Critical role for Gab2 in transformation by BCR/ABL

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Summary

The *BCR/ABL* oncogene causes chronic myelogenous leukemia (CML) in humans and a CML-like disease, as well as lymphoid leukemia, in mice. p210 BCR/ABL is an activated tyrosine kinase that phosphorylates itself and several cellular signaling proteins. The autophosphorylation site tyrosine 177 binds the adaptor Grb2 and helps determine the lineage and severity of BCR/ABL disease: Tyr177 mutation (BCR/ABL-Y177F) dramatically impairs myeloid leukemogenesis, while diminishing lymphoid leukemogenesis. The critical signal(s) from Tyr177 has remained unclear. We report that Tyr177 recruits the scaffolding adaptor Gab2 via a Grb2/Gab2 complex. Compared to BCR/ABL-expressing Ba/F3 cells, BCR/ABL-Y177F cells exhibit markedly reduced Gab2 tyrosine phosphorylation and association of phosphatidylinositol-3 kinase (PI3K) and Shp2 with Gab2 and BCR/ABL, and decreased PI3K/Akt and Ras/Erk activation, cell proliferation, and spontaneous migration. Remarkably, bone marrow myeloid progenitors from *Gab2* (−/−) mice are resistant to transformation by BCR/ABL, whereas lymphoid transformation is diminished as a consequence of markedly increased apoptosis. BCR/ABL-evoked PI3K/Akt and Ras/Erk activation also are impaired in *Gab2* (−/−) primary myeloid and lymphoid cells. Our results identify Gab2 and its associated proteins as key determinants of the lineage and severity of BCR/ABL transformation.

Introduction

Several forms of human leukemia are caused by the Philadelphia chromosome (Ph) translocation [t(9,22) (q34;q11)], which fuses the *BCR* and c-*ABL* genes. Three different translocation breakpoints result in the production of distinct BCR/ABL fusion proteins that share the same segment of c-ABL, but differ in BCR content. The most common fusion protein, p210 BCR/ABL, causes chronic myelogenous leukemia (CML), whereas p190 BCR/ABL is found primarily in a subset of patients with acute lymphocytic leukemia (ALL), and p230 BCR/ABL is found in chronic neutrophilic leukemia (Ben-Neriah et al., 1986; Chan et al., 1987; Clark et al., 1987; Fainstein et al., 1987; Heisterkamp et al., 1983, 1985; Kurzrock et al., 1987; Pane et al., 1996; Shtivelman et al., 1985; Walker et al., 1987). Despite intensive study, the mechanism underlying the different lineage associations of the three BCR/ABL proteins remains largely unknown.

The BCR region of the fusion proteins probably plays a major role in specifying both the virulence and the lineage of the leukemia. When introduced into murine bone marrow cells by retroviral transduction followed by bone marrow transplantation (BMT), all of the BCR/ABL fusion proteins can cause a chronic myeloid leukemia (CML)-like myeloproliferative disease, but p190 BCR/ABL is more potent at inducing lymphoid leukemia (Li et al., 1999; Quackenbush et al., 2000). Recent studies of *BCR/ABL* oncogenes with mutations of a single autophosphorylation site in BCR, Tyr177, have linked BCR signaling to lineage determination and disease latency (Million and Van Et-

SIGNIFICANCE

The BCR/ABL oncogene encodes an activated tyrosine kinase that causes myeloid and lymphoid leukemias. Mutation of the autophosphorylation site at Tyrosine 177 in BCR (BCR/ABL-Y177F) dramatically impairs myeloid leukemogenesis, while diminishing lymphoid leukemogenesis. We report that Tyr177 recruits the scaffolding adapter Gab2 via a Grb2/Gab2 complex. Cells expressing BCR/ABL-Y177F exhibit reduced association of PI3K and Shp2 with Gab2 and BCR/ABL, decreased PI3K/Akt and Ras/Erk activation, and reduced cell proliferation and migration. Remarkably, bone marrow cells from Gab2 (−/−) mice are highly resistant to myeloid, and partially resistant to lymphoid transformation by BCR/ABL, most likely as a consequence of increased apoptosis. Our results identify Gab2 signaling as a key determinant of the lineage and severity of BCR/ABL transformation.

ten, 2000; Zhang et al., 2001). Tyr177 lies within a Grb2 binding motif (Y-X-N-X) in BCR, and is essential for Grb2 binding to BCR/ABL (Pendergast et al., 1993; Puil et al., 1994). The importance of direct binding of Grb2 to BCR/ABL has been controversial. Pendergast et al. reported that p190 BCR/ABL-Y177F is defective for transactivation of a Ras-responsive reporter gene in Rat-1 fibroblasts, transformation of Rat-1 cells to anchorage-independence, and ex vivo transformation of primary bone marrow pre-B lymphocytes. These workers concluded that phosphorylation of Tyr177 was necessary for Ras activation and BCR/ABL transformation (Pendergast et al., 1993). Consistent with this hypothesis, expression of a Grb2 SH3 domain dominant-negative mutant (Gishizky et al., 1995) or interference with the Ras pathway by expression of activated RasGAP (Sawyers et al., 1995) or dominant-negative Ras (Cortez et al., 1995; Sakai et al., 1994) inhibits BCR/ABL transformation. However, subsequent studies demonstrated that BCR/ABL-Y177F could transform primary B lymphocytes (Goga et al., 1995) and cytokine-dependent hematopoietic cell lines ex vivo (Cortez et al., 1995; Goga et al., 1995). Furthermore, unlike in Rat-1 cells, Ras activation by BCR/ABL-Y177F was normal in hematopoietic cell lines (Cortez et al., 1995), suggesting that Ras could be activated through alternative pathways (e.g., via tyrosine phosphorylated Shc) in these cells.

Three recent studies have begun to clarify this controversy. In the murine bone marrow transduction/transplantation model, p210 BCR/ABL-Y177F is profoundly defective for induction of CML-like myeloproliferative disease; instead, recipients of p210 BCR/ABL-Y177F-transduced marrow succumb to B- and T-lymphoid leukemias of prolonged latency (He et al., 2002; Million and Van Etten, 2000; Zhang et al., 2001). These results demonstrate that Tyr177, and presumably Grb2 binding, contribute to both myeloid and lymphoid leukemogenesis by BCR/ABL, although Tyr177 is more important for the former. However, the nature of the signaling pathway downstream of Tyr177 has remained elusive. Because BCR/ABL can stimulate Ras through other mechanisms, at least in hematopoietic cell lines, activated Ras may not be the only or even the principal effector of Grb2 in BCR/ABL-expressing cells. Instead, Tyr177, acting via Grb2, may send another signal(s) required for myeloid transformation and important for determining the latency of lymphoid leukemogenesis.

Here we identify the scaffolding adaptor Gab2 as an essential mediator of BCR/ABL transformation. Gab2, a member of the family of PH-domain containing adaptor proteins that includes mammalian Gab1 (Holgado-Madruga et al., 1996) and Gab3 (Wolf et al., 2002) and *Drosophila* DOS (daughter of sevenless) (Herbst et al., 1996; Raabe et al., 1996), becomes tyrosine phosphorylated in response to a multiple cell stimuli (Gu et al., 1997, 1998, 2001; Lynch and Daly, 2002; Miyakawa et al., 2001; Nishida et al., 1999; Wickrema et al., 1999; Zhao et al., 1999). Reverse genetic and/or genetic analyses implicate Gab2 as a positive (signal-enhancing) component of growth factor, cytokine, and antigen receptor signal transduction (Gu et al., 1998, 2000, 2001; Liu et al., 2001; Lynch and Daly, 2002), although it may have a negative regulatory role in T cell receptor signaling (Pratt et al., 2000; Yamasaki et al., 2001). We reported previously that Gab2 is constitutively tyrosine phosphorylated in BCR/ABL-transformed cells (Gu et al., 1997, 1998). However, its mechanism of phosphorylation and role in BCR/ABL transformation have remained unclear.

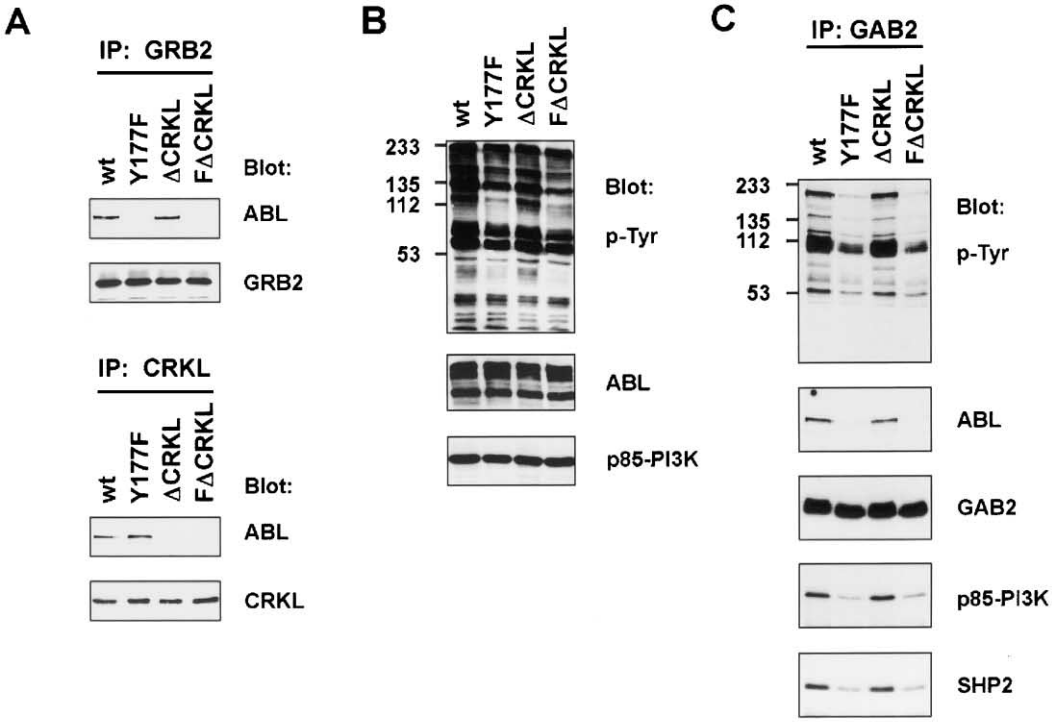
We have found that tyrosine phosphorylation of Gab2 in BCR/ABL-transformed cells requires Y177 of BCR/ABL and the Grb2 SH3 binding site in Gab2; thus, Gab2 phosphorylation by BCR/ABL is mediated by a Grb2/Gab2 complex. This interaction is required for full activation of the PI3K/Akt and Ras/Erk pathways and for optimal proliferation and migration of Ba/F3 cells in response to BCR/ABL. More importantly, BCR/ABL cannot transform primary myeloid cells from *Gab2* (−/−) mice, whereas *Gab2* (−/−) B lymphoblasts can be transformed, but exhibit defective migratory capacity and proliferation. The latter is due, at least in large part, to a marked increase in apoptosis. Moreover, primary myeloid and lymphoid cells from *Gab2* (−/−) mice transduced with BCR/ABL-WT-expressing retroviruses have comparable signaling defects to Ba/F3 cells expressing BCR/ABL-Y177F. Our results implicate Gab2 as a key mediator of signals critical for determining the lineage and severity of transformation by BCR/ABL.

Results

The Grb2 binding site in BCR/ABL regulates tyrosine phosphorylation of Gab2

The binding site for the Grb2 SH2 domain in BCR/ABL (Tyr177) regulates multiple aspects of BCR/ABL action, including ex vivo transformation of fibroblasts or pre-B lymphocytes and efficient proliferation of factor-dependent hematopoietic cell lines (Cortez et al., 1995; Pendergast et al., 1993; Puil et al., 1994), and the latency and type (i.e., lineage) of leukemia caused by BCR/ABL in the murine bone marrow transduction/transplantation model (Million and Van Etten, 2000; Zhang et al., 2001; He et al., 2002). To identify signaling defects specifically associated with Tyr177, we generated pools of interleukin-3 (IL-3)-dependent murine Ba/F3 cells expressing either wild-type p210 BCR/ABL (BCR/ABL-WT) or a Tyr177Phe substitution (Y177F) in the BCR part of the fusion protein. For comparison, a *BCR/ABL* construct with a mutation in its CRKL binding site (ΔCRKL), a proline-rich region in the ABL part of the fusion protein (Senechal et al., 1996), and a Grb2/Crkl binding site double mutant (FΔCRKL), were also used to generate Ba/F3 cell pools. Prolonged growth of BCR/ABL-expressing Ba/F3 cells in the absence of IL-3 can lead to progressive changes in patterns of signal transduction, possibly from the mutagenic effects of BCR/ABL kinase activity (Nieborowska-Skorska et al., 2000). We were concerned that some of the discrepancies in the reported effects of BCR/ABL-Y177F (see Introduction) might be caused by such secondary mutations. Therefore, to avoid this potential problem and control for clonal variation, we studied polyclonal cell populations (pools) selected for G418 resistance rather than cytokine-independence, and maintained these pools in the presence of IL-3 and the ABL kinase inhibitor STI571 (*Gleevec*). Cell activations were initiated by removal of STI571 and IL-3.

To ensure that each mutant had the expected phenotype, we measured the interaction between Grb2 or Crkl and BCR/ABL in the Y177F, ΔCRKL, and FΔCRKL mutants by immunoprecipitation followed by immunoblotting (Figure 1A). As expected, Grb2 did not coimmunoprecipitate with the BCR/ABL-Y177F and -FΔCRKL mutants, whereas Crkl failed to coprecipitate with BCR/ABL-ΔCRKL or -FΔCRKL mutants. We next explored the effects of the Y177F mutation on total cellular tyrosine phosphorylation. Although overall tyrosine phosphorylation was similar in BCR/ABL-WT- and mutant-expressing cells, cells expressing BCR/ABL-



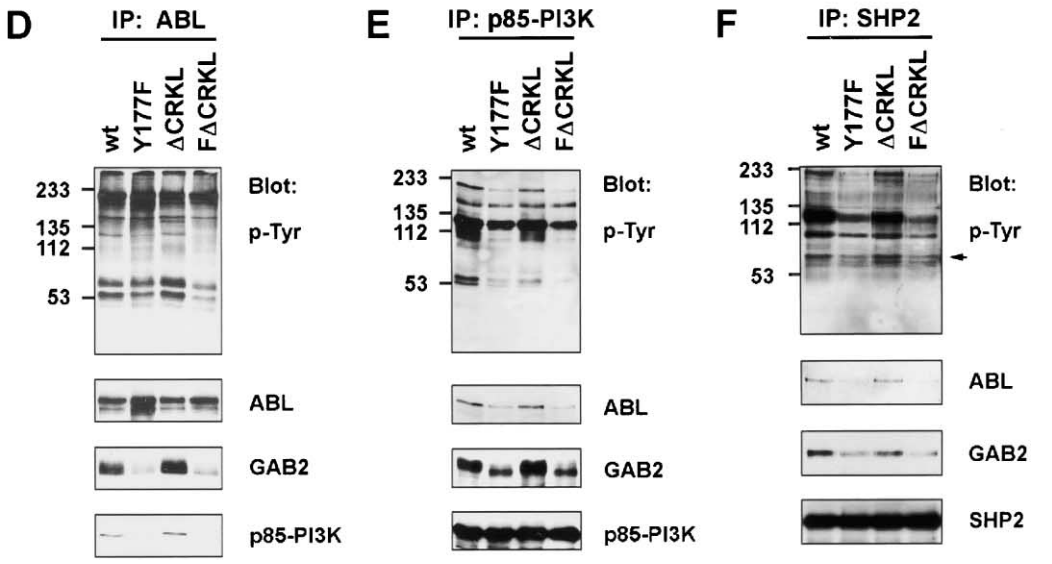


Figure 1. Grb2 binding site in BCR/ABL regulates tyrosine phosphorylation of Gab2

A–F: Ba/F3 cells expressing BCR/ABL-WT or the indicated BCR/ABL mutants were used. **A:** Lysates from 20×10^6 cells were subjected to immunoprecipitation with anti-Grb2 or anti-CRKL antibodies, and coimmunoprecipitation of BCR/ABL was assessed by immunoblotting as indicated. The blots were reprobed with anti-Grb2 or anti-CRKL antibodies, as indicated, to control for equal loading. **B:** The indicated cell lysates (from 0.25×10^6 cells) were subjected to anti-phosphotyrosine immunoblotting. The blot was reprobed with anti-Abl and anti-p85-PI3K antibodies, as indicated. Note equal loading of protein (as assessed by p85-PI3K blot) and comparable expression of each BCR/ABL protein. **C:** Anti-Gab2 immunoprecipitates were prepared from cell lysates as in **A**, and successively immunoblotted with antibodies against phosphotyrosine, Abl, Gab2, Shp2, and p85-PI3K, as indicated. **D–F:** Lysates from 20×10^6 cells were subjected to immunoprecipitations with antibodies against Abl (**D**), p85-PI3K (**E**), or Shp2 (**F**), followed by immunoblotting with the indicated antibodies. For all panels, the migration positions of molecular mass markers (in kDa) are indicated.

Y177F exhibited distinctly reduced tyrosine phosphorylation of proteins with apparent molecular weights of 40, 72, 100, 120, and 150 kDa. In ΔCRKL mutant-expressing cells, there was less of an effect on cellular phosphotyrosine proteins, although proteins with apparent molecular weights of 30–40 kDa and a 160 kDa species showed consistent decreases (Figure 1B, top panel). The pattern of phosphotyrosine-containing proteins in BCR/ABL-FΔCRKL-expressing cells was the sum of the individual single mutants. Reprobing these immunoblots with anti-ABL and anti-p85-PI3K antibodies confirmed that all of the cell lines expressed comparable levels of BCR/ABL compared to these internal controls (Figure 1B, middle and bottom panels).

The size and appearance of the 100 kDa phosphotyrosine protein that exhibited decreased phosphorylation in BCR/ABL-Y177F cells were reminiscent of the scaffolding adaptor Gab2. Since Gab2 is phosphorylated in multiple BCR/ABL-WT transformed cell lines, including BCR/ABL-transformed Ba/F3 cells (Gu et al., 1997, 1998), we asked whether the 100 kDa species was Gab2. Indeed, tyrosine phosphorylation of Gab2 was reduced markedly (~70% as determined by scanning densitometry) in cells expressing BCR/ABL-Y177F or BCR-ABL-FΔCRKL, but not in cells expressing BCR/ABL-ΔCRKL (Figure 1C). Gab2 also coimmunoprecipitated with BCR/ABL-WT and BCR/ABL-ΔCRKL, but to a much lesser extent with BCR/ABL-Y177F (or BCR/ABL-FΔCRKL) (Figure 1C and 1D). Taken together, these results indicate that Tyr177 is required for binding of Gab2 to BCR/ABL and for efficient BCR/ABL-evoked tyrosine phosphorylation of Gab2. Notably, some residual BCR/ABL-evoked Gab2 tyrosine phosphorylation was observed even in the absence of Tyr177 (and in the absence of cytokines). Therefore, at least one additional pathway from BCR/ABL must be capable of causing low levels of Gab2 phosphorylation.

Reduced binding of Gab2-associated signaling proteins in cells transformed by BCR/ABL-Y177F

Gab2 contains binding sites for the SH2 domains of the p85 subunit of PI3K (p85-PI3K) and Shp2. Like Gab2, Shp2 (but not p85-PI3K) is tyrosine phosphorylated constitutively in BCR/ABL-transformed cells (Gu et al., 1997; Tauchi et al., 1994). In cells transformed by BCR/ABL-WT or BCR/ABL-ΔCRKL, p85-PI3K and Shp2 were detected in anti-Gab2 (Figure 1C) and in anti-ABL (Figure 1D) immune complexes. Likewise, Gab2 was found in anti-p85-PI3K (Figure 1E) and anti-Shp2 (Figure 1F) immunoprecipitates. In contrast, Gab2/p85-PI3K and Gab2/Shp2 association, as well as Shp2 tyrosine phosphorylation, were reduced dramatically in cells transformed by BCR/ABL-Y177F, consistent with the substantially decreased Gab2 tyrosine phosphorylation in these cells (Figures 1D–1F). Thus, the Y177F mutation causes a marked reduction in Gab2 binding to BCR/ABL, decreased tyrosine phosphorylation of Gab2 and the Gab2-associated signaling protein, Shp2, and reduced binding of p85-PI3K to Gab2 and BCR/ABL. These findings indicate that Gab2 mediates, at least in part, interaction of Shp2 with BCR/ABL. Moreover, a significant amount of p85-PI3K, and by inference, PI3K (see below) must associate with BCR/ABL via Gab2.

Gab2 is recruited to BCR/ABL via Grb2

Since Tyr177 of BCR is a Grb2 SH2 domain binding site (Pendergast et al., 1993; Puil et al., 1994), and the Grb2 SH3 domains bind Gab2 (Gu et al., 2000; Lock et al., 2000), we asked whether

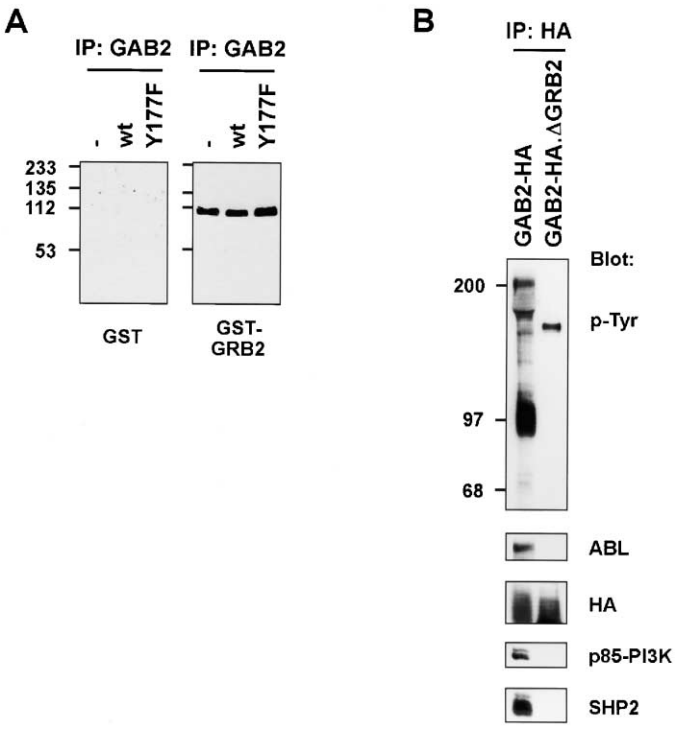


Figure 2. BCR/ABL-evoked tyrosine phosphorylation of Gab2 requires Grb2

A: Grb2 binds directly to Gab2. Gab2 immunoprecipitates (20×10^6 cells/sample) from randomly growing Ba/F3 cells (−) or cell pools expressing BCR/ABL-WT or BCR/ABL-Y177F, as indicated, were resolved by SDS-PAGE and transferred to a PVDF-membrane. Direct binding of GST or a GST-Grb2 fusion protein was assessed by protein overlay assay. Note that Grb2 binds to Gab2 directly, specifically, and constitutively.

B: Ba/F3.BCR/ABL-WT cells were transfected with vectors that express Gab2-HA or a Gab2 mutant containing deletions of its Grb2 binding sites (Gab2-HA.ΔGRB2). Lysates from 20×10^6 cells were subjected to anti-HA immunoprecipitations and tyrosine phosphorylated proteins (p-Tyr), or HA-tagged Gab2 (HA), Abl, Shp2, and PI3K detected by immunoblotting, as indicated. For all panels, the migration positions of molecular mass markers (in kDa) are indicated.

Grb2 mediates the interaction of Gab2 with BCR/ABL. Ba/F3, Ba/F3.BCR/ABL-WT, and Ba/F3.BCR/ABL-Y177F cell lysates were subjected to immunoprecipitations with anti-Gab2 antibodies, and direct binding of Grb2 was assessed by a protein overlay assay. Notably, Gab2 bound to Grb2, and this interaction was independent of BCR/ABL-Y177 (Figure 2A).

Gab2 contains two proline-rich domains that can bind to the Grb2 SH3 domains (Lock et al., 2000). To determine if the Grb2 SH3 domain binding sites in Gab2 are required for the tyrosine phosphorylation of Gab2, BCR/ABL-WT-expressing Ba/F3 cells were transfected transiently with a vector expressing HA-tagged wild-type Gab2 (Gab2-HA) or a Gab2 mutant lacking Grb2 SH3 domain binding sites (Gab2-HA.ΔGrb2). Whereas Gab2-HA was robustly tyrosine phosphorylated in response to BCR/ABL, BCR/ABL-evoked tyrosine phosphorylation of Gab2-HA.ΔGrb2 was undetectable. The mutant Gab2 protein also failed to bind to BCR/ABL, Shp2, and PI3K (Figure 2B). Thus, via its SH3 domain(s), Grb2 binds to Gab2 constitutively. The Grb2/Gab2 complex is then recruited to BCR/ABL via binding of the Grb2 SH2 domain to phosphorylated Tyr177 of BCR/ABL.

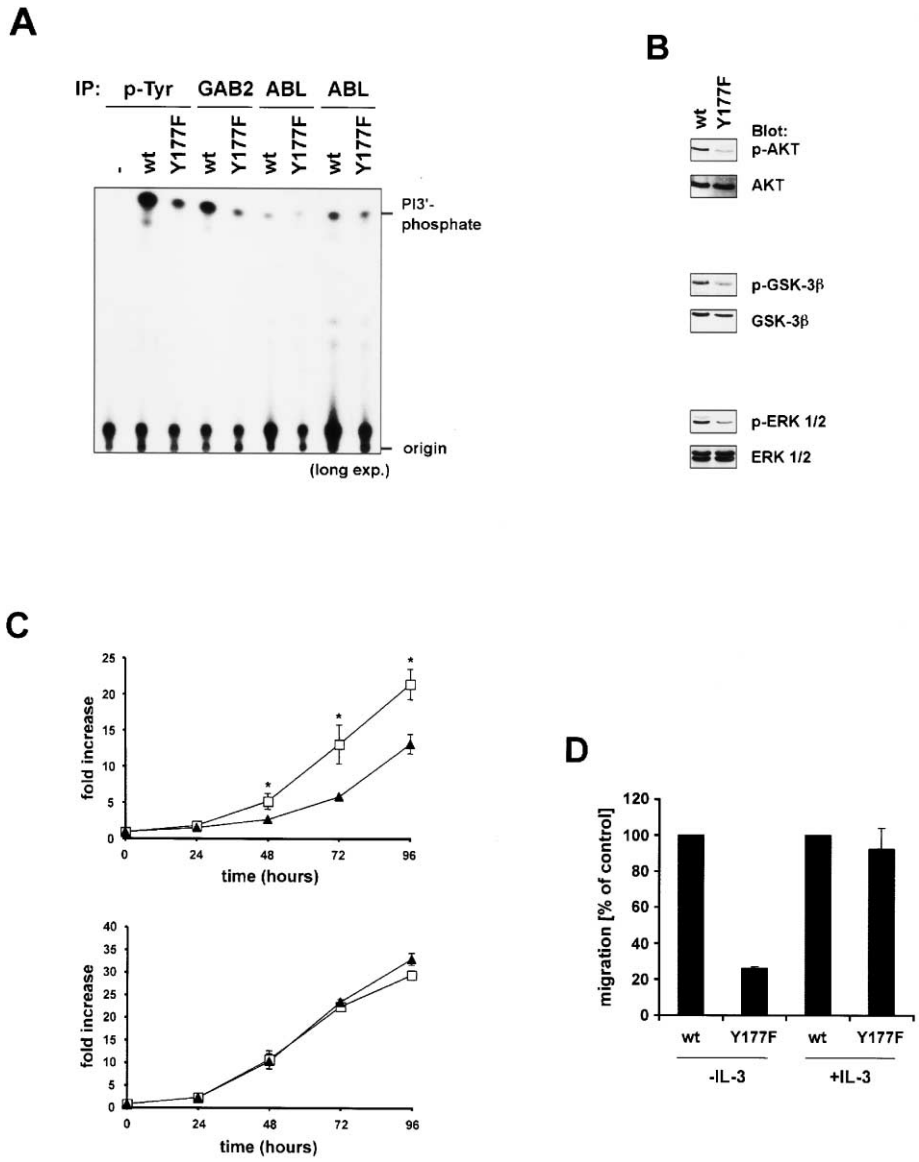


Figure 3. Effect of Tyr177 mutation on BCR/ABL-evoked biochemical and biological events

A: Tyr177 regulates PI3K activation by BCR/ABL. Lysates (25×10^6 cells) from Ba/F3 cells or Ba/F3 cells expressing BCR/ABL-WT or BCR/ABL-Y177F, as indicated, were immunoprecipitated with the indicated antibodies, and the resultant immune complexes were subjected to in vitro PI3K kinase assays, followed by thin-layer chromatography and autoradiography. The position of migration of phosphatidylinositol-3 phosphate (as determined by iodine staining of nonradioactive standards) is indicated. PIP3 production, as quantified by scanning densitometry (see Experimental Procedures), was decreased 60%–70% in anti-phosphotyrosine immunoprecipitates, 70%–80% in anti-Gab2 immunoprecipitates, and 65%–75% in anti-Abl immunoprecipitates (corresponding to 2–4 experiments with each antibody).

B: Tyr177 regulates Akt and Erk activation. Lysates from the indicated cells (0.25×10^6) were subjected to immunoblotting with phosphospecific antibodies to Akt (p-Akt), Gsk-3β (p-Gsk-3β), and Erk proteins (p-Erk). To control for loading, the blots were stripped and reprobed with antibodies against total Akt (AKT), Gsk-3β (GSK-3β), or Erk1 and Erk2 (ERK1/2).

C: Tyr177 regulates BCR/ABL-evoked Ba/F3 cell proliferation. Proliferation of the indicated Ba/F3 cell lines was monitored in the absence (top) or the presence (bottom) of IL-3 and STI571. □ = Ba/F3.BCR/ABL-WT, ▲ = Ba/F3.BCR/ABL-Y177F. Data are expressed as fold increase in cell number (determined by Coulter counting) over the time of the assay. Shown is one of three representative experiments. *Significant differences ($p < 0.02$) were observed between Ba/F3.BCR/ABL-WT and Ba/F3.BCR/ABL-Y177F cells.

D: Optimal BCR/ABL-evoked haplotaxis requires Tyr177. Ba/F3 cells expressing BCR/ABL-WT or BCR/ABL-Y177F were used in transwell migration assays. The number of viable cells in the lower chamber was determined after 3 hr by trypan blue exclusion. Error bars indicate the standard error of the mean (n=3).

Upon recruitment, Gab2 becomes tyrosine phosphorylated and recruits additional signal relay molecules, such as Shp2 and p85-PI3K.

Tyr177 of BCR/ABL regulates multiple downstream signaling pathways

PI3K is known to be activated by BCR/ABL, and is required for BCR/ABL transformation of hematopoietic cells (Skorski et al., 1995). The reduced binding of p85-PI3K to Gab2 and BCR/ABL in BCR/ABL-Y177F-expressing cells suggested that Tyr177 might be involved in the regulation of PI3K activity. Therefore, we measured the PI3K activity associated with anti-phosphotyrosine, anti-Gab2, and anti-ABL immune complexes (Figure 3A). As reported previously (Sattler et al., 1996), PI3K activity in phosphotyrosine immunoprecipitations was increased in cells transformed by BCR/ABL-WT compared to untransformed cells. Significant PI3K activity also was detected in anti-Gab2 and anti-ABL immune complexes in BCR/ABL-WT cells. However, in cells transformed by BCR/ABL-Y177F, PI3K activity associated with anti-phosphotyrosine, anti-ABL, or anti-Gab2 immune complexes was reduced by 60%–80% (see Figure 3A legend). These results show that Tyr177 is involved in the regulation of PI3K and implicate Gab2 in regulating this activity.

We next asked if Y177 regulates the activation of PI3K effectors. Indeed, the serine phosphorylation of Akt and Gsk-3β was reduced in cells expressing BCR/ABL-Y177F (Figure 3B; top and middle panels). Erk 1 and Erk2 activation were also diminished in these cells (Figure 3B, bottom panels). Thus, Tyr177-derived signals contribute substantially to the regulation of multiple downstream signaling pathways known to be critical for BCR/ABL transformation.

Tyr177 in BCR/ABL regulates proliferation and migration

Previous studies of the effects of BCR/ABL Y177F on hematopoietic cells have been somewhat contradictory (see Introduc-

tion and Discussion). At least some of these discrepancies may have been due to different protocols used to generate BCR/ABL-expressing hematopoietic cell lines. As indicated above, we made a special effort to limit the exposure of our cell pools to BCR/ABL kinase activity, thereby minimizing potentially mutagenic effects of BCR/ABL. Using this protocol, we compared the effects of BCR/ABL-WT and BCR/ABL-Y177F on several different biological responses of Ba/F3 cells. Consistent with previous results (Cortez et al., 1995), both of these proteins induced factor-independent growth of Ba/F3 cells. However, Ba/F3 cell proliferation in the absence of added cytokines was reduced in cells expressing BCR/ABL-Y177F, compared to BCR/ABL-WT (Figure 3C, top panel). Addition of IL-3 restored growth of the BCR/ABL-Y177F mutant-expressing cell line to levels comparable to that found in BCR/ABL-WT cells (Figure 3C, bottom panel), indicating that BCR/ABL-Y177F cells do not have a defect in proliferation per se, but only in BCR/ABL-evoked proliferation. BCR/ABL-WT-transformed Ba/F3 cells demonstrate a significant level of spontaneous migration, as measured in a transwell migration assay (Salgia et al., 1999). BCR/ABL-Y177F also evoked lower levels of spontaneous migration than BCR/ABL-WT (Figure 3D) and, as in the proliferation experiments, the addition of IL-3 rescued the BCR/ABL-Y177F migration defect. Analogous results were obtained in studies in which BCR/ABL-WT or BCR/ABL-Y177F was expressed in 32Dcl3 cells (another IL-3 dependent hematopoietic cell line; data not shown). Thus, signals from Tyr177 contribute to both BCR/ABL-evoked proliferation and migration of hematopoietic cell lines.

Gab2 is required for transformation of primary murine myeloid cells by BCR/ABL

The above studies suggest that Gab2-associated signals may be critical for BCR/ABL transformation. Therefore, we compared the ability of BCR/ABL to transform primary myeloid cells from bone marrow of wild-type *Gab2* (+/+) and *Gab2* (−/−) mice. Importantly, *Gab2* (−/−) mice have normal steady state hematopoiesis and are otherwise healthy, although their allergic response is defective (Gu et al., 2001). Normal myeloid progenitors require cytokines to form colonies in methylcellulose. Consistent with previous work (Gishizky and Witte, 1992), *BCR/ABL*-WT, introduced by retroviral transduction, transformed a significant number of progenitors from *Gab2* (+/+) mice, leading to cytokine-independent myeloid colonies (mainly CFU-GM, but a few CFU-M and CFU-GEMM). However, BCR/ABL-WT was completely unable to transform bone marrow cells from *Gab2* (−/−) mice (Figure 4A). Transduction of bone marrow cells from mice of either genotype with the parental retrovirus did not cause cytokine-independent myeloid colony growth. Notably, in the presence of IL-3, transduced bone marrow cells from *Gab2* (−/−) and *Gab2* (+/+) mice gave rise to comparable numbers of myeloid colonies, confirming that the bone marrow CFU-C content of *Gab2* (−/−) mice is normal and demonstrating that BCR/ABL is not toxic to *Gab2* (−/−) progenitors. Moreover, experiments using a green fluorescent protein (GFP)-expressing retrovirus demonstrated that *Gab2* (+/+) and *Gab2* (−/−) bone marrow cells had comparable susceptibility to retroviral transduction (Figure 4B). Compared to BCR/ABL-WT, BCR/ABL-Y177F was only partially defective at inducing colony outgrowth of *Gab2* (+/+) myeloid cells (Figure 4A). Importantly, *Gab2* (−/−) myeloid cells are not completely refractory to transformation, since their ability to be transformed by the *TEL/JAK2* oncogene was reduced only slightly (by at most 2-fold; data not shown). Overall, our data indicate that Gab2 is required for transformation of murine bone marrow myeloid progenitors by BCR/ABL in a colony outgrowth assay, and suggest that at least some of the transforming defects associated with the Y177F mutant are due to failure to transmit appropriate signals through Gab2.

Since *Gab2* (−/−) myeloid cells cannot be stably transformed by BCR/ABL, extensive biochemical studies of the effects of BCR/ABL in these cells were not possible. Nevertheless, by making use of retrovirus that coexpresses BCR/ABL-WT and green fluorescence protein (GFP), we were able to infect primary myeloid cells from WT and *Gab2* (−/−) bone marrow and recover enough transduced cells for limited analyses (see Experimental Procedures). Remarkably, Akt and Erk activation were substantially reduced in BCR/ABL-transduced *Gab2* (−/−) myeloid cells (Figure 4C); notably, these defects are qualitatively similar to those observed in BCR/ABL Y177F-expressing Ba/F3 cells (compared to BCR/ABL-WT.Ba/F3) and therefore support the notion that Gab2 is a key transducer of signal(s) from Tyr177.

Gab2 determines the severity of lymphoid transformation by BCR/ABL

In the murine bone marrow transduction/transplantation model, BCR/ABL-Y177F exhibits a relative defect in lymphoid leukemogenic ability (in contrast to its more profound defect in myeloid leukemogenesis; see Introduction). Therefore, we assessed the susceptibility of *Gab2* (−/−) B lymphoid cells to transformation by BCR/ABL. Bone marrow cells from *Gab2* (+/+) and *Gab2* (−/−) mice were transduced with *BCR/ABL*-WT retroviruses and cultured under conditions that favor outgrowth of transformed B lymphoid cells (Whitlock-Witte cultures [McLaughlin et al., 1987]; see Experimental Procedures). Unlike *Gab2* (−/−) myeloid cells, BCR/ABL-transformed *Gab2* (−/−) B lymphoid cells were observed. These cells had the same surface phenotype (B220+/CD24+/BP-1+) as BCR/ABL-transformed *Gab2* (+/+) B lymphoblasts (data not shown). However, when similar numbers of transduced *Gab2* (+/+) and *Gab2* (−/−) bone marrow cells were plated, it took significantly longer for the latter to reach confluence (Figure 5A). Transduction efficiency of *Gab2* (+/+) and *Gab2* (−/−) bone marrow was equivalent by determination of proviral copy numbers by Southern blot, and comparable numbers of pre- and pro-B cells from wild-type and mutant bone marrow were GFP positive (data not shown). Thus, *Gab2* (−/−) lymphoid cells have a cell autonomous defect in the establishment and/or maintenance of lymphoid transformation. Direct evidence for the latter was provided by measuring the biological properties of pools of BCR/ABL-transformed *Gab2* (+/+) and *Gab2*(−/−) B lymphoblasts. BCR/ABL-transformed *Gab2* (−/−) cells showed a substantial defect in cell proliferation (Figure 5B), as well as in spontaneous (i.e., haplotaxis) and chemokine-induced migration (Figure 5C). Remarkably, BCR/ABL-evoked Akt and Erk activation also were defective in transformed *Gab2* (−/−), compared to *Gab2* (+/+), B lymphoblasts (Figure 5D).

To gain further insight into the reason for the defective proliferation of BCR/ABL transformed *Gab2* (−/−) B lymphoblasts, we performed cell cycle analysis. Although Gab2 deficiency did not affect the distribution of cells in G1, S, and G2/M, there was a marked increase (~5-fold) in the percentage of cells with sub-G1 DNA content (Figures 6A and 6B). Apoptosis was

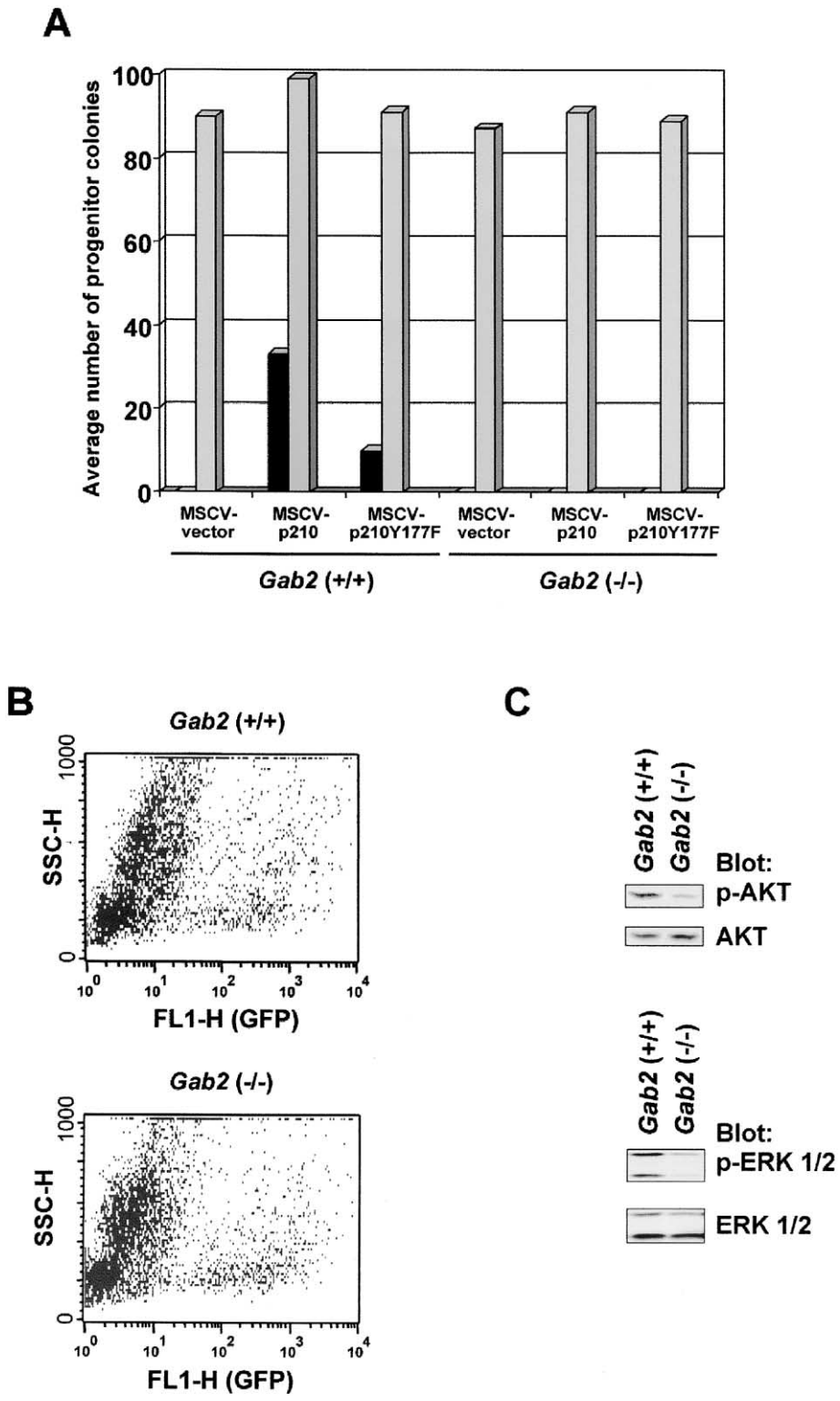


Figure 4. Gab2 is required for BCR/ABL-evoked myeloid colony outgrowth

A: Bone marrow cells from *Gab2* (−/−) and littermate *Gab2* (+/+) mice were transduced with retroviruses expressing BCR/ABL-WT (MSCV-p210), BCR/ABL-Y177F (MSCV-p210Y177F), or the parental control virus (MSCV-vector). The average number of colonies (from triplicate platings) was determined in the absence (black columns) or presence (gray columns) of 100 pg/ml murine IL-3. Shown is a representative experiment from four independent assays that yielded comparable results. In these experiments, no colonies were generated from *Gab2* (−/−) bone marrow cells transduced with *BCR/ABL*-WT (total of ~10^6 bone marrow cells transduced in the four experiments). Parallel experiments yielded a total of approximately 300 colonies following transduction of *Gab2* (+/+) bone marrow cells.

B: Bone marrow cells from *Gab2* (−/−) and littermate wild-type (WT) mice were transduced with a retrovirus expressing the *EGFP* cDNA and analyzed by flow cytometry. Note that *Gab2* (+/+) (15%) and *Gab2* (−/−) (13%) cells are comparably susceptible to retroviral transduction.

C: *BCR/ABL*-transduced myeloid cells were isolated by FACS, and Akt and Erk activation were assessed as described in Experimental Procedures.

confirmed by the presence of characteristic DNA laddering in the *Gab2* (−/−) cells (data not shown). These data indicate that inability of BCR/ABL to prevent apoptosis probably accounts for the decreased proliferation of *Gab2* (−/−) B lymphoblasts. Our biochemical analyses indicate that Gab2 (−/−) cells exhibit defective BCR/ABL-evoked Erk and Akt activation. To gain insight into which of these pathways might contribute to the increased apoptosis in the absence of Gab2, we treated BCR/ABL-transformed *Gab2* (+/+) B lymphoblasts with inhibitors of these signaling pathways. The PI3K inhibitor LY294002 and the

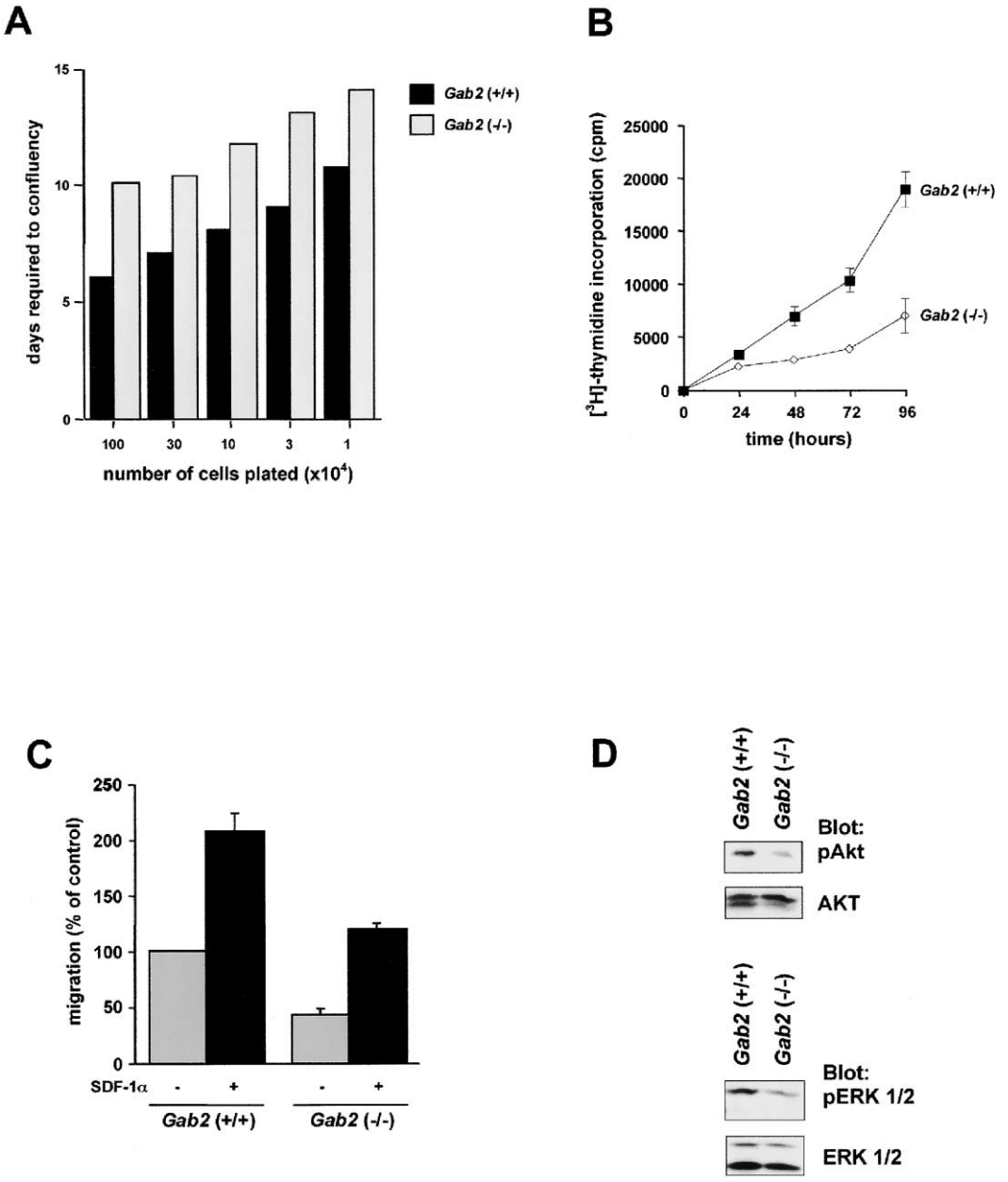


Figure 5. Gab2 contributes to, but is not required for, lymphoid transformation

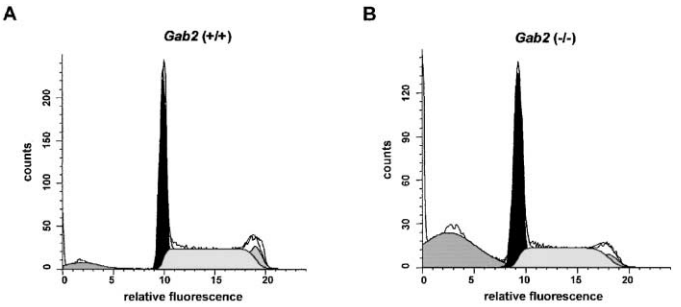
A: The indicated number of bone marrow cells from *Gab2* (+/+) and *Gab2* (−/−) mice were transduced with *BCR/ABL*-WT retrovirus and cultured under conditions that favor outgrowth of transformed B lymphoblasts (Whitlock-Witte cultures; see Experimental Procedures). The time required for the culture to reach confluence (corresponding to 10^6 B lymphoblasts) is shown. Each bar represents three independent wells seeded with the indicated number of bone marrow cells. Note that BCR/ABL can transform *Gab2* (−/−) B cells, but with decreased efficiency. When lower numbers of cells were plated (1–3 × 10^3/well), BCR/ABL-evoked transformation was observed in some *Gab2* (+/+) wells, but not in *Gab2* (−/−) wells. Shown is a representative experiment from three independent assays that yielded comparable results.

B: Proliferation of BCR/ABL-transformed *Gab2* (+/+) and *Gab2* (−/−) B lymphoblasts, as measured by [^{3}H]-thymidine incorporation.

C: BCR/ABL-transformed *Gab2* (+/+) and *Gab2* (−/−) B lymphoblasts were subjected to transwell migration assays in the presence or absence of the chemokine SDF-1α.

D: Akt and Erk activation in BCR/ABL-transformed *Gab2* (+/+) and *Gab2* (−/−) B lymphoblasts was measured by immunoblotting with the indicated phospho-specific antibodies, followed by reprobing for levels of the respective proteins to control for loading.

MEK inhibitor U0126 markedly increased apoptosis of these cells (Figure 6C). Similar results were obtained with the MEK inhibitor PD98059 (data not shown). These data suggest that both the PI3K/Akt and Ras/Erk pathways transmit survival signals in BCR/ABL transformed B lymphoblasts, and that decreases in these signaling pathways contribute to the increased apoptosis and consequently decreased proliferation observed in the absence of Gab2.



C

Genotype	Inhibitor	% of viable cells in phase: G_1	S	G_2/M	Total % viable cells
Gab2 (-/-)	-	50.8	42.6	6.6	66.8
Gab2 (+/+)	-	40.6	49.8	9.6	94
Gab2 (+/+)	U0126 (10 μM)	56.3	37.3	6.4	79
Gab2 (+/+)	LY294002 (10 μM)	76.6	19	4.4	57.8

Figure 6. Lack of Gab2 results in increased apoptosis of BCR/ABL-transformed B lymphoblasts

A and B: Flow cytometric profiles of propidium-iodide-stained BCR/ABL-transformed *Gab2* (+/+) (**A**) and *Gab2* (−/−) (**B**) B lymphoblasts. Note the similar cell cycle distribution, but the marked increase in apoptotic cells (indicated by sub-G1 DNA content) in *Gab2* (−/−) cells.

C: Shown are the percentages of cells in the indicated cell cycle stages, as well as the percentage of viable cells (as determined by excluding cells with sub-G1 DNA content) in BCR/ABL-transformed *Gab2* (+/+) and *Gab2* (−/−) B lymphoblasts, and BCR/ABL-transformed *Gab2* (+/+) cells treated with the indicated inhibitors.

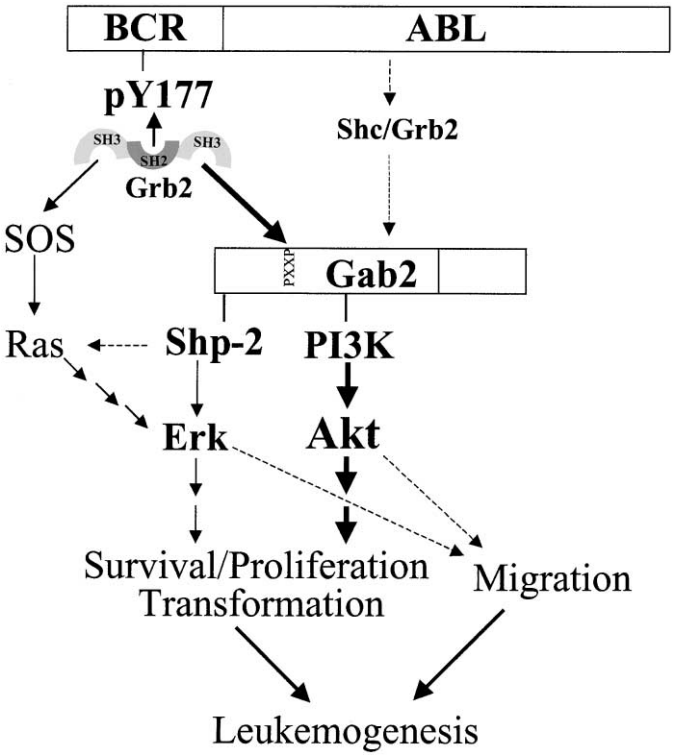


Figure 7. Model for the role of Gab2 in BCR/ABL transformation

Autophosphorylation of BCR/ABL generates phosphorylated Tyr177, which binds to the SH2 domain of Grb2. This leads to recruitment and tyrosine phosphorylation of Gab2, which binds constitutively to the Grb2 SH3 domain. Tyrosine phosphorylated Gab2 binds to signal relay molecules, including PI3K, which is required for Akt activation, and Shp2, which may be important for Erk activation. Akt and Erk both send antiapoptotic signals, and may also contribute to BCR/ABL-evoked migration. Together, these and perhaps other Gab2-generated signals are required for myeloid transformation and contribute to lymphoid transformation, and ultimately leukemia. The thicker lines indicate that the PI3K/Akt pathway may be more important for promoting survival. Multiple arrows indicate the existence of several intervening steps in a pathway, whereas dashed lines represent potential connections that have not yet been demonstrated.

Discussion

Increasing evidence suggests that the BCR component of BCR/ABL fusion proteins determines the aggressiveness and lineage of BCR/ABL-induced leukemia. The BCR autophosphorylation site Tyr177, a Grb2 binding site, is required for transformation of fibroblasts (Pendergast et al., 1993) and for efficient induction of CML-like myeloproliferative disease by BCR/ABL (Million and Van Etten, 2000; Zhang et al., 2001; He et al., 2002). Tyr177 also plays a significant, although less profound, role in lymphoid leukemogenesis (Million and Van Etten, 2000; Zhang et al., 2001; He et al., 2002). Whether Grb2 is, indeed, the key Tyr177 binding protein, as well as the nature of the signaling pathways activated by direct binding of Grb2 to BCR/ABL, has been controversial (see Introduction). Here, using a combined biochemical and genetic approach, we have identified the scaffolding adaptor protein Gab2, which binds to Grb2, as a critical mediator of the Tyr177 signal.

We showed previously that, whereas Gab2 becomes tyrosine phosphorylated in response to a wide variety of cell stimuli, including several hematopoietic cell growth factors and cytokines, it is constitutively tyrosine phosphorylated and associated with signal relay molecules in multiple BCR/ABL-transformed cell lines (Gu et al., 1997, 1998). The results herein show that Gab2 also associates with BCR/ABL, and efficient Gab2 tyrosine phosphorylation and BCR/ABL association require Tyr177 phosphorylation (Figures 1C and 1D). Since Tyr177 is a binding site for the Grb2 SH2 domain (Pendergast et al., 1993; Puil et al., 1994), and the Grb2 SH3 domains associate constitutively with proline-rich motifs in Gab2 (Gu et al., 1998, 2000; Lock et al., 2000; Figure 2A), these findings suggested that Gab2 is recruited to, and subsequently phosphorylated by, BCR/ABL (or a BCR/ABL-associated kinase) via a Grb2/Gab2 complex (Figure 7). Consistent with this model, a Gab2 mutant lacking Grb2 binding sites is defective in BCR/ABL association, tyrosine phosphorylation, and binding to Shp2 and PI3K (Figure 2B). Although a previous study suggested that Shp2 associates directly with BCR/ABL (Tauchi et al., 1994), our results argue that Shp2 tyrosine phosphorylation and BCR/ABL association are mediated mainly via Gab2.

Recruitment via Grb2 is likely to be a general mechanism for activating Dos/Gab family members. In response to βc cytokines, and possibly cytokines that signal via the IL-2 receptor β chain, Gab2 tyrosine phosphorylation is mediated primarily by a Shc-Grb2-Gab2 complex (Gu et al., 2000). The Gab2 relative Gab1 is also recruited to some growth factor receptors via Grb2 (Lock et al., 2000; Schaeper et al., 2000), and a recent

study indicates that Gab1 binds to polyoma middle T antigen through a Shc/Grb2 complex (Ong et al., 2001). Since Dos/Gab proteins activate multiple downstream signaling pathways, signals emanating from Dos/Gab family scaffolds may be as important for Grb2 function as is its ability to recruit Sos to activated receptors. Transforming proteins such as BCR/ABL (and perhaps middle T antigen and others) can subvert this normal Grb2/Gab2 (or Shc/Grb2/Gab2) signaling pathway. Conceivably, Gab2 plays an important role in the pathogenesis of other types of neoplasms as well; notably, a high percentage of breast cancer cell lines and tumors overexpress Gab2 (Daly et al., 2002).

Our work provides compelling evidence that Gab2 is an essential mediator of leukemogenic signals emanating from Tyr177. Cell proliferation and spontaneous migration are impaired significantly in BCR/ABL-Y177F Ba/F3 (and 32Dcl3) cells compared to BCR/ABL-WT cells (Figure 3). *Gab2* (−/−) and *Gab2* (+/+) bone marrow cells yield similar numbers of myeloid colonies in the presence of cytokines (Figure 4A), but whereas BCR/ABL-WT gives rise to cytokine-independent myeloid colonies when introduced into *Gab2* (+/+) progenitors (Gishizky and Witte, 1992; Figure 4A), *Gab2* (−/−) progenitors are refractory to BCR/ABL transformation. Several studies indicate a good correlation between the factor-independent myeloid colony assay and myeloid leukemogenesis by BCR/ABL mutants (Gross and Ren, 2000; Nieborowska-Skorska et al., 1999; Skorski et al., 1997). Myeloid leukemogenesis studies (which require crossing the Gab2 mutation onto Balb/c background) are in progress in our laboratories.

Gab2 may be particularly important for myeloid transformation by BCR/ABL, compared to other oncogenic tyrosine kinase fusions. Like BCR/ABL, TEL/JAK2 evokes Gab2 tyrosine phosphorylation and association with Shp2 and PI3K (Nguyen et al., 2001) (J. Franstve, H.G., D. Sternberg, M.G.M., B.G.N., and D.G. Gilliland, unpublished data). However, in contrast to BCR/ABL, TEL/JAK2 evokes factor-independent myeloid colonies from *Gab2* (−/−) bone marrow cells (data not shown; J. Franstve, H.G., D. Sternberg, M.G.M., B.G.N., and D.G. Gilliland, unpublished data). Interestingly, BCR/ABL and TEL/JAK2 also have contrasting requirements for Stat5: Stat5 is dispensable for BCR/ABL transformation (Sexl et al., 2000), but essential for leukemogenesis by TEL/JAK2 (Schwaller et al., 2000). Thus, different oncogenic-tyrosine kinase fusion proteins may have distinct transformation requirements, with obvious therapeutic implications.

Gab2 also is an important mediator of lymphoid transformation signals from Tyr177. Remarkably, just as lymphoid leukemogenesis is affected less severely than myeloid leukemogenesis by Y177F mutation (Million and Van Etten, 2000; Zhang et al., 2001; He et al., 2002), the defect in BCR/ABL-evoked transformation of *Gab2* (−/−) B lymphoblasts is milder than the (absolute) defect in myeloid transformation by BCR/ABL. Nevertheless, multiple aspects of lymphoid leukemogenesis may be altered in the absence of Gab2. BCR/ABL-transformed B lymphoblasts clearly have a cell autonomous proliferation defect (Figure 5B), most likely due to increased apoptosis (Figure 6); obviously, such a defect could contribute to the increased latency of lymphoid leukemias evoked by BCR/ABL-Y177F. There probably is an independent defect in establishment of lymphoid transformation in *Gab2* (−/−) mice. If lower numbers of BCR/ABL retrovirus-transduced bone marrow cells are plated in experiments such as those in Figure 5A, *Gab2* (+/+) cells can yield lymphoid cell transformation, whereas no transformation was observed using *Gab2* (−/−) bone marrow despite equal levels of transduction and prolonged culture (data not shown). Furthermore, *Gab2* (−/−) B lymphoblasts have defective haplotaxis and chemotaxis (Figure 5C), which also should affect leukemic phenotype.

We cannot conclude that Gab2 is the only essential downstream mediator of the Tyr177 signal; other Grb2 binding proteins may be recruited via Y177 and might be required for BCR/ABL transformation. The role of such proteins in BCR/ABL transformation, and whether their recruitment is dependent or independent of Gab2, remains to be determined by genetic analysis. Nevertheless, we favor the notion that Gab2 is the major, if not the only, transforming signal from Tyr177. Whereas *Gab2* (−/−) myeloid progenitors are refractory to BCR/ABL transformation, the transforming ability of BCR/ABL-Y177F (for wild-type myeloid cells) is only reduced, not absent. These findings parallel the marked reduction (but not absence) of Gab2 tyrosine phosphorylation, and the decreased, but not absent, PI3K, Akt, and Erk activation in BCR/ABL-Y177F-expressing cells, suggesting that a small amount of Gab2 can be tyrosine phosphorylated by alternative means in the absence of Tyr177. Shc is tyrosine phosphorylated in BCR/ABL-expressing cells (Goga et al., 1995); conceivably, the residual Gab2 phosphorylation in BCR/ABL-Y177F cells could be generated via a Shc/Grb2/Gab2 complex, as in βc cytokine signaling (Gu et al., 2000). The striking similarity between the effect of Tyr177 mutation on BCR/ABL leukemogenesis in vivo and the consequences of Gab2-deficiency on myeloid and lymphoid transformation ex vivo also argue that Gab2 is the physiologically relevant target of Tyr177. Additional support for this conclusion is provided by the comparable increase in apoptosis in *Gab2* (−/−) B cells transformed by BCR/ABL and BCR/ABL-transformed *Gab2* (+/+) cells treated with inhibitors of two pathways (PI3K/Akt and Ras/Erk, respectively) affected by Gab2 deficiency (Figure 6).

Two pathway(s) downstream of Gab2, the PI3K/Akt and Ras/Erk pathways, appear to be critical, at least for lymphoid transformation (Figures 5 and 6). PI3K activity is required for BCR/ABL transformation (Skorski et al., 1995), but the mechanism of PI3K activation by BCR/ABL has been controversial. BCR/ABL has a consensus binding site (Y-X-X-M) for p85-PI3K, but mutation of this site does not abrogate PI3K activation or association with BCR/ABL (Jain et al., 1996). Instead, Skorski et al. reported that activation of PI3K by BCR/ABL requires the ABL SH2 domain. These workers found no effect of Tyr177 mutation on BCR/ABL-evoked PI3K activity (Skorski et al., 1997). In contrast, Roumiantsev et al. found that the SH2 domain of BCR/ABL was dispensable for PI3K activation (Roumiantsev et al., 2001). The reason for this discrepancy is not clear. However, our results show that coimmunoprecipitation of p85-PI3K with BCR/ABL-Y177F is reduced markedly, at least in part because of failure of BCR/ABL-Y177F to bind to Gab2 (Figure 1). Moreover, PI3K activity is reduced in anti-phosphotyrosine, anti-ABL, and anti-Gab2 immunoprecipitates in cells expressing BCR/ABL-Y177F, and at least two critical targets of the PI3K pathway, Akt and Gsk-3β, exhibit decreased activation in these cells (Figure 3). These data suggest that Tyr177 is critically involved in the regulation of PI3K binding and biological activity and implicate Gab2 in BCR/ABL activation of the PI3K/Akt pathway. Consistent with this notion, BCR/ABL-evoked Akt activa-

tion is diminished in primary myeloid (Figure 4) and lymphoid (Figure 5) cells from *Gab2* (−/−) mice. Notably, Gab2 is also essential for PI3K activation in normal signaling pathways, such as signaling from the high affinity IgE receptor and the IL-3 receptor in mast cells and factor dependent hematopoietic cell lines (Gu et al., 2000, 2001). Since PI3K activation is only partially defective in BCR/ABL-Y177F cells, other BCR/ABL-evoked pathways to PI3K activation must exist. Also, Gab2 may not be the only route from Tyr177 to the PI3K pathway; preliminary studies indicate that Cbl tyrosine phosphorylation is also impaired by the Y177F mutation (M.S. and J.D.G., unpublished data), and Cbl interacts with PI3K in BCR/ABL-transformed cells (Sattler et al., 1996). Nevertheless, our data suggest that the fraction of PI3K activation mediated via Gab2 is of key pathophysiological importance. Decreased PI3K activation is likely not the sole cause of impaired transformation by BCR/ABL-Y177F. Impaired Erk activation (Figure 5B), which may reflect reduced recruitment of Grb2/Sos and/or reduced signaling through Gab2 via Shp2-dependent or independent pathways, also contributes (Figure 6C).

Overall, our results strongly support the concept that the profound defects in transformation associated with the Y177F mutation of BCR/ABL are due, in part or in total, to the failure of this mutant to recruit Grb2/Gab2 complex(es), and the subsequent reduction in the ability of BCR/ABL to activate the PI3K/Akt, Ras/Erk, and perhaps other, as yet undefined, pathways. These defects would significantly impair viability and motility signals from BCR/ABL (Figures 5 and 6), consistent with the ex vivo and in vivo effects of BCR/ABL-Y177F. An additional implication of our results is that key transforming signals from BCR/ABL are mediated by a pathway that is dispensable for normal, basal hematopoiesis. The development of the ABL inhibitor STI571 has had a dramatic effect on the therapy of CML (BCR/ABL disease) (Mauro et al., 2002). However, it is clear that resistance to STI571 will limit its therapeutic efficacy (Barthe et al., 2001; Gorre et al., 2001; Hochhaus et al., 2001). Identifying targets for other signal transduction inhibitors that are complementary to STI571 without adding toxicity to normal blood cells is critical; Gab2-dependent pathways provide a source of such targets. Intriguingly, pharmacological inhibition of two of these pathways, the PI3K and Erk pathways, evokes apoptosis of BCR/ABL-transformed B lymphoblasts (Figure 6). Inhibitors of these pathways, most likely in combination with STI571, may be useful for treatment of CML blast crisis. Finally, it will be important to define the role of Gab2 and Gab2-dependent pathways in transformation by other tyrosine kinase oncogenes.

Experimental procedures

Cell culture

The murine hematopoietic line Ba/F3 was grown in RPMI 1640 with 10% (v/v) fetal calf serum (FCS) and 10% (v/v) WEHI-3B conditioned medium (as a source of murine IL-3), plus antibiotics (Penicillin/Streptomycin). Pools of Ba/F3 cell lines transfected to express BCR/ABL-WT, BCR/ABL-Y177F, BCR/ABL-ΔCRKL, and BCR/ABL-FΔCRKL (all in the backbone of p210 BCR/ABL) were selected for G418 resistance (2 mg/ml). The G418-resistant cells were maintained in Ba/F3 culture medium in the presence of the ABL kinase inhibitor STI571 (1 μg/ml) to limit the long-term mutagenic effects of BCR/ABL. For biochemical experiments, these polyclonal cell lines were grown for 18 hr in the absence of STI571 and WEHI-3B conditioned medium. The number of viable cells was determined by trypan blue exclusion, or total cell number was determined using a Coulter particle counter (Coulter Counter Z2). Following their generation as described below, BCR/ABL-transformed *Gab2* (+/+) and *Gab2* (−/−) B lymphoblasts were maintained in RPMI plus 20% FCS, antibiotics, and 50 μM 2-mercaptoethanol (2-ME).

Expression constructs

BCR/ABL-WT, *BCR/ABL-Y177F*, *BCR-ABL-ΔCRKL*, and *BCR/ABL-FΔCRKL* cDNAs (kindly provided by Dr. Brian Druker, Oregon Health Sciences Center) were subcloned into the EcoRI site of the pcDNA3 expression vector (Clontech Laboratories, Palo Alto, CA), and used for transfection by electroporation into Ba/F3 cells. pEBB expression vectors containing HA-tagged murine *Gab2* cDNA (Gab2-HA) (Gu et al., 1998) or *Gab2* cDNA with mutations in the Grb2 binding sites (Gab2-HA.ΔGRB2, kindly provided by Drs. Lisa Lock and Morag Park, McGill University, Canada) were used for electroporation into Ba/F3.BCR/ABL-WT cells. There are two potential Grb2 binding sites on Gab2 (Lock et al., 2000). In Gab2-HA.ΔGRB2, one of the sites (S^{344}AIAPPPRPP^{353}) was deleted, and the other was mutated (P^{498}PPVNR^{503} to P^{498}APVNA^{503}).

Bone marrow transduction and transformation assays

High-titer, helper virus-free retroviral stocks of MSCVneo p210 (*BCR/ABL*), MSCVneo p210 (*BCR/ABL*)-*Y177F*, and MSCVneo were prepared by transient transfection of 293T cells using the *kat* ecotropic packaging system, as described previously (Million and Van Etten, 2000). All viral stocks had titers of 3 to 5 × 10^6 neomycin-resistant colony-forming units/ml in NIH 3T3 cells and gave rise to equivalent proviral copy numbers in transduced NIH3T3 or primary bone marrow cells, as determined by Southern blotting. *Gab2* (−/−) and littermate *Gab2* (+/+) mice (129Sv X C57Bl6/J) were described previously (Gu et al., 2001).

For ex vivo colony formation assays, bone marrow cells harvested from 6–10 week old mice were prestimulated at 37°C in IMDM with 15% (v/v) heat-inactivated FCS, 5% (v/v) WEHI-3B conditioned medium, 1 μg/ml ciprofloxacin, and murine IL-3 (6 ng/ml, Upstate Biotechnology), IL-6 (10 ng/ml, R&D Systems), and SCF (50 ng/ml, R&D Systems). The next day, cells were transferred into 6-well plates, exposed to viral supernatants in prestimulation medium in the presence of 10 mM HEPES (pH 7.5) and 2 μg/ml polybrene, and centrifuged at 2500 rpm for 90 min at 30°C. After adsorption for 4 hr at 37°C, the medium was changed with fresh prestimulation medium. At 48 hr, a second round of transduction and cosedimentation was performed. After a 3 hr adsorption period, the cells were washed once with IMDM, counted, and plated in triplicate in MethoCult M3234 medium (StemCell Technologies, Vancouver) at 8 × 10^4 cells/35 mm dish in the presence or absence of murine IL-3 (100 pg/ml) at 37°C, 5% CO_2, as indicated. Colonies were scored at day 12. In some experiments, bone marrow cells were transduced with MSCV-*GFP*, and the efficiency of transduction of Gab2 (+/+) and Gab2 (−/−) cells was determined by flow cytometry for the GFP fluorophore.

For primary B lymphoid transformation assays (McLaughlin et al., 1987), bone marrow cells were treated with NH_4Cl solution to lyse erythrocytes, and then transduced by cosedimentation with p210 BCR/ABL-expressing retroviruses for 90 min at 30°. After adsorption for 3 hr at 37°C, cells were plated (1 × 10^6 total cells/well) into 24-well plates at different densities in the absence (only at the highest density) or presence of feeder cells (untransduced bone marrow cells) in 1 ml of Whitlock/Witte culture medium (RPMI 1640, 20% fetal calf serum, 1 μg/ml ciprofloxacin, and 50 μM 2-mercaptoethanol). Cultures were scored as transformed when the number of nonadherent cells exceeded 10^6 per ml of culture medium. Similar results were obtained when feeder cells were supplied by *Gab2* (+/+) or *Gab2* (−/−) bone marrow cells. Transformed cells were expanded from these wells and maintained as described above.

Immunoprecipitation and immunoblotting

Cells were lysed in a buffer containing Tris-HCl (50 mM, pH 8.0), NaCl (150 mM), NP40 (1% v/v), deoxycholic acid (0.5% w/v), sodium dodecyl sulfate (0.1% w/v), NaF (100 mM), and glycerol (10% v/v). Immunoprecipitation and immunoblotting using a chemiluminescence technique were performed using standard methods. Tyrosine phosphorylated proteins were detected using the monoclonal antibody 4G10 (kindly provided by Dr. Brian Druker, Oregon Health Science University, Portland, OR). Polyclonal rabbit antisera against Gab2, Cbl, and Shp2 (Santa Cruz Biotech, Santa Cruz) and monoclonal anti-HA (12CA5) and ABL (3F12) antibodies were used for immunoblotting and/or immunoprecipitation. In some studies, phospho-specific antibod-

ies reactive with Ser473 of Akt or Ser9 of Gsk-3β (Cell Signaling Technology, Beverly, MA) or Tyr204 of Erk1 and Erk2 (Santa Cruz Biotech) were used.

For biochemical analysis of primary myeloid cells, bone marrow was transduced with MSCV-*p210 BCR/ABL-IRES-GFP*, and transduced cells were purified by FACS for the GFP fluorophore. Sorted cells were cultured overnight in a cytokine cocktail (mIL-3, mIL-6, and mSCF), and an equal number of live *Gab2* (+/+) and *Gab2* (−/−) cells were depleted of cytokines for 6 hr before cell lysates were prepared for immunoblotting. BCR/ABL-transformed primary B lymphoid cells obtained from in vitro lymphoid transformation assays were lysed and subjected to immunoblot analysis as described above.

Protein overlay assays

Direct in vitro binding of glutathione S-transferase (GST) or a GST-Grb2 fusion protein (2 μg protein/mL) was determined using a previously described method (Sattler et al., 1996).

PI3K assays

Lysates from Ba/F3, Ba/F3.BCR/ABL-WT, and Ba/F3.BCR/ABL-Y177F cells were prepared in NP40 buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% [v/v] NP40, 100 mM NaF, and 10% [v/v] glycerol), and PI3K activity in anti-phosphotyrosine, anti-Gab2, and anti-ABL immunoprecipitates was determined as described previously (Auger et al., 1989). Radiolabeled lipid reaction products were visualized by autoradiography (BIOMAX MR Film, Kodak, Rochester, NY) and compared to nonradiolabeled standards, visualized by iodide staining. Autoradiograms were scanned (ScanMakerIII, Mikrotek) and the images analyzed with NIH Image 1.62 software (for MacOS by Wayne Rasband at the National Institute of Health). The relative changes in phosphatidylinositol-3′-phosphate (PIP3) produced in the PI3K assays were calculated as a percentage of PIP3 production in BCR/ABL-WT-expressing cells.

Transwell migration assays

The membranes of transwell chambers (8 μm pore size polycarbonate membrane, Corning Costar Corp., Cambridge, MA) were coated with 10 μg/ml fibronectin (Life Technologies, GibcoBRL) for 18 hr. Cells (0.2×10^6 in 100 μl) were transferred to the upper chamber in medium without IL-3. After 3 hr, living cells in the lower compartment were quantified (by trypan blue exclusion). Spontaneous migration was expressed as a "% of control" (% of migrating cells expressing BCR/ABL-Y177F compared to cells expressing BCR/ABL-WT or % of BCR/ABL-transformed *Gab2* [−/−] B lymphoblasts migrating compared to *Gab2* [+/+] cells, respectively). In some experiments, migration was measured in the presence of the chemokine SDF-1α (100 ng/ml). The standard error of the mean was calculated from the migration indices of at least three independently performed experiments.

Proliferation, cell cycle, and apoptosis assays

BCR/ABL-transformed *Gab2* (+/+) and *Gab2* (−/−) primary B lymphoblasts (1×10^4 cells/well) were plated into 96-well round bottom plates in RPMI medium containing 20% FCS and 50 μM 2-ME for 24, 48, 72, and 96 hr. [^{3}H]-thymidine (1 μCi/well) was added for 4 hr before harvesting, and [^{3}H]-thymidine incorporation was determined using a Cell Harvester (Skatron, Sterling, VA).

Live cells from randomly growing BCR/ABL-transformed B lymphoblasts were isolated using Histopaque (Sigma). After washing twice, the cells were cultured for 4 days in RPMI medium containing 20% FCS and 50 μM 2-mercaptoethanol, harvested, and fixed in 70% ethanol for 3 hr at −20°. Fixed cells were stained with propidium iodide, and cell cycle parameters were analyzed by using FACScan and Modfit LT software. In some experiments, the PI3K inhibitor LY 294002 (Biomol Research Laboratories, Plymouth Meeting, PA) or the MEK inhibitor U0126 (Calbiochem) was added at the indicated concentrations prior to [^{3}H]-thymidine incorporation and cell cycle analysis.

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