein (lamin, data not shown). In an independent yeast screen utilizing a cDNA library obtained from a CML cell line (K562), we again identified the SH2 domain of a human splice variant of Grb10 (hGrb10svIR) (data not shown). Thus, in two independent yeast two-hybrid screens, one of which using a human CML cell line cDNA library, Grb10 was identified as a binding partner of Bcr-Abl. To determine whether this interaction was autophosphorylation-dependent, a kinase-defective Bcr-Abl (Bcr-AblKD) was assessed for its interaction with the Grb10 SH2 domain (clone B17) along with the Grb2-SH2 domain as negative and the Crk-SH3 domain as positive control (Figure 2a). While the wt-Bcr-Abl bound to all three constructs, the

kinase defective Bcr-Abl failed to induce histidine autotrophy in yeast with the Grb2-SH2 and the Grb10-SH2 domains demonstrating the phosphotyrosine-dependency of these interactions. The interaction of Grb10 with Bcr-Abl in yeast was comparable in strength both by histidine autotrophy (Figure 2a) and

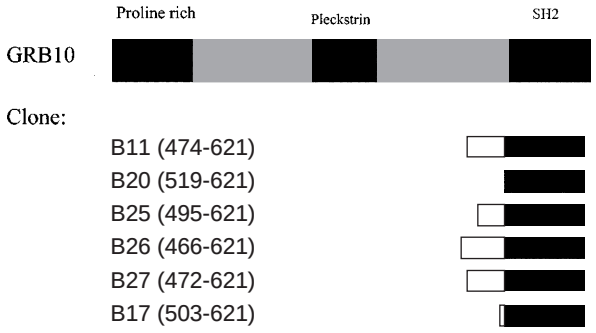

Figure 1 Bcr-Abl selects the SH2 domain of Grb10 in a yeast two-hybrid screen. The Grb10 full-length structure with a N-terminal proline rich region, a middle pleckstrin domain and a C-terminal SH2 domain is shown. Bcr-Abl selected six independent clones of Grb10 all coding for the SH2 domain of Grb10. The aa-numbers for the individual clones are indicated

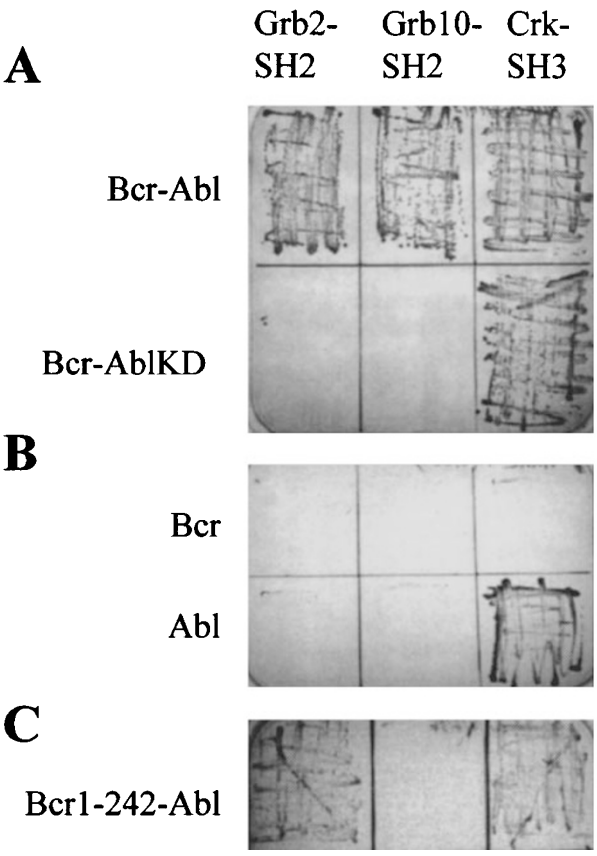

Figure 2 Grb10-SH2 domain binds to a Bcr-Abl autophosphorylation site located between Bcr aa243-509. (**a**) Bcr/1-509-AblΔSal (Bcr-Abl) and a kinase-defective Bcr/1-509-AblΔSal (Bcr-Abl KD) or (**b**) Bcr/1-509 and Abl or (**c**) Bcr/1-242-AblΔSal were transfected into yeast together with the SH2 domains of Grb2 and Grb10 and the SH3 domain of Crk. Co-transfected yeast were plated onto agar lacking histidine. Growth on this medium indicate interaction of the co-transfected constructs

β-galactosidase activity (data not shown). As expected, the Crk-SH3 domain which is known to bind to a proline rich sequence in the Abl C-terminus (Feller *et al*., 1994a; Ren *et al*., 1994), still showed interaction with the kinase-defective Bcr-Abl in yeast (Figure 2a). Thus, binding of Grb10 to Bcr-Abl is mediated by an interaction between the Grb10-SH2 domain and a Bcr-Abl autophosphorylation site.

To define the region of interaction more closely, the mutants listed in Figure 2b and c were expressed in yeast and examined for their interaction with the Grb10-SH2 domain. Bcr alone (Bcr aa1-509), as well as Abl alone (Abl aa1-1097), failed to interact with Grb10 suggesting that the interaction took place at a phosphorylation site in Bcr within Bcr-Abl (Figure 2b). As expected the Grb2-SH2 domain also failed to interact with Abl or Bcr alone while the Crk-SH3 domain bound to the Abl construct. Figure 2c shows the interaction of Bcr1-242/Abl which carries a deletion of Bcr aa243-509 but still possesses the known autophosphorylation site Tyr(P)177. Therefore, this construct retains the ability to bind to the Grb2-SH2 and the Crk-SH3 domains but is not capable of binding to the Grb10-SH2 domain (Figure 2c). Thus, the autophosphorylation site for the Grb10-SH2 domain differs from that for the Grb2-SH2 domain (Tyr(P)177) and is located between Bcr aa243-509.

Bcr-Abl specifically interacts with the Grb10 SH2 domain in vitro

To confirm the yeast binding results *in vitro*, an *in vitro* binding assay was performed. Bcr-Abl mutants were *in vitro*-translated and autophosphorylated and assessed for their ability to bind to a bacterially purified GST-GRB10-SH2 fusion protein. As expected, the Bcr-Abl mutants harboring Bcr sequences aa243-509 bound to the Grb10 SH2 domain (Figure 3a, middle panel, lanes 1, 3 and 9), whereas the Bcr-Abl mutants carrying a deletion of this region (Bcr1-242/Abl, Bcr1-175/Abl), failed to bind to the Grb10-SH2 domain (Figure 3a, middle panel, lanes 5 and 7). The Bcr1-446/Abl mutant bound Grb10 and therefore the interaction region could be further delineated to Bcr aa243-446. Consistent with the fact that the Grb2 binding site is at Tyr177, the Grb2-SH2 domain bound to Bcr/1-509-Abl, Bcr/1-446-Abl and Bcr/1-242-Abl but not to Bcr/1-175-Abl or Bcr/177FAbl (Figure 3a, upper panel, lanes 1, 3, 5, 7 and 9). All binding reactions were specific in as much as GST alone did not bind to any Bcr-Abl construct (Figure 3a, lower panel). Furthermore, the Grb10-Bcr-Abl interactions appeared to be at least as strong as the Grb2-Bcr-Abl interaction *in vitro* corroborating the results obtained in yeast (Figure 3a, compare the bound and flow-through fractions of the upper panel and the middle panel, lanes 1–4). The Bcr region 243–446 contains seven potential autophosphorylation sites. In an attempt to identify the exact tyrosine residue responsible for binding to the Grb10-SH2 domain phosphopeptide competition experiments with ten amino acid peptides encompassing the seven autophosphorylation sites were performed. However, none of the peptides even at high concentration (400 μM) was able to block the binding of the Grb10-SH2 domain to Bcr-Abl (data not shown). We also constructed a series of tyrosine to phenylalanine Bcr-Abl mutants including

Bcr-Abl231F, Bcr-Abl246F, Bcr-Abl276F, Bcr-Abl283F, Bcr-Abl316F, Bcr-Abl328F, Bcr-Abl360F and Bcr-Abl436F. None of these mutants lost the ability to bind to the Grb10-SH2 domain *in vitro* (Figure 3b and data not shown). The inability to exactly determine a single Bcr-Abl autophosphorylation site responsible for Grb10 binding was also reported by several other investigators (Frantz *et al*., 1997; O'Neill *et al*., 1996). Therefore, the binding site of the Grb10-SH2 domain seems to be more complex and might involve several autophosphorylation sites or additional sequences in the Brc region aa243-446.

Absorption of endogenous Bcr-Abl from CML cells to Grb10

Binding of Grb10 to Bcr-Abl requires autophosphorylation of a new autophosphorylation site *in vitro* and in yeast. To test whether this binding also occur *in vivo*, we examined Bcr-Abl-Grb10 complex formation in CML cells (K562).

Binding of full length Bcr-Abl (210kd Bcr-Abl) could be demonstrated with the GST Grb10-SH2 domain selecting Bcr-Abl from these cell lysates (Figure 4a, left panel), while an unrelated SH2 domain (Nck-SH2) or a control protein (GST) showed no binding (Figure 4a, left panel).

Grb10/Bcr-Abl complex formation in CML cells in vivo

To determine whether a Bcr-Abl/Grb10 complex existed *in vivo* in leukemic cells co-immunoprecipitation experiments were performed with CML cells obtained from a patient in blast crisis (K562). As shown in Figure 4b, the Grb10 antibody is able to precipitate Bcr-Abl from these CML cells. No endogenous c-Abl was detectable in the Grb10 co-immunoprecipitation analysis (Figure 4b, left panel). In line herewith, the Grb10 antibody failed to precipitate any endogenous c-Abl in a Bcr-Abl negative leukemic cell line (HL-60) (Figure 4b, middle panel) though both cell lines do express Grb10 (Figure 4b, right panel). These experiments confirm the binding results in yeast and *in vitro* and suggest that the *in vivo* interaction of Bcr-Abl and Grb10 occurs at an autophosphorylation site within the Bcr sequences. Co-

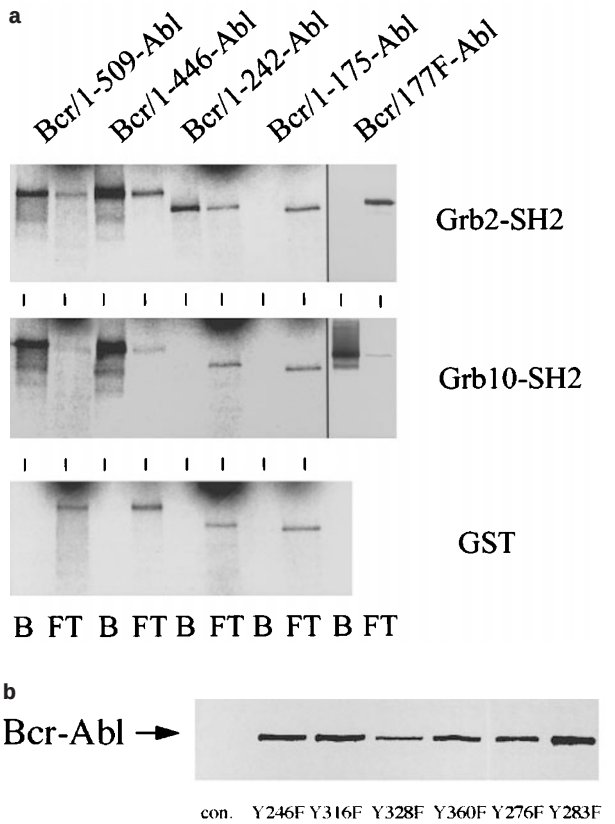

Figure 3 Grb10-Bcr-Abl complex formation *in vitro*. (**a**) Bcr-Abl mutants as indicated were *in vitro* translated and ^{35}S-methionine-labeled. 50 μl of the translation mix was first allowed to autophosphorylate *in vitro* and then incubated with 5 μg purified GST-Grb2-SH2 (upper panel), 5 μg purified GST-Grb10-SH2 (middle panel) or GST protein alone (lower panel). The bound (B) and the flow through fractions (FT) were separated by SDS–PAGE and the Bcr-Abl mutants visualized by autoradiogram. (**b**) Bcr-Abl tyrosine to phenylalanine mutants as indicated were *in vitro* translated and ^{35}S-methionine-labeled. 50 μl of the translation mix was first allowed to autophosphorylate *in vitro* and then incubated with 5 μg purified GST-Grb10-SH2 and GST-Nck-SH2 (Con.) as control. The bound fractions were separated by SDS–PAGE and the Bcr-Abl mutants visualized by autoradiography

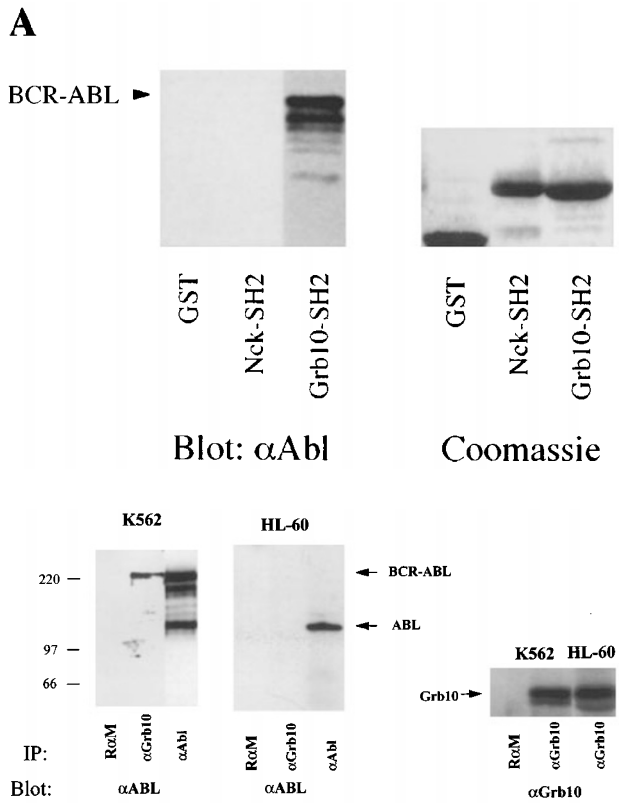

Figure 4 (**a**) The Grb10-SH2 domain selects Bcr-Abl from CML cells. Lysates from 1×10^7 K562 cells were incubated either with 5 μg of GST-Grb10-SH2, GST-NCK-SH2 or GST at 4°C for 1 h. The bound protein complexes were collected with glutathione-beads, washed thoroughly and resolved by SDS–PAGE. Immunoblotting was performed with an anti-Abl antibody(8E9) detecting both Abl and Bcr-Abl (left panel). An identical gel was also stained with Coomassie blue to indicate the amount of GST fusion protein in each binding reaction. (**b**) Bcr-Abl forms complexes with Grb10 *in vivo*. Lysates of K562 cells (left panel) or HL-60 cells (middle panel) or both (right panel) were immunoprecipitated with anti-Abl or anti-Grb10 antiserum or normal rabbit serum. Samples were subsequently immunoblotted with anti-Abl or grb10 antibodies. The position of Bcr-Abl and c-Abl are indicated with arrows

immunoprecipitation of Grb10 with an anti-Abl antibody was not successful since the Grb10 antibody recognizes the denatured protein very poorly.

Association of Grb10 and Bcr-Abl is kinase activation dependent in vivo

To examine whether the complex formation of Bcr-Abl with Grb10 is dependent on an activated kinase, a temperature-sensitive mutant of Bcr-Abl (tsBcr-Abl) was introduced into the IL-3 dependent hematopoietic cell line Ba/F3. Following antibiotic selection and single cell cloning by limited dilution, one clone was further characterized for its tsBcr-Abl expression and kinase activity by Western blotting. Cell lysates from tsBcr-Abl transfected Ba/F3 cells were kept at the restrictive or permissive temperature for 3 h and cell lysates were analysed with an Abl-specific and a Tyr(P)-specific antibody. These cells express tsBcr-Abl at the permissive (32°C) and restrictive (39°C) temperature at comparable levels (Figure 5a, left panel). Three hours after a temperature shift to 32°C the cells contained enhanced levels of tyrosine-phosphorylated tsBcr-Abl demonstrating activation of tsBcr-Abl at the permissive temperature (Figure 5a, right panel). To investigate whether this kinase activation leads to enhanced Grb10 binding, lysates of cells grown at restrictive or permissive temperatures were incubated with a GST-Grb10-SH2 fusion protein. As shown in Figure 5b, left panel, a little Bcr-Abl protein could be pulled down from cells kept at the restrictive temperature probably reflecting leaky kinase activity even in cells grown at 39°C (see Figure 5a, right panel). However, at the permissive temperature increased amounts of tsBcr-Abl bound to the Grb10-SH2 domain demonstrating that complex formation is kinase-dependent. Under the same conditions tsBcr-Abl could not be precipitated with GST beads alone neither at the restrictive nor at the permissive temperatures (Figure 5b, left panel).

Kinase-dependency of the Bcr-Abl/Grb10 complex could also be demonstrated *in vivo* by co-immunoprecipitation analysis. As shown in Figure 5b, right panel, the Grb10 antibody failed to precipitate Bcr-Abl protein at the restrictive temperature when the kinase was inactive. However, after shifting to the permissive temperature, tsBcr-Abl could be immunoprecipitated by the Grb10 antibody. Thus, complex formation of Brc-Abl and Grb10 is kinase-dependent *in vitro* and *in vivo*.

Analysis of the transforming abilities of Bcr-Abl mutants

We used Ba/F3 cells as a model system to analyse the transforming abilities of Bcr-Abl mutants. Ba/F3 cells have been shown to gain growth factor independence upon transfection of Bcr-Abl (Daley and Baltimore, 1988). This transforming capability of Bcr-Abl has been shown to require an active tyrosine kinase and an oligomerization signal within the first 63 AA of Bcr (Daley and Baltimore, 1988; McWhirter *et al*., 1993). Other functional domains which have been shown to be essential for transformation of fibroblasts, however, are dispensable for abrogation of IL3-dependent growth of Ba/F3 cells, e.g. the Grb2 binding site and the SH2 domain (Cortez *et al*., 1995; Goga *et al*., 1995; McWhirter and Wang, 1993). The biological function

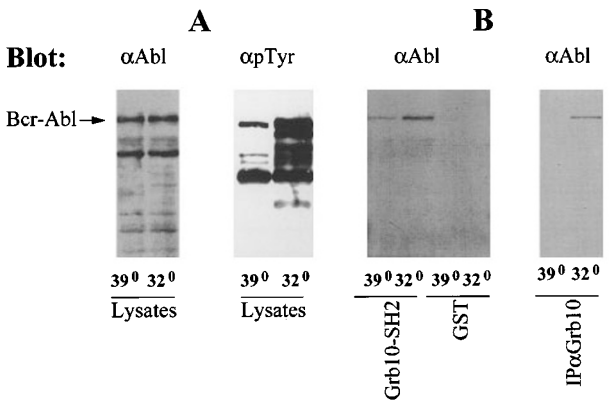

Figure 5 (**a**) tsBcr-Abl expression and autophosphorylation in tsBcr-Abl transfected Ba/F3 cells. tsBcr-Abl transfected Ba/F3 cells were kept at 39°C or shifted to 32°C for 4 h. Cells lysates were run on SDS–PAGE and immunoblotted with an anti-Abl antibody (8E9 left panel) or an anti-phosphotyrosine antibody (PY20) (right panel). The position of Bcr-Abl is indicated by an arrow. (**b**) The Grb10-SH2 domain and Grb10 forms complexes with activated Bcr-Abl. Cell lysates from 1×10^7 tsBcr-Abl transfected Ba/F3 cells kept at the permissive (32°C) or restrictive (39°C) temperature were incubated with GST-Grb10-SH2 or GST-fusion proteins (left panel) or immunoprecipitated with an anti-Grb10 antibody (right panel). Protein complexes were washed thoroughly and resolved by SDS–PAGE followed by immunoblotting with anti-Abl antibody (8E9)

of Grb10 is so far unknown. However, since Grb10 lacks a catalytic domain and consists of domains known to be involved in protein complex formation, it was hypothesized, like Grb2, the Grb10 molecule could also be an adapter molecule linking Bcr-Abl to downstream signaling targets (Ooi *et al*., 1995). To test whether presumed downstream targets are important for IL-3-independent growth of Ba/F3 cells, the Bcr/1-509-Abl as well as the Bcr/1-242-Abl mutant which lacks the ability to interact with GRB10 and a kinase-defective Bcr-Abl mutant as control, were transfected into Ba/F3 cells. After antibiotic selection of the transfected clones, protein expression was verified by Western blotting analysis (data not shown). Cells were then kept in medium lacking IL-3 and the growth of cells was monitored over a period of 5 weeks to determine the progression to IL-3 independence. As shown in Figure 6 both Bcr/1-509-Abl and Bcr/1-242Abl could render Ba/F3 cells IL-3-independent. However, the Bcr/1-242 mutant had a weaker transforming potential and cells transfected with this construct had significantly delayed onset of IL-3-indpendent growth demonstrated in two independent experiments. Since the Bcr/1-242-Abl mutant can not form a complex with Grb10, these experiments indicate that this interaction is important but not absolutely required for induction of growth factor independence in these cells. Additionally, McWhirter *et al*. have shown by using the same Bcr/1-242-Abl mutant that the ability of this mutant to induce focus formation in Rat-1 fibroblasts is impaired (McWhirter *et al*., 1993). Taken together, the data indicate that like other important functional domains of Bcr-Abl (SH2, Tyr(P)177), the Grb10 binding site is important but not sufficient for the transforming activity of Bcr-Abl which redundantly activates the mitogenic pathway by utilizing several autophosphorylation sites.

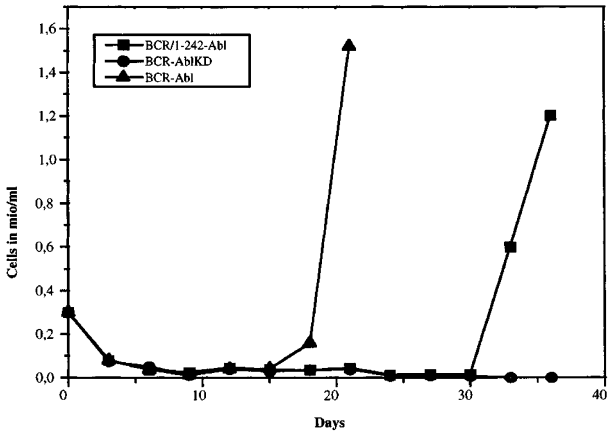

Figure 6 Abrogation of IL-3 dependence in Ba/F3 cells by Bcr-Abl proteins. Ba/F3 cells were transfected with the indicated Bcr-Abl mutants as described in Materials and methods. Cells were cultured in medium without IL-3 and the ability to grow was monitored over a period of 5 weeks. One of two independent representative experiments is shown

Discussion

Bcr-Abl is an oncogenic tyrosine kinase capable of transforming myeloid cells and certain fibroblasts (Daley and Baltimore, 1988; Lugo *et al*., 1990; Lugo and Witte, 1989). A large body of evidence indicates that the activated and upregulated tyrosine kinase of Bcr-Abl is responsible for its transformation potential. Mutational analysis have shown that autophosphorylation of Bcr-Abl is important for its transforming properties (Pendergast *et al*., 1993a,b; Puil *et al*., 1994). One autophosphorylation site of Bcr-Abl (Tyr(P)177) could be identified and was subsequently shown to interact with the Ras adapter Grb2 thereby possibly linking Bcr-Abl to the mitogenic Ras pathway (Pendergast *et al*., 1993b; Puil *et al*., 1994). It could be demonstrated that this autophosphorylation site was necessary to transform Rat-1 fibroblasts. However, this site appeared to be dispensable for the transformation of myeloid cells or induction of tumors in nude mice suggesting the presence of additional autophosphorylation sites binding to additional adapter molecules which contribute to the redundant transforming potential of Bcr-Abl (Cortez *et al*., 1995; Goga *et al*., 1995). To address this possibility, we have performed a yeast two-hybrid screen which allows interchain phosphorylation of Bcr-Abl on tyrosine residues in yeast. By screening a mouse embryo library and a K562 library, we have identified a series of signaling molecules already known to bind to Bcr-Abl in a phosphotyrosine-dependent and -independent manner. Among them were the SH2 domains of Grb2, PI3kinase and the SH3 domain of Crk. These signaling molecules have been previously identified to interact with Bcr-Abl and to be of importance for the biological function of Bcr-Abl (Feller *et al*., 1994b; Pendergast *et al*., 1993b; Puil *et al*., 1994; Ren *et al*., 1994). This demonstrates that autophosphorylation of Bcr-Abl in yeast resembles the autophosphorylation of tyrosine residues occurring *in vivo*. Therefore, this is a useful system to screen for Tyr(P)-dependent binding partners of Bcr-Abl. Among the clones identified was a new SH2-containing adapter molecule, Grb10, which was independently isolated six times from a mouse embryo library. In addition, a human splice variant of Grb10 (hGrb10svIR) (Liu *et al*., 1995; O'Neill *et al*., 1996) could be isolated in the yeast two-hybrid screen with a library obtained from a CML cell line (K562). Grb10 belongs to an emerging family of SH2-containing adapter molecules including Grb7 and the recently identified Grb14 (Ooi *et al*., 1995). These adapter molecules share a general feature in that they consist of a N-terminal proline rich region, a central pleckstrin domain and a C-terminal SH2 domain (Ooi *et al*., 1995). The exact function of pleckstrin domains has not been fully elucidated as yet. Studies in *C. elegans* which contain a homologous region, called F10E9.6, indicate a function in cell migration (Wilson *et al*., 1994). In addition, pleckstrin domains were found in a variety of proteins known to exert important functions in signal transduction (Lemmon *et al*., 1996). It has been proposed that pleckstrin domains are involved in specific protein–protein interactions. The general structure of highly conserved domains reveals that Grb10 functions as an adapter molecule for signal transduction control. It was speculated that Grb10 couples activated receptor tyrosine kinases via its SH2 domain to more downstream signaling which may bind to the proline-rich region or to the pleckstrin domain of Grb10. Hereby, Grb10 is perhaps able to deliver a pro-mitogenic signal. Experiments with a putative dominant-negative mutant of Grb10 (the isolated SH2 domain) expressed in fibroblasts show inhibition of DNA synthesis (O'Neill *et al*., 1996). Furthermore, overexpression of Grb7 in certain types of breast cancer and Grb14 in estrogen receptor-positive breast cancer cells and prostate cancer suggest a role for this adapter family in oncogenesis (Stein *et al*., 1994; Daly *et al*., 1996).

Sequence analysis of the six independent Grb10 clones isolated by two-hybrid screening indicated that the interaction with Brc-Abl indeed required the SH2 domain of Grb10. Interaction involved novel Bcr-Abl autophosphorylation sites different from Tyr(P)177 located between Bcr aa242-446. We were not able to identify a single tyrosine residue responsible for Grb10-SH2 binding to Bcr-Abl. Previous studies have also failed to detect a single Grb10-SH2 binding site for the insulin receptor (O'Neill *et al*., 1996; Frantz *et al*., 1997). Therefore, it is reasonable to speculate that the Grb10-SH2 domain requires more than a single phosphorylated tyrosine for interaction with Bcr-Abl. Complex formation of Grb10 with Bcr-Abl was demonstrated *in vivo* by co-immunoprecipitation of the endogenous proteins in CML cells. Quantitative analysis revealed that about 5% of the endogenous Bcr-Abl in these cells is complexed to Grb10. Furthermore, we showed that an upregulated tsBcr-Abl tyrosine kinase is necessary for this interaction. Recently, a paper described *in vitro* binding of the Abl-SH3 domain to a proline rich stretch in Grb10 (Frantz *et al*., 1997). None of our Grb10 clones isolated in yeast contained this proline rich region. In addition our data with the tsBcr-Abl expressing Ba/F3 cells demonstrated Grb10 complex formation with Bcr-Abl to be strictly kinase dependent. Thus, the SH2 domain of Grb10 rather than the proline rich region seems to control complex formation with

activated Bcr-Abl *in vivo*. Since only the activated Bcr-Abl tyrosine kinase transforms cells, we have investigated whether the Grb10/Bcr-Abl complex is important to induce IL-3 independence in Ba/F3 cells. Interestingly, a Bcr-Abl mutant which lost the ability to interact with Grb10 could still render Ba/F3 cells IL-3-independent however, with a significantly lower efficiency. In different biological assay systems, different Bcr-Abl domains are required for transformation. For example, it was shown that the Abl SH2 domain is required for fibroblasts transformation by Bcr-Abl, but not for Ba/F3 growth factor-independence (McWhirter *et al*., 1993). Similarly, the Tyr(P)177 is required to transform fibroblasts, but not to render Ba/F3 cells IL-3-independent (Pendergast *et al*., 1993b). It was shown that in hematopoetic cells, combinations of mutations are required to abolish the transforming activities of Bcr-Abl and that these functional domains act in concert to render Ba/F3 cell growth factor-independent (Cortez *et al*., 1995; Goga *et al*., 1995). In this light, our findings showing that mutation of the Grb10 interacting domain alone in Bcr-Abl does not completely diminish Bcr-Abl transforming activity in the Ba/F3 assay was not surprising. In line herewith, data of McWhirter *et al*. suggest that the Grb10-Bcr-Abl complex also seems to be important to transform fibroblasts. It appears reasonable to assume that Bcr-Abl activates different signaling pathways, e.g. activation of Ras and STATS, modulation of the cytoskeleton, inhibition of apoptosis or induction of cytokine release (Anderson and Mladenovic, 1996; Bedi *et al*., 1995; Carlesso *et al*., 1996; Cortez *et al*., 1995; Gotoh *et al*., 1995; Sawyers *et al*., 1995). For the development of leukemia, a combination of these Bcr-Abl functions seems to be required. It will be necessary to study the biological function of Grb10 in greater detail to define its role in the pathogenesis of CML. The fact that Grb10 binding to Bcr-Abl is strong (at least as strong as that of Grb2) and that it can be easily co-immunoprecipitated from endogenous proteins in CML cells, along with the observation that the Bcr-Abl/Grb10 complex is important for the transformation of both fibroblasts and hematopoietic cells, strongly support the hypothesis that Grb10 plays an important, though yet unidentified role, in the development of CML. Association of the Grb10 adapter family with certain types of malignancies (O'Neill *et al*., 1996) as well as experimental data supporting a role of Grb10 in mitogenesis encourage this hypothesis.

Materials and methods

Cell culture and transfections

IL-3-dependent Ba/F3 cells were cultured in RPMI 1640 supplemented with 10% FCS and 2 ng/ml murine IL-3. K562 and HL-60 were cultured in RPMI 1640 supplemented with 10% FCS.

Expression plasmids used in this study have been previously described (McWhirter *et al*., 1993; McWhirter and Wang, 1991, 1993; Renshaw *et al*., 1995). Tyrosine to phenylalanine mutants Bcr/231F-Abl, Bcr/246F-Abl, Bcr/276F-Abl, Bcr/283F-Abl, Bcr/316F-Abl, Bcr/328F-Abl, Bcr/360F-Abl and Bcr/436F-Abl were made by site directed mutagenesis using overlapping oligonucleotides and *Pfu* DNA polymerase (Stratagene, Heidelberg, Germany). Bcr/177F-Abl was kindly provided by O Witte, Los Angeles, USA. The Bcr-Abl temperature sensitive mutant (tsBcr-Abl) was generated by subcloning a temperature sensitive v-Abl mutant (DP) (Kipreos *et al*., 1987) into pSLXBcr-Abl (McWhirter *et al*., 1993). Ba/F3 subclones expressing tsBcr-Abl, Bcr-Abl, Bcr-Abl/KD (kinase defective) and Bcr/1-242-Abl were generated by transfection of the corresponding pSLXCMV vector into Ba/F3 cells by electroporation as previously described (McWhirter and Wang, 1993) using a gene pulser (IBI geneZapper 450/2500, Eastman Kodak, New Haven, USA) and selecting for G418-resistant clones. Transfected cells were resuspended at 0.5×10^6 cells per ml in medium lacking a source of IL-3. The number of viable cells in each culture was determined at various time points using trypan blue staining and a hemocytometer.

Yeast two-hybrid system

The cDNA for Bcr-AblΔSal (the actin-binding domain of Bcr-Abl was deleted to avoid detection of actin coding clones) was fused to LexA sequences in the yeast expression vector BTM116 (Weidner *et al*., 1996). Different hybrid cDNAs with different spacing between the LexA and Bcr-Abl coding sequences were transfected into the yeast strain L40 (which contains a His3 and LacZ reporter gene) and assayed for Bcr-Abl tyrosine kinase autophosphorylation activity. The yeast clone displaying the highest autokinase activity was subsequently used and assayed for interaction with proteins encoded by a VP16 activation domain cDNA library from E10.5 mouse embryos (Behrens *et al*., 1996). Alternatively, a cDNA library prepared from K562 cells was used (purchased from Clontech, Heidelberg, Germany). Mutants of Bcr-AblΔSal including Bcr-AblΔSalKD, Bcr (Bcr aa1-509), AblΔSal (Abl aa1-934) and Bcr1-242/AblΔSal were subcloned into the BTM-Bcr-AblΔSal Vector used for the yeast two-hybrid screen.

GST-Fusion proteins and pull down assay

The Grb10 SH2 domain (aa519-621), the Grb2 SH2 domain (aa54-164) and the Nck SH2 domain (aa270-377) were cloned in frame into the vector pGex2TK to make GST (glutathione S-transferase)-fusion proteins (Smith and Johnson, 1988). Wt- and mutant-Bcr-Abl proteins were *in vitro*-translated and ^{35}S-radiolabeled using the TNT system (Promega). The translation mix was diluted to a final concentration of 25 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 1 mM dithiothreitol and 100 μM cold ATP and incubated for 30 min at 4°C to allow for autophosphorylation of the translated proteins. Reactions were stopped by dilution to a final concentration of 10 mM Tris·HCl (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml of each phenantroline, aprotinin, leupeptin and pepstatin (lysis buffer). 5 μg of GST-fusion proteins were added and incubated for 1 h at 4°C. Protein complexes were collected on glutathione-agarose beads (Pharmacia, Freiburg, FRG), washed thoroughly with lysis buffer and subjected to SDS–PAGE. *In vitro* translated proteins were visualized by autoradiography.

For binding experiments with cell extracts, 1×10^7 cells were solubilized in lysis buffer, precleared with glutathione-beads and incubated with 5 μg of GST-fusion proteins for 1 h at 4°C. Protein complexes were collected on glutathione-beads and subjected to SDS–PAGE. Bcr-Abl and Abl was detected by immunoblotting using an Abl-specific antibody 8E9 (Duyster *et al*., 1995).

Immunoprecipitation and immunoblotting

Immunoprecipitation was done as previously described (Duyster *et al*., 1995; McWhirter and Wang, 1991). Briefly, 1×10^7 cells were solubilized in lysis buffer containing 10 mM Tris·HCl (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 and 10 μg/ml of each phenantroline, aprotinin, leupeptin and pepstatin. After clarification by centrifugation, Grb10 was precipitated with anti-Grb10 antibody (Santa Cruz, USA), and Bcr-Abl and Abl were precipitated with anti-Abl antibody 8E9. Immunoprecipitations were analysed by SDS–PAGE followed by immunoblotting with anti-Abl (8E9) and monoclonal antiphosphotyrosine antibody (PY20) as previously described (Duyster *et al*., 1995; McWhirter and Wang, 1991).

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