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Differential interaction and activation of Rho family GTPases by p210^{bcr-abl} and p190^{bcr-abl}

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The p210^{bcr-abl} and p190^{bcr-abl} fusion proteins, respectively responsible for chronic myelogenous leukemia and acute lymphoblastic leukemia, present deregulated tyrosine kinase activity and abnormal localization. The Dbl homology domain of Bcr, activating Rho GTPases, is present in p210^{bcr-abl}, but absent in p190^{bcr-abl}. We investigated the interaction of Bcr-Abl chimeras and Rho proteins by coimmunoprecipitation, pull-down experiments and GEF activity measurement. RhoA, Rac1 and Cdc42 interact *in vivo* with p210^{bcr-abl} only. Moreover, the three types of GTPases are activated *in vitro* and *in vivo* by p210^{bcr-abl}. Nevertheless, Rac1 and Cdc42, but not RhoA, are activated by p190^{bcr-abl} *in vitro* and *in vivo*. Part of this GEF activity of p190^{bcr-abl} is probably attributable to p95^{av}, which is complexed with both p190^{bcr-abl} and p210^{bcr-abl} in an activated form. p160^{bcr}, also in complex with Bcr-Abl, presents no GEF activity in p190^{bcr-abl}-expressing cells. These results suggest that differential activation of Rho proteins should play a major role in Bcr-Abl-induced leukemogenesis.

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Keywords: Bcr-Abl, Rho GTPases, signal transduction

Introduction

Bcr-Abl is a chimeric oncogene generated by a reciprocal translocation between chromosomes 9 and 22 (Philadelphia chromosome, Ph⁺) that leads to the fusion of the 5' region of the bcr gene with the 3' region of the abl gene. Bcr is a multidomain cytosolic protein whose function in signal transduction has not yet been precisely defined. Bcr is composed of an oligomerization N-terminal domain, a serine kinase domain, a DH/PH domain specific for RhoA and Cdc42 and a C-terminal GAP domain active on Rac. The only function attributed to Bcr, the deactivation of neutrophil NADPH oxidase, relies on this latter domain (Voncken

et al., 1995). Abl is a nonreceptor tyrosine kinase that contains, in addition to other Src family proteins, a long C-terminal domain regulating its localization and consisting of several nuclear localization signals, a nuclear export signal, a DNA binding domain and a C-terminal F-actin binding domain (Van Etten, 1999). Different types of chimeras comprising varying regions of Bcr and the greater part of Abl are found in several hematological diseases. Among them, p210^{bcr-abl} is the most frequent and responsible for chronic myelogenous leukemia (CML) (Daley *et al.*, 1990). Less frequent, p190^{bcr-abl} is associated with acute lymphoid leukemia (ALL) (Clark *et al.*, 1988). Both proteins exhibit enhanced and constitutive tyrosine kinase activity (Lugo *et al.*, 1990; Ilaria and Van Etten, 1996). Transformation by p210^{bcr-abl} is a complex mechanism, implicating enhanced proliferation and inhibition of apoptosis, but conserved differentiation. In this respect, the chronic phase cells cannot be characterized as fully transformed. In the absence of treatment, a fatal acute phase follows after 3–5 years, which can take different forms (myeloid, erythroid or even lymphoid). In contrast, p190^{bcr-abl} induces an immediately acute disease with undifferentiated blasts and inhibition of normal hematopoiesis. Interestingly, CML is associated with modifications of the actin cytoskeleton. Bcr-Abl, unlike c-Abl, is preferentially associated with F-actin (McWhirter and Wang, 1993), and its distribution changes in migrating cells (Skourides *et al.*, 1999). In fact, many substrates of Bcr-Abl are focal adhesion proteins such as paxillin, vinculin, talin and FAK (Salgia *et al.*, 1995).

The small GTPases of the Rho family (i.e. RhoA, Rac1 and Cdc42) are known regulators of the actin cytoskeleton (Tapon and Hall, 1997). In addition to this action, they modulate apoptosis (Nishida *et al.*, 1999), proliferation (Olson *et al.*, 1998) and myeloid differentiation (Ohguchi *et al.*, 1997; for a review, see Zohn *et al.*, 1998). Interestingly, the isolated recombinant Dbl-homology domain (DH) of Bcr is an activator of Rho GTPases (Chuang *et al.*, 1995). It should be noted that the only difference between p190^{bcr-abl} and p210^{bcr-abl} resides in the absence of this DH domain in p190. Neither p190^{bcr-abl} nor p210^{bcr-abl} possess the RacGAP domain. Overexpression of Bcr in fibroblasts

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expressing p190^{bcr-abl} diminishes the latter's transformation potential (Wu *et al.*, 1999), which implies that some Bcr activities may regulate Bcr-Abl oncogenicity. Different substrates or partners of p210^{bcr-abl} are known modulators of Rho activity, as p190^{rhoGAP} (Druker *et al.*, 1992; Cheng *et al.*, 1995). Moreover, Bcr-Abl phosphorylates p95^{vav} (Matsuguchi *et al.*, 1995). Vav, a GTP exchange factor (GEF) for Rac activated by tyrosine phosphorylation (Crespo *et al.*, 1996) has been shown to be active toward Rac in Bcr-Abl-transformed cells (Skorski *et al.*, 1998). Vav may also activate Cdc42 (Olson *et al.*, 1996; Han *et al.*, 1997). Finally, PI 3-kinase activity is enhanced by Bcr-Abl (Skorski *et al.*, 1995) leading to an antiapoptotic pathway necessary for the survival of Ph⁺ cells (Skorski *et al.*, 1997). Among Rho family members, at least Rac is activated directly by PI 3-kinase products (Missy *et al.*, 1998).

On the basis of these elements, it seems likely that for proliferative, antiapoptotic and motility pathways, Bcr-Abl transduces signals through Rho proteins. We have thus decided to study the relations between Bcr-Abl and Rho proteins.

Here we show for the first time a difference between the pathways used by p190^{bcr-abl} and p210^{bcr-abl}. In contrast to p190^{bcr-abl}, p210^{bcr-abl} binds to small GTPases of the Rho family both *in vitro* and *in vivo*. The immunoprecipitated p210^{bcr-abl} complex activates RhoA, Rac1 and Cdc42, whereas the immunoprecipitated p190^{bcr-abl} complex, despite the absence of a DH domain in p190, is able to activate Rac1 and Cdc42 but not RhoA. Vav can form a complex with both forms of Bcr-Abl and may be responsible for the GEF activity of p190^{bcr-abl}. This differential activation of RhoA and Rac1/Cdc42 by p210^{bcr-abl} and p190^{bcr-abl} should have implications in the disease phenotype observed in each case.

Results

Coimmunoprecipitation of Rho proteins with p210^{bcr-abl}

In order to show a putative interaction between RhoA, Rac1 or Cdc42 and p210^{bcr-abl}, we first performed immunoprecipitation experiments. We used 8E9 monoclonal anti-Abl antibody to purify Abl and Bcr-Abl complexes from Ba/F3wt, Ba/F3p190 and Ba/F3p210 cells. As expected, in Ba/F3wt cells, only c-Abl (145 kDa) was immunoprecipitated (Figure 1). Immunostaining of the Western blot with anti-RhoA, anti-Rac1 or anti-Cdc42 antibodies showed no associated Rho GTPase in c-Abl complexes. In Ba/F3p190 cells, both p145^{abl} and p190^{bcr-abl} were precipitated. Neither RhoA, Rac1 nor Cdc42 was present in a complex with p190^{bcr-abl} in Ba/F3p190 cells. In contrast, in Ba/F3p210 cells, these GTPases were found associated in a complex with p210^{bcr-abl}. In all, 9.1% of total RhoA, 7.9% of total Rac1 and 7.0% of total Cdc42 were found associated with p210^{bcr-abl}.

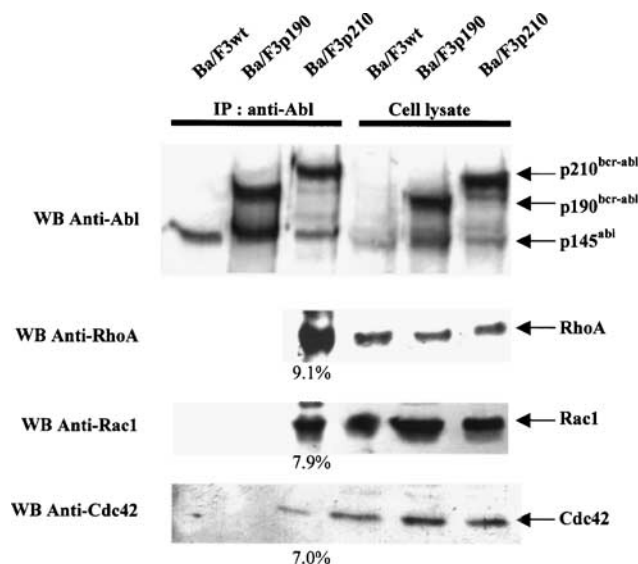


Figure 1 Coimmunoprecipitation of Rho GTPases with p210^{bcr-abl}. Cells were lysed and immunoprecipitation was carried out with anti-Abl antibody (8E9) as described in Materials and methods. Immunoprecipitates from Ba/F3wt, Ba/F3p190 and Ba/F3p210 were divided into four equal parts. The first part was loaded onto a 7% SDS-PAGE apparatus and the three remaining parts were loaded separately onto a 13% gel and then Western blotted. The presence of Abl, Bcr-Abl, RhoA, Rac1 and Cdc42 was revealed using anti-Abl (8E9), anti-Rho (26C4), anti-Rac (23A8) or anti-Cdc42 (P1G) antibodies, respectively. Total cell lysates (20 μ g of protein) are migrated in parallel and revealed with the same antibodies. Percentage of coimmunoprecipitated GTPases is shown, obtained by densitometric analysis of bands revealed by anti-RhoA, anti-Rac1 or anti-Cdc42. Results are representative of three independent experiments

GDP- and GTP-bound Rho proteins bind p190^{bcr-abl} and p210^{bcr-abl} differentially *in vitro*

A characteristic of small GTPases is to bind *in vitro* to their GEFs in a GDP-bound or in a nucleotide-free state. In contrast, they bind to their effectors only in a GTP-bound form. To determine which form of Rho proteins bind Bcr-Abl, we have performed pull-down experiments using RhoA, Rac1 and Cdc42 as GST fusion proteins bound to glutathione-sepharose beads and preloaded with GDP β S or GTP γ S, or nucleotide-free (Figure 2). The GST protein alone retained no protein revealed by 8E9 antibody from any cell type tested. Interestingly, only p210^{bcr-abl} was retained on glutathione-sepharose beads carrying RhoA, Rac1 or Cdc42 in the GTP-bound form. Similarly, in the GDP-bound and the nucleotide-free forms, p210^{bcr-abl} was retained by each Rho family GTPase. In contrast, p190^{bcr-abl} associated with Rac1 and Cdc42 in their nucleotide-free and GDP-bound forms, but was unable to bind to RhoA. *In vitro*, p210^{bcr-abl} forms a direct or indirect complex with the GTP-bound and the GDP-bound forms of RhoA, Rac1 and Cdc42. In contrast, p190^{bcr-abl} binds directly or indirectly only to the GDP-bound forms of Rac1 and Cdc42, which is a characteristic of GEFs. As p190^{bcr-abl} possesses no DH domain, this characteristic should be carried by one or more protein partners of p190^{bcr-abl}.

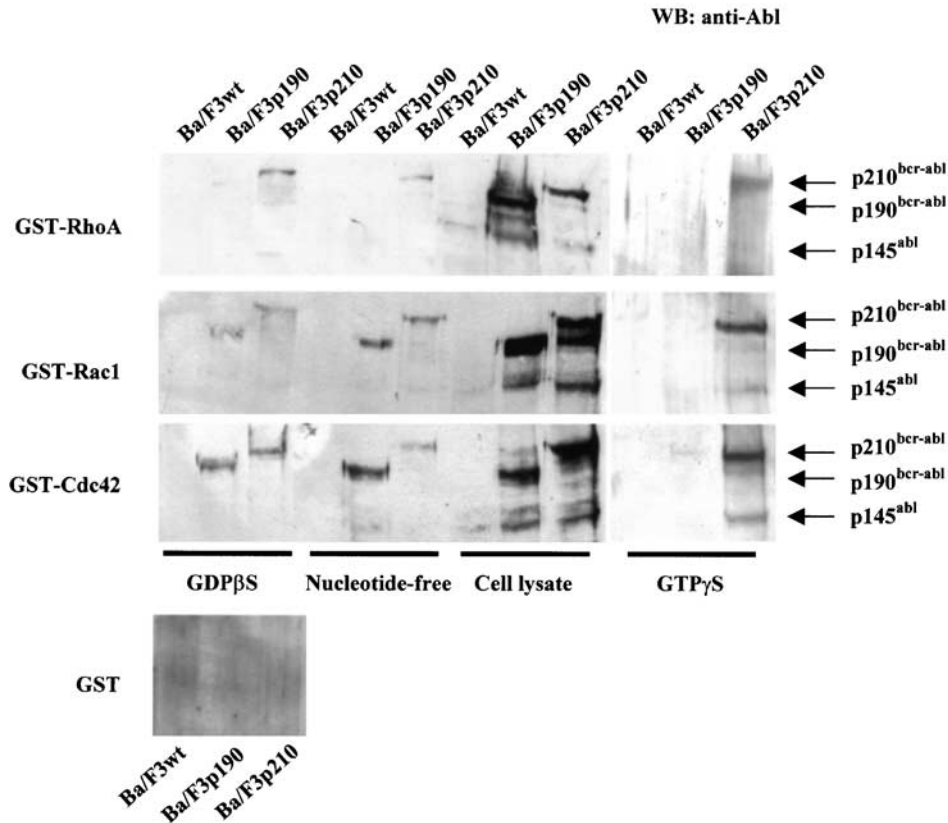


Figure 2 Pull-down experiments with Rho GTPases as baits. GSH-sepharose-bound GST-Rho GTPases (2 μ g) were preloaded with GTP γ S, GDP β S or emptied by EDTA as described in Materials and methods. They were incubated with 1 ml of cell lysate corresponding to 2×10^6 Ba/F3wt, Ba/F3p190 or Ba/F3p210 cells. Bead-bound complexes were loaded onto a 8% SDS-PAGE apparatus followed by Western blotting. The presence of Bcr-Abl was revealed using anti-Abl (8E9) antibody. Total cell lysates (20 μ g of protein) are migrated in parallel for control

GEF activity of p190^{bcr-abl} and p210^{bcr-abl} immunoprecipitated complexes toward Rho proteins

The isolated DH domain of Bcr expressed as a GST-fusion protein shows a GEF activity preferentially toward Cdc42 and RhoA (Chuang *et al.*, 1995). In contrast, it activates Rac1 weakly. It should be noted that these experiments were done with a DH domain coupled with the N-terminal half of the adjacent PH domain. Thus, it is possible that this truncated PH domain was in part or totally inactive, and the activity observed may be different *in vivo* (Aghazadeh *et al.*, 2000; Das *et al.*, 2000). Furthermore, p190^{bcr-abl} has no DH domain and should not be able to activate any of the Rho family GTPase. To investigate this activity, we performed anti-Abl immunoprecipitations from Ba/F3wt, Ba/F3p190 and Ba/F3p210 cells, and tested these immunoprecipitates for GEF activity toward Rho family GTPases (Figure 3). Cdc42 and Rac1 were activated by both p190^{bcr-abl} and p210^{bcr-abl} immunoprecipitates. In contrast, RhoA was activated only by p210^{bcr-abl} immunoprecipitates, and p190^{bcr-abl} immunoprecipitates had no GEF activity toward RhoA. Despite the lack of a DH domain, immunoprecipitated p190^{bcr-abl} was able to activate Cdc42 and Rac1. As a control, immunoprecipitated Vav from Ba/F3p190 or Ba/F3p210, which has been shown to be activated by

Bcr-Abl (Matsuguchi *et al.*, 1995), presents a lower exchange rate than p190 or p210 immunoprecipitates toward Rac1.

Characterization of DH domain-containing molecular partners of Bcr-Abl in Ba/F3 cells

The GEF activity observed with anti-Abl immunoprecipitates from Ba/F3p190 cells is obviously not due to p190^{bcr-abl} itself, since no DH domain is present in its structure. Several reports show that in different cell types other DH domain-containing proteins interact with Bcr-Abl (Puil *et al.*, 1994; Matsuguchi *et al.*, 1995). The presence of an oligomerization domain at the N-terminus of Bcr allows the tetramerization of Bcr-Abl (McWhirter *et al.*, 1993). It is possible that heterotetramers between Bcr-Abl and Bcr exist. Campbell *et al.* (1990) have shown that in a strain of K562 cells containing both p210^{bcr-abl} and p190^{bcr-abl}, p160^{bcr} is complexed with Bcr-Abl. To assess this possibility in our cell line, we performed immunoprecipitations with anti-Abl antibody (8E9) in Ba/F3p190 and Ba/F3p210 cells. P160^{bcr} forms a complex with both p190^{bcr-abl} and p210^{bcr-abl}, as revealed by anti-Bcr antibody on the Western blot (Figure 4).

p95^{vav} has been recently characterized as a GEF for Rac1 and Cdc42 (Han *et al.*, 1997). It is activated,

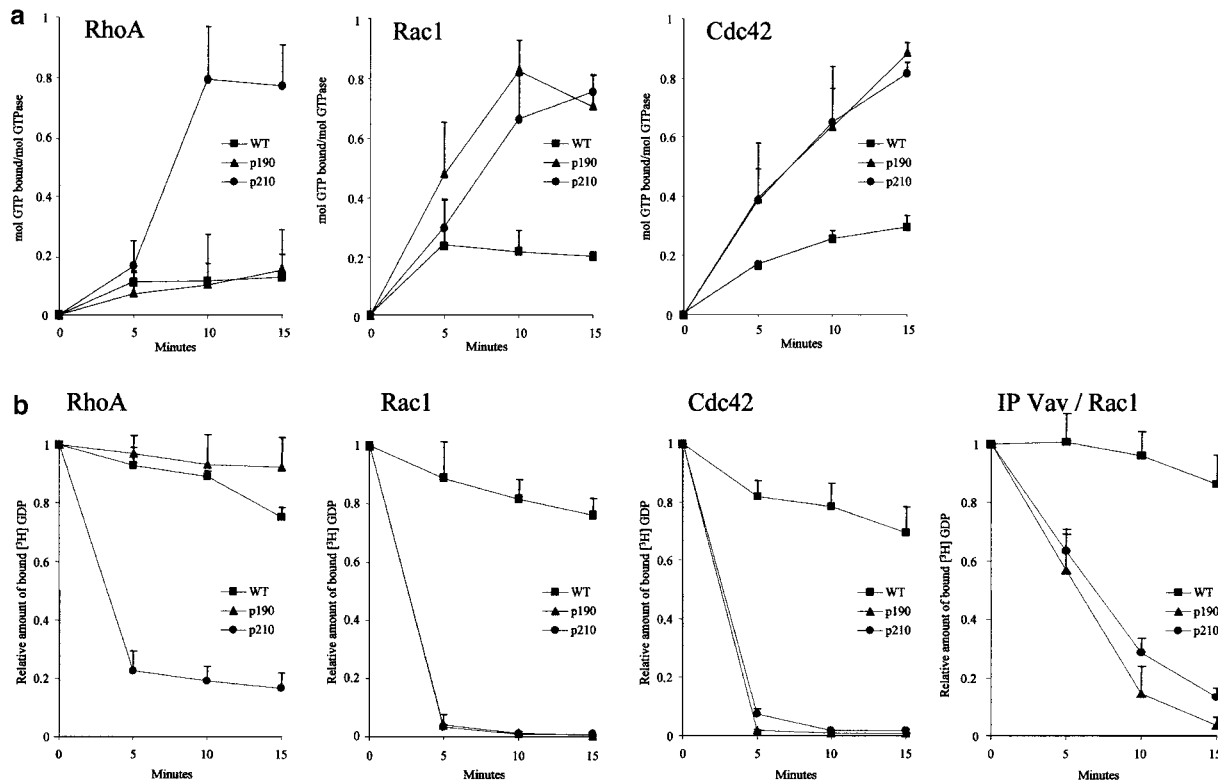


Figure 3 The GEF activity of p190^{bcr-abl} and p210^{bcr-abl} toward Rho GTPases. p145^{abl}, p190^{bcr-abl}, p210^{bcr-abl} and p95^{vav} were immunoprecipitated by monoclonal anti-Abl antibody (8E9) or anti-Vav antibody from 10⁷ Ba/F3wt, Ba/F3p190 or Ba/F3p210 cells, respectively. GEF activity of these immunoprecipitates was measured by [³⁵S]GTP-binding (a) or [³H]GDP-releasing assays (b) on purified GST-fused Rho GTPases (i.e. GST-RhoA, GST-Rac1 and GST-Cdc42) as described in Materials and methods. (●) p210^{bcr-abl} immunoprecipitates. (▲) p190^{bcr-abl} immunoprecipitates. (■) p145^{abl} immunoprecipitates from Ba/F3wt cells. Bars represent the s.d. calculated from three independent experiments for each GTPase

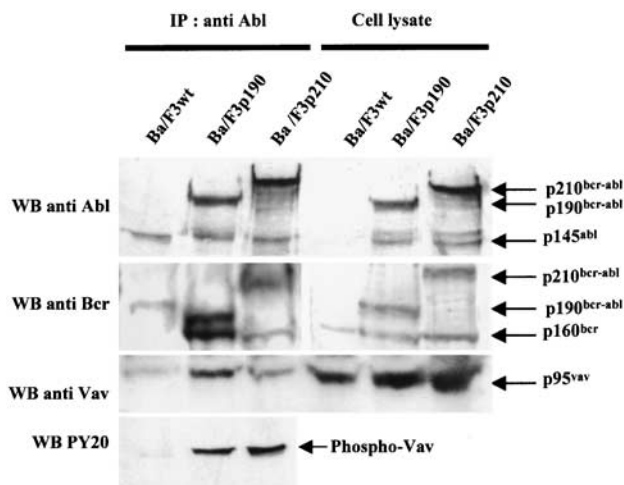


Figure 4 Coimmunoprecipitation of Bcr–Abl (or Abl) with Bcr and p95^{vav} in Ba/F3 cells. Immunoprecipitation with anti-Abl (8E9) antibody was carried out as described in Materials and methods. Immunoprecipitates were loaded onto a 8% SDS–PAGE apparatus followed by Western blotting. The presence of Abl, Bcr–Abl, Bcr and Vav was revealed using anti-Abl (8E9) (upper), anti-Bcr (G6) (middle) or anti-Vav (lower) antibodies, and tyrosine phosphorylation of Vav was revealed using PY20 (lower) antibody. Results are representative of four independent experiments

among other mechanisms, by tyrosine phosphorylation (Aghazadeh *et al.*, 2000). Interestingly, Matsuguchi *et al.* (1995) showed that in 32Dcl3 cells expressing a p210^{bcr-abl} temperature-sensitive mutant for kinase activity, Vav was phosphorylated on tyrosine, only at the permissive temperature. Furthermore, Skorski *et al.* (1998) performed GEF assays on Rac1 with immunoprecipitated Vav from 32Dcl3 cells expressing p210^{bcr-abl}, and showed that Vav is able to activate Rac1 in these cells. To explore the role of Vav in the GEF activities observed in the previous experiments (Figure 3), we performed anti-Abl immunoprecipitations from Ba/F3wt, Ba/F3p190 and Ba/F3p210 cells (Figure 4). We show that p95^{vav} is present complexed with p145^{abl}, p190^{bcr-abl} and p210^{bcr-abl}. Immunostaining with PY20 antibody reveals a band corresponding to the Mr of Vav only in p190 and p210 immunoprecipitates. Together with previous reports (Matsuguchi *et al.*, 1995), these results suggest that Vav is in an activated form not only in Ba/F3p210 cells, but also in Ba/F3p190 cells.

Inactivation of p160^{bcr} in p190^{bcr-abl}-expressing cells

Vav is known to activate Rac1 and Cdc42, but to have a weak activity toward RhoA (Han *et al.*, 1997). In

contrast, Bcr shows GEF activity toward Cdc42 and RhoA, but a weak activity toward Rac1 (Chuang *et al.*, 1995). If Bcr is complexed with both p190^{bcr-abl} and p210^{bcr-abl}, why might p190^{bcr-abl} not be able to activate RhoA? To clarify this point, we performed anti-Bcr immunoprecipitation of Ba/F3wt and Ba/F3p190 cells (Figure 5). The respective amounts of p160^{bcr} immunoprecipitated from each cell type was almost the same as determined by densitometric analysis of the band revealed by anti-Bcr antibody. Using these anti-Bcr immunoprecipitates from Ba/F3wt and Ba/F3p190 cells, we then performed GEF activity measurements toward RhoA. In parallel, anti-Abl immunoprecipitates from Ba/F3wt and Ba/F3p190 cells were also performed and tested for GEF activity toward RhoA (Figure 5). As previously shown in Figure 3, no GEF activity toward RhoA can be associated with anti-Abl immunoprecipitates, either from Ba/F3wt cells or from Ba/F3p190 cells. In contrast, anti-Bcr immunoprecipitates from Ba/F3wt cells showed a classic GEF activity toward RhoA, in agreement with the results obtained by

Chuang *et al.* (1995). Anti-Bcr immunoprecipitates from Ba/F3p190 cells showed no GEF activity toward RhoA. The same experiment was done in Ba/F3p210, but as expected no difference in GEF activity toward RhoA was seen between anti-Abl and anti-Bcr immunoprecipitates (data not shown).

Localization of Rho proteins in Ba/F3 cells

We have shown that p210^{bcr-abl} and p190^{bcr-abl} present a differential GEF activity toward Rho proteins. To explore this activity in our cell lines, we performed confocal microscopy experiments in order to visualize the localization of Rho proteins. It has been demonstrated that Rho proteins, when activated, are translocated from a cytosolic compartment to a submembrane localization (Adamson *et al.*, 1992). In Ba/F3wt cells, RhoA, Rac1 and Cdc42 are cytosolic (Figure 6a–c). Interestingly, a large amount of GTPase was concentrated in a perinuclear area. In Ba/F3p190 cells, RhoA is also cytosolic (Figure 6d). Nevertheless, in these cells, Rac1 and Cdc42 are in part localized in a membrane or submembrane compartment (Figure 6e, f). This differential localization indicates that RhoA is not activated in Ba/F3p190 cells, but that Rac1 and Cdc42 are partly activated. In contrast to Ba/F3p190 cells, in Ba/F3p210 cells RhoA, Rac1 and Cdc42 show a partial membrane or submembrane localization, suggesting that they are in an activated form (Figure 7 g–i).

Activation state of Rho proteins in different Ba/F3 cell types

A characteristic of Rho GTPases is to bind to their effectors in an activated GTP-bound form. This property was used to measure the relative amount of GTP-bound GTPases contained in a cell by pull-down experiments with the GTPase binding (GB) domain of PAK (PAK-CD) or Rhotekin (C21) (Sander *et al.*, 1998). Equal quantities (10⁷ cells) of untreated Ba/F3wt, Ba/F3p190 or Ba/F3p210 cells were lysed as described in Materials and methods, and the lysates were incubated with purified GB domains (PAK-CD or C21) fused with GST and immobilized on GSH-sepharose beads. Bound proteins, corresponding to GTP-bound forms of the GTPases, were separated on SDS-PAGE and visualized by immunoblotting analysis using anti-RhoA, anti-Rac1 or anti Cdc42 antibodies (Figure 7). The major part of RhoA was in inactivated form in Ba/F3wt and Ba/F3p190 cells. In contrast, a significant proportion of RhoA was in the activated GTP-bound form in Ba/F3p210 cells. The activated forms of Rac1 and Cdc42 were significantly more abundant in Ba/F3p190 and Ba/F3p210 cells, as compared to Ba/F3wt cells. These experiments show that RhoA is activated only in Ba/F3p210 cells, but that Rac1 and Cdc42 are activated in both Ba/F3p190 and Ba/F3p210 cells.

Discussion

In this study, we have examined the involvement of small GTP-binding proteins of the Rho family in the

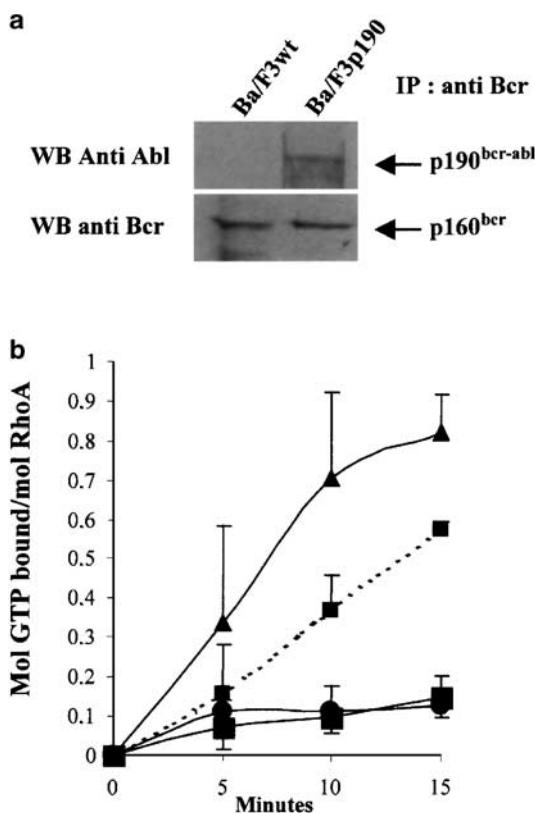


Figure 5 The GEF activity of Bcr in Ba/F3wt and Ba/F3p190 cells. Immunoprecipitation was made with anti-Bcr (G6) antibody in the same conditions as in Figure 1. (a) Immunoprecipitates from Ba/F3wt and Ba/F3p190 were first loaded onto a 7% SDS-PAGE apparatus and then Western blotted. The presence of Abl, Bcr–Abl, Bcr was revealed using anti-Abl (8E9) (upper) or anti-Bcr (G-6) (lower) antibodies, respectively. (b) GEF activity toward RhoA was measured as described for Figure 4. (–●–) Anti-Abl immunoprecipitates from Ba/F3wt cells. (–■–) Anti-Abl immunoprecipitates from Ba/F3p190 cells. (–▲–) Anti-Bcr immunoprecipitates from Ba/F3wt cells. (–◼–) Anti-Bcr immunoprecipitates from Ba/F3p190 cells. Bars represent the s.d. calculated from three independent experiments

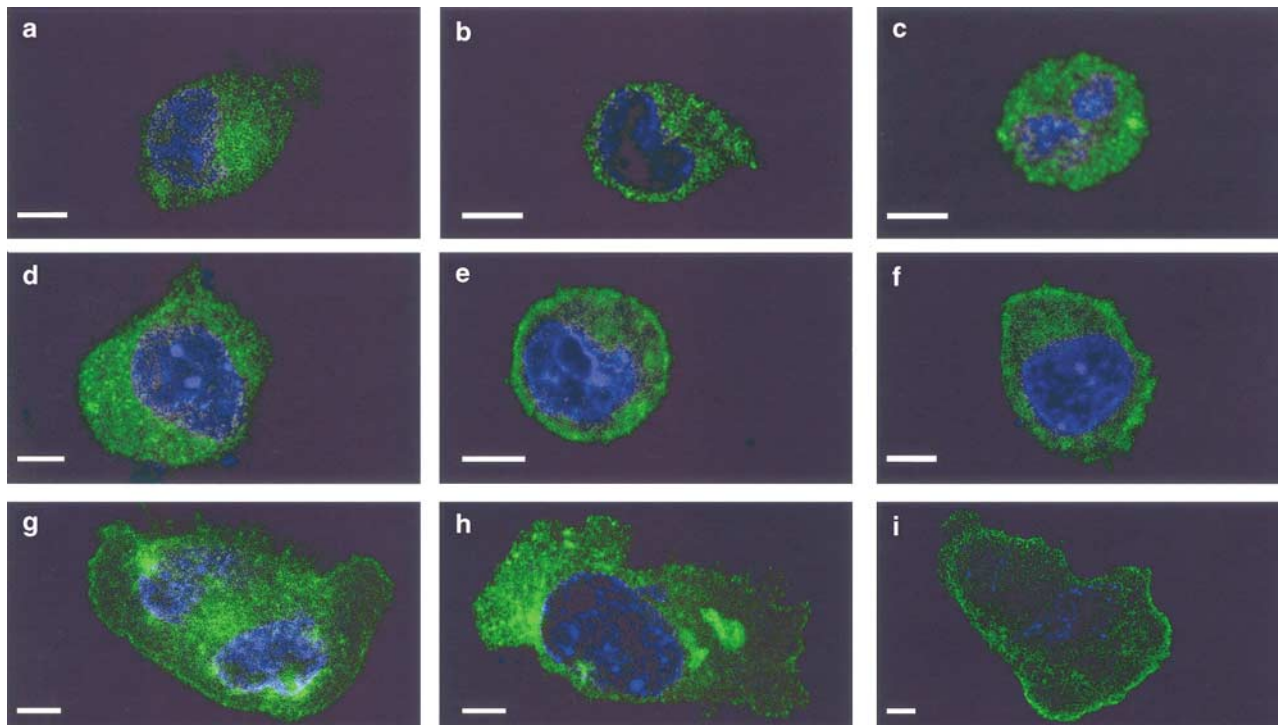


Figure 6 Localization of Rho GTPases in Ba/F3wt, Ba/F3p190 and Ba/F3p210 cells. Wild type Ba/F3 cells (a–c) cultured with rm-IL3 (10 ng/ml), Ba/F3p190 (d–f) and Ba/F3p210 (g–i) were plated on coverslips coated with ECM gel and labeled with anti-RhoA (a,d,g), anti-Rac1 (b,e,h) or anti-Cdc42 (c,f,i), and nuclei were counterstained with TO-PRO-3. Scale bar, 5 μ m

signal transduction induced by Bcr–Abl. We have shown that these GTPases form a stable complex with p210^{bcr–abl}. We have also shown that p210^{bcr–abl} is able to activate RhoA, Rac1 and Cdc42, while p190^{bcr–abl} activates only Rac1 and Cdc42. Ba/F3 cell lines have been widely used in different laboratories to characterize Bcr–Abl-related events. These IL3-dependent hematopoietic cell lines are transformed by each Bcr–Abl chimera (i.e. p190, p210 and p230) (Daley and Baltimore, 1988; Li *et al.*, 1999; Quackenbush *et al.*, 2000).

The signaling of Bcr–Abl through actin cytoskeleton disorganization has been intensely studied in recent years (Verfaillie *et al.*, 1995; Bazzoni *et al.*, 1996; Salgia *et al.*, 1997), but the involvement of Rho GTPases in these pathways has been investigated only indirectly (Skorski *et al.*, 1998). We established that a complex exists between Rho GTPases and p210^{bcr–abl}, but not between Rho GTPases and p190^{bcr–abl}. This association was shown by coimmunoprecipitation assays, using anti-Abl antibody to immunoprecipitate Bcr–Abl. It should be noted that when an anti-RhoA antibody was used, only p210^{bcr–abl} was coimmunoprecipitated (data not shown). The most likely hypothesis to explain this association would be the presence of a DH domain on p210 that is absent in p190. Nevertheless, no data in the literature support the idea that small G proteins form stable complexes with their activators, whereas many reports show stable association with effectors in the GTP-bound state (Eda *et al.*, 2001; Hansen and Nelson, 2001; Rudolph *et al.*, 2001). Pull-down assays, using Rho GTPases in GDP, GTP and nucleotide-free forms

as baits, show that p210^{bcr–abl} is able to bind *in vitro* to both active and inactive RhoA, Rac1 and Cdc42. On the other hand, p190^{bcr–abl}, which does not coimmunoprecipitate with Rho GTPases in a whole cell system, binds only with the GDP and nucleotide-free forms of Rac1 and Cdc42 in a cell-free system. One can then postulate that the complex revealed by immunoprecipitation arises through the GTP-bound form of GTPases.

Confocal microscopy analysis of Rho family protein localization pointed out the differential activation of Rho GTPases in Ba/F3p190 and Ba/F3p210. In Ba/F3wt cells, Rho GTPases are cytosolic and therefore not activated (Adamson *et al.*, 1992). In Ba/F3p190 cells, Rac1 and Cdc42 are in part translocated to the plasma membrane, a localization characteristic of activated GTP-bound forms of these GTPases. However, RhoA is not translocated to the membrane and persists as a cytosolic unactivated protein. In contrast, in Ba/F3p210 cells, all three Rho GTPases display a partial redistribution to the plasma membrane. These observations are confirmed by pull-down experiments using Rho GTPase-binding domain of effectors (Rhotekin or PAK) as bait to trap GTP-bound forms of these proteins. A significant part (3.6 – 6.4%) of Rac1 and Cdc42 is in activated form in both Ba/F3p190 and Ba/F3p210 cells. In contrast, a part of RhoA (4.5%) is in activated form only in Ba/F3p210 cells. In Ba/F3wt cells, the major part (>99%) of RhoA, Rac1 and Cdc42 is in the GDP-bound inactive form. This activation of Rho GTPases in Ba/F3p190 and Ba/F3p210 cells is obviously due to the presence of Bcr–Abl chimeras in these cells. In fact, Bcr–Abl chimeras form multiprotein

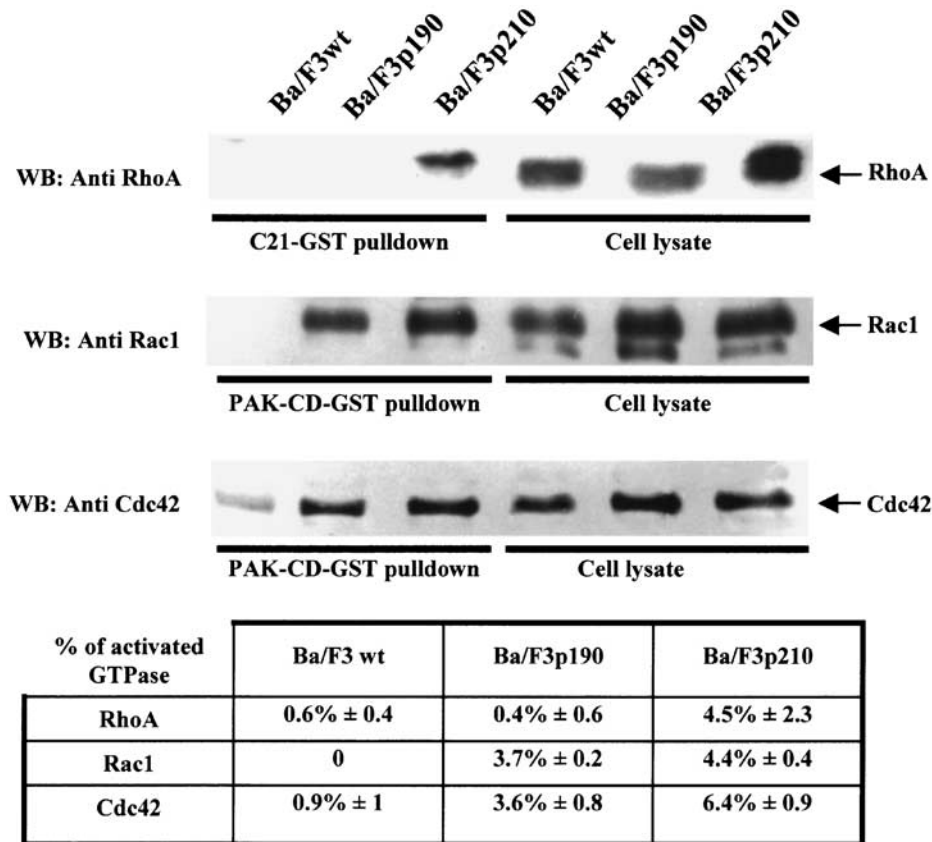


Figure 7 The activation state of Rho GTPases in Ba/F3 cells. Wild-type Ba/F3, Ba/F3p190 and Ba/F3p210 cells were lysed as described in Materials and methods. Lysates were incubated with the sepharose-bound GST-fused Rho-binding domain of Rhotekin-C21 (upper panel) or with sepharose-bound GST-fused PAK-CD (middle and lower panels). Bead-bound complexes comprising activated (GTP-bound) forms of Rho GTPases were revealed by western blot using anti-RhoA (upper panel), anti-Rac1 (middle panel) or anti-Cdc42 (lower panel) antibodies. For comparison, the relative amount of RhoA, Rac1 and Cdc42 present in 20 μ g of total lysate from each cell line is shown. Results are representative of four independent experiments for each GTPase. The table shows the percentage of activated GTPase in each cell type measured after densitometric analysis of bands revealed by anti-RhoA, anti-Rac1 or anti-Cdc42 antibodies

complexes with their molecular partners that may contain GEF activity toward Rho GTPases. In complete agreement with the above results, immunoprecipitated p210^{bcr-abl} complexes showed a GEF activity toward recombinant RhoA, Rac1 and Cdc42. Similarly, only recombinant Rac1 and Cdc42 were activated by immunoprecipitated p190^{bcr-abl} complexes, and no GEF activity toward RhoA could be measured. The DH domain of Bcr is absent in p190^{bcr-abl}, and the activation of Rac1 and Cdc42 observed in this case must thus be indirect. Our results show that p95^{vav}, a GEF for Rac1 and Cdc42 precisely activated by Bcr-Abl (Matsuguchi *et al.*, 1995), is present in a stable complex with p190^{bcr-abl} and p210^{bcr-abl}. Vav, phosphorylated by Bcr-Abl may be responsible for this latter activity. The activation of Rac1 by p210^{bcr-abl} should also be indirect. A recent report confirms this hypothesis, showing that Vav, through its SH2 domain, is in complex with Bcr-Abl, and is responsible of the Rac1 activation induced by Bcr-Abl (Bassermann *et al.*, 2002). Also present in both complexes, p160^{bcr} is inactivated. Our results show that immunoprecipitated p160^{bcr} from Ba/F3wt cells has GEF activity toward RhoA, but that immunoprecipi-

tated p160^{bcr} from Ba/F3p190 cells has no more or at least a largely inhibited activity. In Ba/F3p210 cells, we were not able to differentiate immunoprecipitated Bcr GEF activity from the one of immunoprecipitated p210^{bcr-abl} by anti-Abl antibody, since a similar amount of p210^{bcr-abl} was present in both preparations.

Interestingly, the exchange rate of GDP activated by immunoprecipitated Bcr-Abl is rapid when compared with results obtained *in vitro* by Chuang *et al.* (1995). We measured the exchange activated by anti-Vav immunoprecipitates toward Rac1 (Figure 3b). Immunoprecipitated Bcr-Abl is more efficient than immunoprecipitated Vav from the same cells. A similar observation was published by Lippé *et al.* (2001) concerning Rab5, who showed that the complex of Rabex-5 (GEF for Rab5) with Rabaptin-5 (effector of Rab-5) was more effective than Rabex-5 alone for GDP-GTP exchange. The presence of different effectors could also potentiate the exchange rate of GDP toward ARF3 (Zhu *et al.*, 2000). The presence of effectors in complex with Bcr-Abl is currently under experimentation.

Cdc42 and Rac1 are central regulators of cell motility (Nobes and Hall, 1999), Cdc42 being required to

establish cell polarity and Rac1 to permit protrusive activity. RhoA is not required for cell movement, although a basal RhoA activity is necessary to maintain cell substrate adhesion. This proadherent effect of RhoA seems to counterbalance the effects of Rac1 and Cdc42, and at least Rac1 must inhibit RhoA to exert its activity toward myosin (Sander *et al.*, 1999). Apart from these cytoskeletal effects, Rho proteins act on other important pathways, such as apoptosis, proliferation and differentiation (for a review, see Zohn *et al.*, 1998). Again, Rac1/Cdc42 and RhoA effects can be distinguished. Rac1 is an important key in Ras transformation (Qiu *et al.*, 1995; Burstein *et al.*, 1998), and mediates cell survival signals through PI3-kinase/Akt pathway activation (Nishida *et al.*, 1999). Cdc42 may have the same proliferative and antiapoptotic activities as Rac1, because isolated inhibition of Rac1 in Bcr-Abl cells induces no change in apoptosis or proliferation status (Skorski *et al.*, 1998). In fact, like Rac1, Cdc42 activates both PI3-kinase and PAK, two regulators of these pathways (Tolias *et al.*, 1995; Roig *et al.*, 2000). In contrast, RhoA is implicated in G1 control of Raf-induced proliferation (Olson *et al.*, 1998) and most likely, through phospholipase D activation, promotes myeloid cell differentiation (Nakashima and Nozawa, 1999).

To summarize, Rac1 and Cdc42 may act preferentially as coactivators of the oncogenicity of Bcr-Abl, whereas RhoA is expected to behave as a regulator; p190^{bcr-abl}, which is responsible for acute leukemia, activates Rac1 and Cdc42, but not RhoA, and this should contribute to the much more aggressive expression of the disease. On the other hand, p210^{bcr-abl} does not fully transform hematopoietic progenitors, terminal myeloid differentiation of Ph⁺ cells being achieved during the chronic phase of CML. In these cells, Rac1 and Cdc42 may contribute to Bcr-Abl-activated proliferation, cell survival and motility, but the concomitant activation of RhoA should permit the differentiation of progenitors and a less-aggressive phenotype. These hypotheses are to be validated, and it would be of interest to determine the activation status of Rho proteins on CML and ALL patient cells.

Materials and methods

Cells

The murine hematopoietic cell line Ba/F3 (Ba/F3wt) was cultured in RPMI 1640 (Gibco BRL) containing 10% fetal calf serum (FCS) (Valbiotech, France) and 10 ng/ml of rm-IL3 (generous gift of Eric Lelièvre, INSERM E99-28, Angers, France) at 37°C with 5% CO₂. For stable transfection, retroviruses were produced by transfecting Phoenix-ampho cells (ATCC, Manassas, VA, USA and Garry Nolan, Stanford University Medical Center, CA, USA) with pGD vector containing the cDNAs of the different forms of Bcr-Abl (gift of George Daley, Whitehead Inst., Cambridge, MA, USA). The Bcr-Abl-expressing cell lines Ba/F3p210 and Ba/F3p190 were generated by infection with retrovirus supernatants as described (Daley and Baltimore, 1988; Pear *et al.*, 1993).

Antibodies

Antibodies against Abl: mouse monoclonal antibody 8E9 (Pharmingen) and rabbit polyclonal Ab-1 (Oncogene Research, Cambridge, MA, USA). Antibodies against Bcr: mouse monoclonal antibody G6 and rabbit polyclonal N-20 (Santa Cruz Biotechnology Inc., CA, USA). Other antibodies used were: anti-RhoA (26C4, mouse monoclonal, Santa Cruz Biotechnology Inc., CA, USA); anti-Rac (mouse monoclonal 23A8, Upstate Biotechnology, Lake Placid, NY, USA); anti-Cdc42 (P1-G rabbit polyclonal and B-8 mouse monoclonal, Santa Cruz Biotechnology Inc., CA, USA); anti-Vav (mouse monoclonal ascites, Upstate Biotechnology, Lake Placid, NY, USA); anti-Vav (H-111 rabbit polyclonal, Santa Cruz Biotechnology Inc., CA, USA); antiphosphotyrosine (PY20 mouse monoclonal, transduction Laboratories, Lexington KY, USA).

Secondary antibodies for immunofluorescence were obtained from Molecular Probes: Alexa488-conjugated goat anti-mouse IgG, Alexa488-conjugated goat anti-rabbit IgG. Secondary antibodies for Western blotting were obtained from Amersham Pharmacia Biotech: HRP-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG, or from Santa Cruz Biotechnology Inc., CA, USA: HRP-conjugated donkey anti-goat IgG.

Recombinant proteins

PGEX-2T vectors containing the cDNAs of RhoA, Rac1 and Cdc42 were obtained from Allan Hall (University College London, UK). Recombinant proteins were prepared as glutathione S-transferase fusion proteins in *Escherichia coli* (BL21 strain), purified using glutathione-sepharose beads (Amersham Pharmacia Biotech), eluted with glutathione and used as GST-fusion proteins. The GST-PAK-CRIB domain and GST-Rhotekin-RBD (C21) were obtained as pGEX-2T fusion genes (gift of JG Collard, Netherlands Cancer Institute, Amsterdam, NL, USA) and produced as described (Sander *et al.*, 1998).

Affinity binding assay

A total of 10⁷ Ba/F3wt, Ba/F3p190 or Ba/F3p210 cells were washed twice in cold PBS and then lysed in 1 ml of lysis buffer (Tris HCl 50 mM pH 7.4, NaCl 100 mM, MgCl₂ 2 mM, 1% NP-40 (w/v), 10% glycerol, PMSF 1 mM, leupeptine 20 μM, aprotinin 0.8 μM, pepstatin 10 μM). Total amount of protein in each lysate was measured and corresponded to 2 μg/μl. Lysates were clarified by centrifugation at 15 000 g for 15 min at 4°C and the supernatant was mixed into 50 μl of GST-fusion protein (C21: Rhotekin-Rho-binding-domain or PAK-CD: PAK-CRIB-domain) corresponding to 5 μg of protein bound to glutathione-sepharose beads. Incubation was conducted at 4°C for 1 h. Bead-bound complexes were washed four times in lysis buffer, boiled in Laemmli sample buffer and fractionated by a 13% SDS-PAGE, followed by Western blotting. For the C21 assay, the presence of RhoA was revealed using anti-RhoA antibody. For the PAK-CD assay, the presence of Rac1 and Cdc42 was revealed using anti-Rac1 or anti-Cdc42 antibodies.

For GST-Rho proteins binding assays, 2 μg of GSH-sepharose bead-bound GTPases were loaded with GDPβS, GTPγS or emptied by EDTA as described in Missy *et al.* (1998). In all, 2 × 10⁶ Ba/F3wt, Ba/F3p190 or Ba/F3p210 cells were washed twice in cold PBS and then lysed in 200 μl of RIPA buffer (Tris-HCl 50 mM pH 7.4, NaCl 150 mM, EDTA 5 mM, MgCl₂ 5 mM, 0.05% NP-40 (w/v), 1% sodium deoxycholate (w/v), 1% Triton X-100 (w/v), 0.1% SDS (w/v),

PMSF 1 mM, leupeptine 20 μ M, aprotinine 0.8 μ M, pepstatine 10 μ M). DNA and F-actin aggregates were disrupted by flushing several times through a syringe needle. Then 800 μ l of NET buffer (Tris-HCl 50 mM pH 7.4, EDTA 5 mM, MgCl₂ 5 mM, NaCl 150 mM, 0.05% NP-40, PMSF 1 mM, leupeptine 20 μ M, aprotinine 0.8 μ M, pepstatine 10 μ M) were added and the lysate was clarified by centrifugation at 15 000 g for 15 min at 4°C. The supernatant was mixed with preloaded (GDP or GTP) or nucleotide-free GTPases in the presence of 10 mM MgCl₂. Incubation was carried out for 1 h at 4°C. Bead-bound complexes were washed four times in NET buffer, boiled in Laemmli sample buffer and fractionated by a 7% SDS-PAGE, followed by Western blotting. The revelation was made using anti-Abl antibody (8E9).

Immunoprecipitation

Cell lysates were obtained from 10⁷ Ba/F3wt, Ba/F3p190 or Ba/F3p210 cells. Cells were washed twice in PBS and then lysed in 1 ml of RIPA buffer. DNA and F-actin aggregates were disrupted by flushing several times through a syringe needle. Then 4 ml of NET buffer was added and the lysate was clarified by centrifugation at 15 000 g for 15 min at 4°C. A measure of 1 ml of the supernatant was incubated overnight with first antibody at 4°C with continuous agitation. For anti-Abl immunoprecipitation, 1.6 μ g of 8E9 antibody was used; for anti-Bcr immunoprecipitation, 2 μ g of G6 antibody was used; for anti-Vav immunoprecipitation, 2 μ g of H211 was used. To precipitate the immune complexes, an incubation with 30 μ l of protein G-sepharose (100 μ g/ml) or protein A-sepharose (Amersham-Pharmacia) was conducted for 1 h at 4°C. Bead-bound complexes were washed four times with a cold lysis buffer containing 10 mM MgCl₂. They were used for GTP-exchange assays or boiled in the Laemmli sample buffer and loaded onto an SDS-PAGE apparatus.

SDS-PAGE and immunoblots

SDS-PAGE was performed according to Laemmli and Favre (1973). Gels were stained with Coomassie brilliant blue R-250. For Western blots, proteins were transferred from gels to nitrocellulose membranes (0.20 μ m pore) (Sartorius, Göttingen, Germany) using a Miniprotein electroblotter (Biorad laboratories, Hercules, CA, USA). Immunoblots were washed three times in PBS containing 0.1% Tween-20 (PBS-Tween) and then probed overnight at 4°C with antibody in PBS-Tween. Following three washes with PBS-Tween, membranes were incubated for 1 h at 4°C with antimouse immunoglobulin (HRP-linked whole antibody from sheep) or antirabbit immunoglobulin (HRP-linked whole antibody from donkey) (Amersham-Pharmacia). The membranes were washed three times for 5 min per wash with PBS-Tween, and bound antibodies were detected using enhanced luminol and oxidizing reagent as specified by the manufacturer (ECL, Amersham-Pharmacia).

Immunofluorescence and confocal microscopy

Ba/F3 cells were washed in PBS twice and resuspended at 10⁶ cells per ml. Cell suspension (100 μ l) was plated on a glass coverslip precoated with ECM-gel (Sigma) and cultured for 24 h. Coverslips were washed with TBS. Cells were fixed in 3% PFA for 10 min, washed with TBS, then permeabilized with a solution of PIPES 80 mM pH 6.4, MgCl₂ 1 mM, EGTA 5 mM and 0.1% saponin for 2 min. For Cdc42 staining,

after PFA, a second fixation in methanol at -20°C was performed for 2 min. Cells were washed with TBS containing 2% BSA and coverslips were incubated on a parafilm with normal goat serum (10% in TBS) for 15 min at room temperature. Then, coverslips were incubated for 1 h with 20 μ l of a mix of antibodies (1% anti-RhoA, 1/80 anti-Rac1 or 1% anti-Cdc42) made up in 2% NGS. Cells were washed twice for 5 min in 2% TBS-BSA, and then incubated for 1 h with secondary antibodies (1% anti-rabbit Alexa488 for Cdc42 or 1% anti-mouse Alexa488 for Rac1 and RhoA) and 1/200 TO-PRO-3 (Molecular Probes). Coverslips were washed three times in TBS-BSA 2%, and then mounted with Vectashield fluorescence medium H-1000 (Vector Lab. Inc., Burlingame, CA, USA). Immunolabeled samples were examined by confocal laser scanning microscopy using a Biorad MRC 1024 ES equipped with an argon-krypton gas laser and deconvoluted using the Imaris software (Bitplane).

GDP releasing and GTP binding assays

[³H]GDP release was measured as described by Hart *et al.* (1991) with slight modification. In brief, 50 pmol of GST-fused GTPases were incubated in 20 mM Tris-HCl pH 7.5, 1 mM MgCl₂, 1 mM DTT, 100 mM NaCl, 40 μ g/ml BSA and 5 μ Ci of [³H]GDP (32.5 Ci/mmol) for 45 min at 37°C. [³H]GDP-bound GTPases (total volume 110 μ l) were then kept on ice. Release reaction started by adding [³H]GDP-bound GTPases to 40 μ l of protein-G-sepharose-bound complexes obtained with anti-Abl or anti-Vav antibody on 10⁷ cells, and GTP (1 mM final). The incubation was conducted with continuous agitation at 25°C for 15 min. At the indicated times, 30 μ l of aliquots were removed and added to 1 ml of ice-cold stop solution (Tris-HCl 25 mM pH 7.4, NaCl 100 mM, MgCl₂ 30 mM, 2 mM DTT) and the content of the tube was filtered under vacuum onto a HA 0.45 μ m nitrocellulose filter. The filter was washed five times with 1 ml of stop solution; then radioactivity fixed on the filter was quantified by liquid scintillation counting. The association of [³⁵S]GTP γ S to the GDP-bound form of GST-GTPases was carried out according to Hoshino *et al.* (1999), with slight modifications. In brief, 80 μ l of protein-G-sepharose-bound complexes obtained with anti-Abl antibody on 10⁷ cells was mixed with 40 pmol of GDP-bound GTPases in 300 μ l of 50 mM Tris-HCl pH 8.0, 1 mM DTT, 10 mM MgCl₂ and 2.9 mM EDTA, and 3 μ l of 100 mM GTP γ S containing GTP γ [³⁵S]. The incubation was conducted with continuous agitation at 25°C for 15 min. At the indicated times, 35 μ l aliquots were removed and added to 1 ml of ice-cold stop solution and the content of the tube was filtered under vacuum onto an HA 0.45 μ m nitrocellulose filter. The filter was washed five times with 1 ml of stop solution; then radioactivity fixed on the filter was quantified by liquid scintillation counting. Results are expressed as mol of GTP bound per mol of GTPase.

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