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Bcr–Abl-mediated resistance to apoptosis is independent of PI 3-kinase activity

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Abstract

The Bcr–Abl tyrosine kinase is responsible for the oncogenic phenotype observed in Philadelphia chromosome-positive leukemia and induces resistance to apoptotic cell death in a variety of cell types. Recent evidence supports the hypothesis that these two properties of Bcr–Abl are derived from cooperative but distinct signaling pathways. Phosphatidylinositol 3-kinase (PI3K), which has been suggested to associate with and become activated by Bcr–Abl, has been shown to be required for Bcr–Abl-mediated cell growth. Also, PI3K has been implicated in resistance to apoptosis induced by some growth factors. We therefore examined the role of PI 3-kinase in the anti-apoptotic effect of Bcr–Abl. First, we confirmed that expression of p185$^{bcr-abl}$ in HL-60 cells, which renders these cells resistant to apoptosis, induces tyrosine phosphorylation of the p85 subunit of PI3K. Consistent with this result, we observed a 20-fold increase in PI3K activity upon immunoprecipitation of tyrosine-phosphorylated proteins from cells expressing Bcr–Abl versus control cells. Nevertheless, treatment of HL-60.p185$^{bcr-abl}$ cells with wortmannin, a potent inhibitor of PI3K, eliminated PI3K activity but did not interfere with the resistance of these cells to apoptosis. Similar results were obtained with the CML line K562 and with the BaF3.p185$^{bcr-abl}$ line. We conclude that while PI3K participates in the anti-apoptotic response mediated by some growth factors and also seems to be important for the growth of Bcr-Abl-positive cells, it does not play any role in Bcr–Abl-mediated resistance to apoptosis.

Keywords: apoptosis; Bcr–Abl; PI 3-kinase; oncogene

Abbreviations: PI3K, Phosphatidylinositol 3-kinase; ALL, acute lymphocytic leukemia; CML, chronic myelogenous leukemia; CHX, cycloheximide; mAb, monoclonal antibody; pAb, polyclonal antibody

Introduction

The Bcr–Abl tyrosine kinase found in Philadelphia chromosome (Ph[1])-positive leukemia occurs in two different forms depending on the breakpoint of the Bcr gene on chromosome 22. In chronic myelogenous leukemia (CML), a 210 kDa Bcr–Abl protein is associated with the proliferation and accumulation of myeloid cells and their precursors (Shtivelman *et al*, 1985), whereas a 185 kDa form of this protein is responsible for the pathogenesis observed in Ph[1]-positive acute lymphocytic leukemia (ALL) (Clark *et al*, 1988). Both forms of Bcr–Abl proteins are able to recapitulate a CML-like disease in mice (Daley *et al*, 1990; Elefanty *et al*, 1990; Gishizky *et al*, 1993; Kelliher *et al*, 1990).

It has been demonstrated that Bcr–Abl not only induces transformation of fibroblasts and hematopoietic cells (Lugo *et al*, 1990; McLaughlin *et al*, 1989) but also confers resistance to a variety of apoptosis-inducing agents (Bedi *et al*, 1994; Laneuville *et al*, 1994; McGahon *et al*, 1994, 1995; Smetsers *et al*, 1994). The CML cell line, K562, was shown to be resistant to CD95-mediated apoptosis (McGahon *et al*, 1995), as well as apoptosis induced by actinomycin D, camptothecin, etoposide and cycloheximide (McGahon *et al*, 1994). Down-regulation of Bcr–Abl by antisense oligonucleotides abolishes the resistance of K562 cells to these insults (McGahon *et al*, 1994, 1995) and induces apoptosis in another Ph[1]-positive cell line, BV173 (Smetsers *et al*, 1994). In addition, ectopic expression of Bcr–Abl renders BaF3 and 32D cells interleukin 3 (IL-3) independent (Cortez *et al*, 1995; Daley *et al*, 1992), such that they no longer undergo apoptosis upon IL-3 withdrawal.

Many proteins have been shown to associate directly or indirectly with Bcr–Abl. Among such proteins are the regulatory transcription factors c-Myc (Afar *et al*, 1994; Sawyers *et al*, 1992) and c-Myb (Ratajczak *et al*, 1992), the adapter molecules Shc (Puil *et al*, 1994), Grb-2 (Pendergast *et al*, 1993), and Crkl (de Jong *et al*, 1995; ten Hoeve *et al*, 1994), p21^{ras} (Goga *et al*, 1995; Mandanas *et al*, 1993; Pendergast *et al*, 1993), p120$^{ras\text{-}gap}$ (Skorski *et al*, 1994), p160^{bcr} (Lu *et al*, 1993), c-Cbl (de Jong *et al*, 1995), and PI3K (Gotoh *et al*, 1994; Skorski *et al*, 1995; Varticovski *et al*, 1991). These molecules represent potential substrates for the Bcr–Abl tyrosine kinase and are likely to be important in determining the transformation or the apoptosis-resistant phenotypes, or both.

PI3K is a heterodimeric molecule composed of an adapter subunit of 85 kDa (p85) which couples the catalytic 110 kDa subunit (p110) to activated protein tyrosine kinases (Carpenter *et al*, 1993; Dhand *et al*, 1994; Parker and Waterfield, 1992). Two isoforms of p85, α and β, have been isolated (Otsu *et al*, 1991; Volinia *et al*,

1992) each containing one SH3 and two SH2 domains. Interestingly, sequence homology analysis identified another domain in p85 with significant similarity to the *Bcr* gene (Otsu *et al*, 1991), which suggests that it may possess GAP activity. PI3K is a bifunctional kinase that expresses lipid kinase activity that phosphorylates the D-3 position of the inositol ring of phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol 4,5-biphosphate and also protein serine kinase activity towards its p85 subunit (Dhand *et al*, 1994).

The 3′-phosphorylated inositides produced upon activation of PI3K are thought to act as second messenger molecules, however, the exact role of this enzyme in signal transduction is yet to be elucidated. PI3K is found associated with growth factor receptors and can be activated by protein tyrosine kinases in a variety of cell types (Panayotou and Waterfiel, 1993). It has also been found complexed with the polyoma middle T antigen/ $pp60^{c\text{-}src}$ transforming complex (Ling *et al*, 1992) and with the activated PDGF receptor (Coughlin *et al*, 1989; Fantl *et al*, 1992; Valius and Kazlauskas, 1993) suggesting a role in mitogenesis and cell transformation. Most importantly, PI3K was shown to be required for both NGF- and PDGF-mediated protection from apoptosis in PC-12 neuronal cells (Yao and Cooper, 1995). It was also demonstrated that the PI3K requirement for anti-apoptotic effect depends upon the survival factor used (Minshall *et al*, 1996). Both IL-3 and IGF-1 stimulate PI3K activity in IL-3-dependent FDCP/Mac-1 murine hematopoietic progenitor cells. Down-regulation of PI3K by wortmannin abrogates IGF-1-mediated inhibition of apoptosis in these cells, but does not interfere with the anti-apoptotic effect of IL-3 (Minshall *et al*, 1996).

Varticovski *et al*. (1991) detected elevated levels of PI3K activity in cells that expressed transforming variants of *abl*, and provided evidence that this was due to an association of oncogenic Abl and PI3K. Similarly, others have observed association of Bcr–Abl and PI3K as well as tyrosine phosphorylation of the latter in $p185^{bcr-abl}$-expressing cells (Gotoh *et al*, 1994). Furthermore, PI3K activity was suggested to be regulated by Bcr–Abl tyrosine kinase and required for the growth of Bcr–Abl-expressing cells, based on the ability of wortmannin and p85α antisense oligonucleotides to inhibit growth of these cells (Skorski *et al*, 1995). In addition, in $p210^{bcr-abl}$-positive hematopoietic cells, PI3K was found to associate with the adapter protein Shc (Harrison Findik *et al*, 1995), which seems to provide an alternative signal from Bcr–Abl to Ras activation (Goga *et al*, 1995), and the latter has been suggested to participate in Bcr–Abl-induced resistance to apoptosis (Cortez *et al*, 1995; Sakai *et al*, 1994). These observations led us to investigate the possible role of PI3K in Bcr–Abl-mediated resistance to apoptosis.

Results

Bcr–Abl triggers PI3K tyrosine phosphorylation in HL-60 cells

Infection of HL-60 cells with a retrovirus containing $p185^{bcr-abl}$ renders these cells resistant to a variety of apoptosis-inducing agents (Amarante-Mendes *et al*, submitted). We used this system to address whether PI3K is involved in Bcr–Abl-mediated resistance to apoptosis. First, we observed that similar amount of the p85 subunit of PI3K could be immunoprecipitated from Bcr–Abl-positive and negative HL60 cells (Figure 1A). Second, ectopic expression of Bcr–Abl in HL-60 cells induced an intense tyrosine phosphorylation of the p85 subunit of PI3K and of other substrates (Figure 1B). Anti-Ptyr immunoprecipitation of Bcr–Abl-expressing HL-60 cells revealed a 20-fold increase of lipid kinase activity in the precipitated fraction when compared to Bcr–Abl negative counterparts (Figure 1C), confirming the

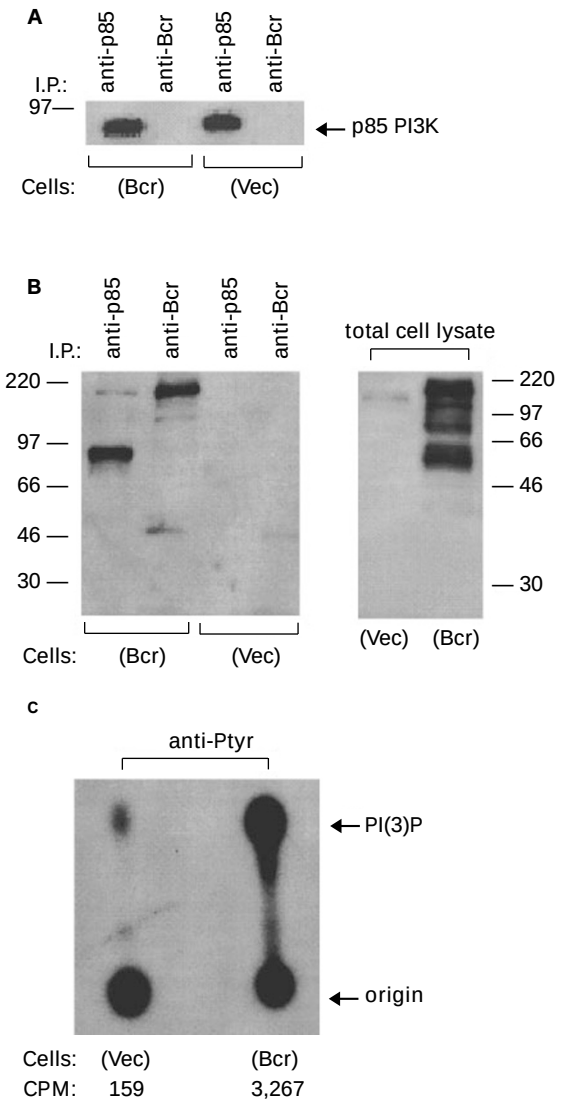

Figure 1 Bcr–Abl triggers PI3K tyrosine-phosphorylation in HL-60 cells. (**A**) Detection by Western-blot of p85 subunit of PI3K in anti-p85 and anti-Bcr IPs. (**B**) Left panel shows tyrosine-phosphorylation of p85 PI3K in HL-60.Bcr–Abl observed in the same blot as in (**A**). Right panel shows tyrosine-phosphorylated proteins observed in total cell lysates. (**C**) PI3K activity in anti-phosphotyrosine (Ptyr) IPs from HL-60 (vec) and HL-60.$p185^{bcr-abl}$ (Bcr) cells. The reactions were performed and visualized as described in Materials and Methods. The numbers below each sample (CPM) correspond to the Cerenkov counts.

phosphorylation state of the PI3K molecule. These results are similar to those reported by others (Gotoh *et al*, 1994; Varticovski *et al*, 1991).

Inhibition of PI3K activity does not impair the anti-apoptotic effect of Bcr–Abl

Since PI3K activity seems to be involved in the growth of Bcr–Abl-expressing cells (Skorski *et al*, 1995) and in the PDGF-, NGF- and IGF-1-mediated resistance to apoptosis (Minshall *et al*, 1996; Yao and Cooper, 1995; Kulik *et al*, 1997), we investigated whether this enzyme plays a role in the ability of Bcr–Abl to inhibit apoptosis. Treatment of HL-60 and HL-60.p185$^{bcr-abl}$ cells with wortmannin substantially decreased the activity of PI3K in both cell lines, in a dose-dependent manner (Figure 2A). This effect was detected in anti-p85 IPs obtained immediately after the 45 min-incubation with wortmannin (Figure 2A and B) and persisted during the 5 h-treatment of the cells with the apoptosis-inducing drugs (Figure 2B). However, this drug had no effect on the ability of Bcr–Abl to confer resistance to either VP-16- or CHX-induced apoptosis, in a 5 h assay (Figure 3). This was true even at very high doses of wortmannin, such as 100 μM (Figure 4). It is important to note that wortmannin was shown to block IGF-1-mediated resistance to apoptosis in a 15 h assay (Kulik *et al*, 1997), indicating that regardless of the short half-life of wortmannin, this drug is able to drive a potent and long-lasting interference with the anti-apoptotic signaling of IGF-1. These data indicate that PI3K activity is not required for Bcr–Abl-mediated resistance to apoptosis in HL-60 cells.

To rule out the possibility that these results were particular to HL-60 cells, two other cell lines were treated with wortmannin and tested for resistance to apoptosis. K562 cells were previously shown to be relatively resistant to a variety of apoptosis-inducing agents and this phenotype could be reverted by treatment with antisense oligonucleotides directed against c-*abl* (McGahon *et al*, 1994). BaF3.Bcr–Abl cells were also shown to be resistant to IL-3 withdrawal and γ-irradiation-induced apoptosis (Cortez *et al*, 1995). Similarly to our results with HL-60 cells, wortmannin induced a marked decrease in PI3K activity in these two cell lines (Figure 5A). Again, inhibition of PI3K activity in K562 and BaF3.Bcr–Abl cells did not interfere with the level of apoptosis observed in these cells (Figure 5B). K562 cells showed a strong resistance to staurosporine and CHX but only a partial protection against 100 μM VP-16. Even in this latter case, where a high enough concentration of VP-16 did induce some cell death,

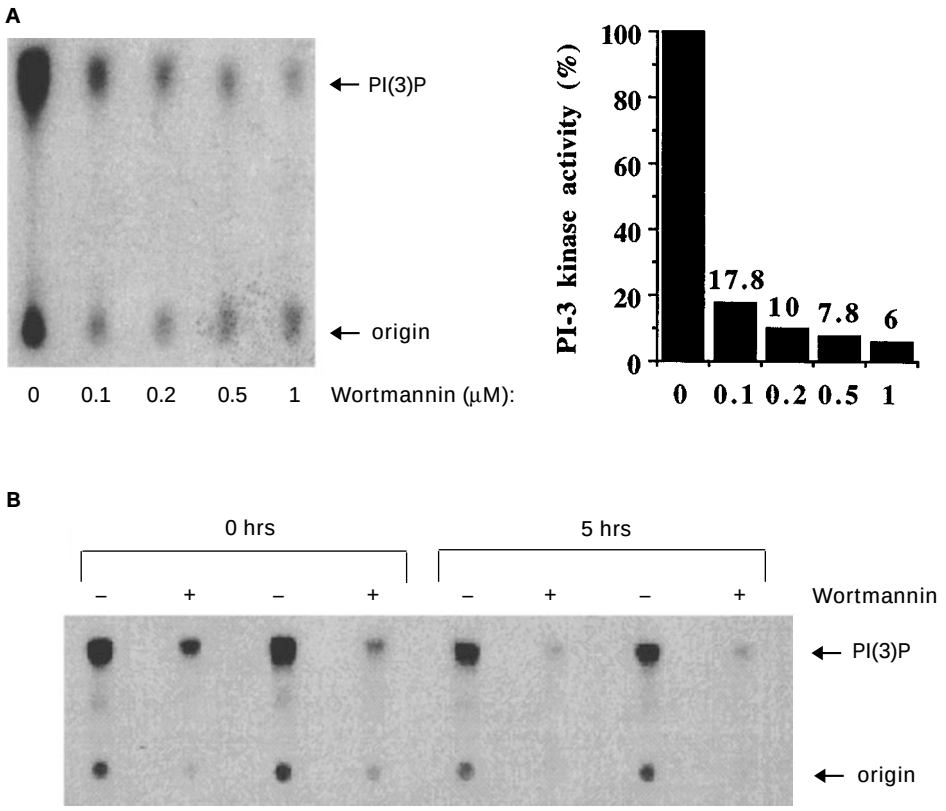

Figure 2 Bcr–Abl-mediated resistance to apoptosis is independent of PI3K activity. (**A**) PI3K activity in anti-p85 IPs from HL-60.Bcr–Abl cells treated with different concentrations of wortmannin for 45 min. The reactions were performed and visualized as described in Materials and Methods. The right panel shows the percentage of PI3K activity in relation to untreated control. (**B**) PI3K activity detected in anti-p85 IPs from HL-60 and HL-60.Bcr–Abl cells treated with 0.5 μM wortmannin for different periods of time. Cells were incubated with wortmannin in serum-free RPMI-1640 for 45 min (t=0 h) and then diluted in RPMI-FCS and incubated for another 5 h in the presence of fresh 0.5 μM wortmannin. For details, see Material and Methods.

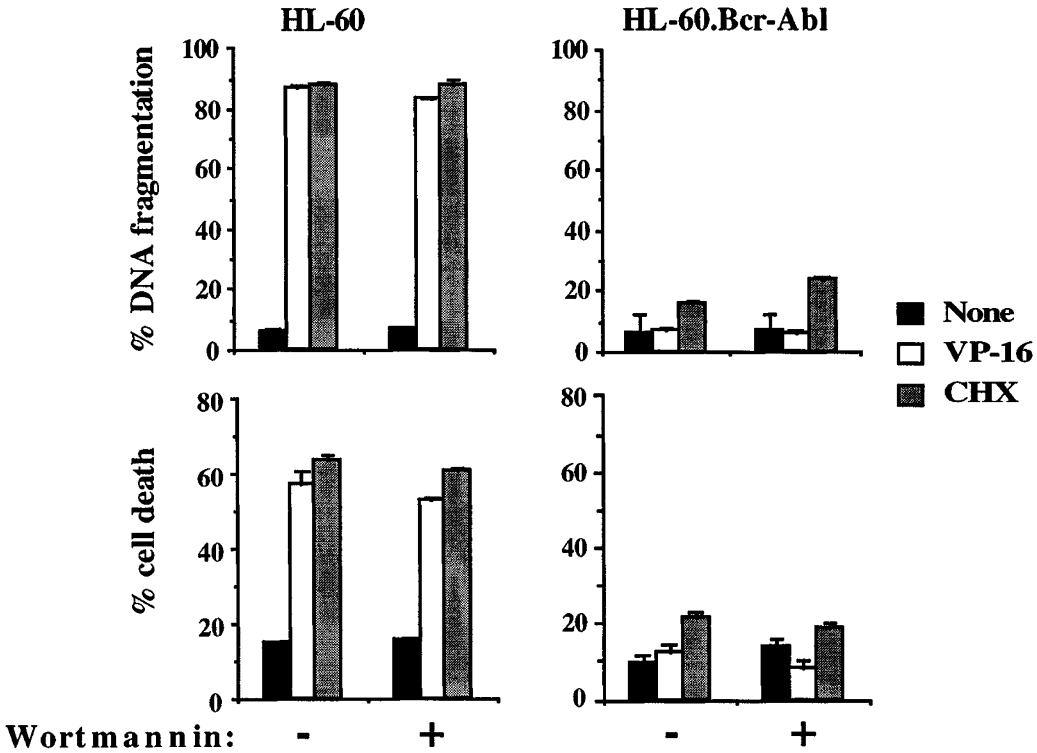

Figure 3 Percentage of DNA fragmentation and cell death in HL-60 and HL-60.Bcr–Abl cells pretreated for 45 min with 0.5 μM wortmannin and incubated for 5 h with either 100 μM VP-16 or 50 μM CHX in fresh medium replenished with 0.5 μM wortmannin. All experiments were done in triplicates.

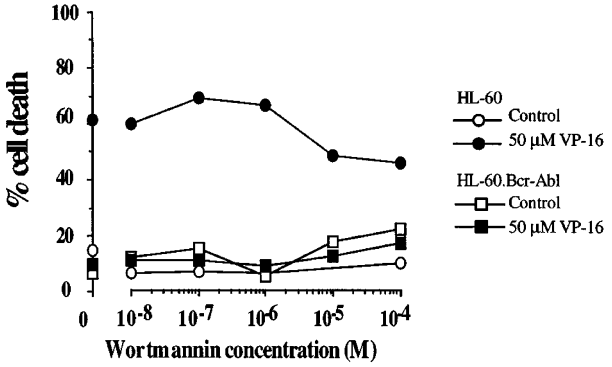

Figure 4 VP-16-induced apoptosis in cells treated with different concentrations of wortmannin. HL-60 and HL-60.Bcr–Abl were pre-treated with wortmannin and then incubated with VP-16 for 5 h in fresh medium replenished with 0.5 μM wortmannin, according to the protocol described in Materials and Methods. Apoptosis were estimated by changes in light scattering properties of the cells.

down regulation of PI3K did not amplified the apoptotic effect of the drug.

Discussion

In this paper we have shown that Bcr–Abl induces tyrosine phosphorylation of the p85 subunit of PI3K in HL60 cells, which suggested a possible role for downstream events in Bcr–Abl-mediated oncogenesis. Nevertheless, inhibition of PI3K activity by treatment with wortmannin did not interfere with the apoptosis-resistant phenotype observed in the Bcr–Abl-positive HL-60, K562 and BaF3 cells.

PI3K activity was found to be required for the anti-apoptotic effect mediated by NGF, PDGF and IGF-1, but not IL-3 (Minshall *et al*, 1996; Yao and Cooper, 1995; Kulik *et al*, 1997). Given the fact that wortmannin inhibits PI3K activity without interfering with Bcr–Abl-mediated resistance to apoptosis, we propose that the Bcr–Abl anti-apoptotic signaling is independent from the 3′-phosphorylated inositides produced upon activation of PI3K. It is possible that PI3K molecules participate in the anti-apoptotic signaling through a non-enzymatic way. The tyrosine phosphorylation of its p85 subunit may specifically direct a different set of substrates to the signaling cascade triggered by Bcr–Abl. Since p85 PI3K is composed of one SH3 and two SH2 domains, it can act as an adapter protein. In this case, tyrosine phosphorylation in specific sites within one or more domains may not change PI3K enzymatic activity but rather direct the molecule to a particular sub-cellular localization where it will interact with different substrates. In this regard, it was shown that the SH2 domain of p85 PI3K binds to tyrosine-phosphorylated SHC in $p210^{bcr-abl}$-expressing cells (Harrison Findik *et al*, 1995) which was shown to provide an alternative signal from Bcr–Abl to Ras activation (Goga *et al*, 1995), and the latter has been suggested to participate in Bcr–Abl-induced resistance to apoptosis (Cortez *et al*, 1995; Sakai

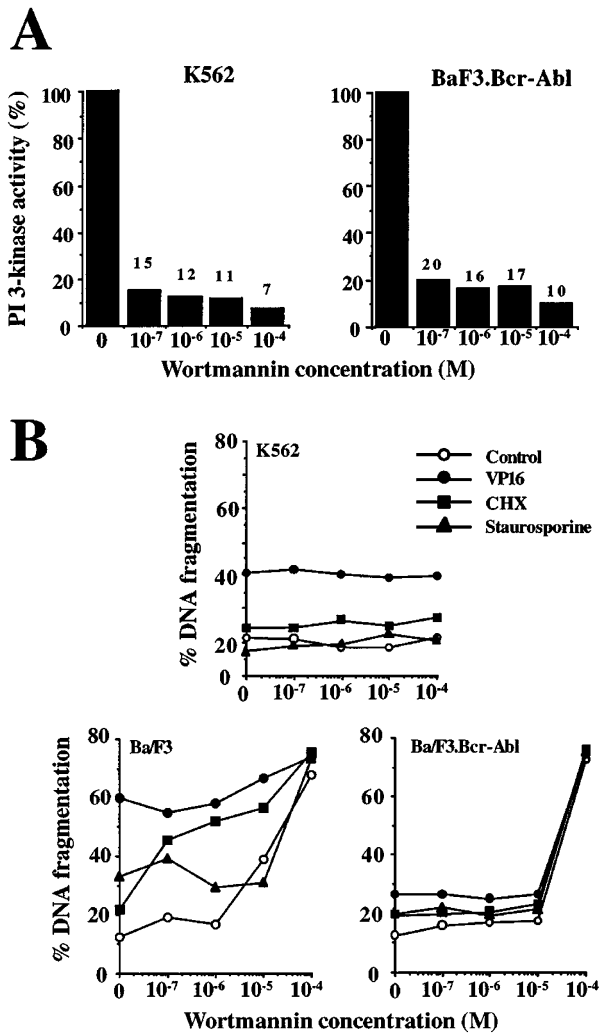

Figure 5 Resistance to apoptosis in K562 and BaF3p185$^{bcr-abl}$ is independent of PI3K activity. (**A**) PI3K activity in anti-p85 IPs from K562 and BaF3p185$^{bcr-abl}$ cells treated with different concentrations of wortmannin for 45 min. The reactions were performed and visualized as described in Materials and Methods. The right panel shows the percentage of PI3K activity in relation to untreated control. (**B**) Percentage of DNA fragmentation in K562, BaF3 and BaF3p185$^{bcr-abl}$ cells pretreated for 45 min with different concentrations of wortmannin and incubated for 5 h with either 100 μM VP-16, 50 μM CHX or 1 μM staurosporine in fresh medium replenished with 0.5 μM wortmannin. All experiments were done in triplicates.

et al, 1994). More recently, Griffin and collaborators, using co-immunoprecipitation and Far-Western blotting approaches, suggested that PI3K binds primarily to p120^{cbl} in Bcr–Abl-expressing cells and that Cbl associates both directly with Bcr–Abl and indirectly via the interaction with CRKL or c-CRK (Sattler *et al*, 1996).

Varticovski *et al*. (1991) found a direct correlation between the ability of Abl variants to transform cells and to increase cellular levels of PIP_3, which strongly suggests that PI3K activity is important in Abl-mediated transformation. Similarly, others have suggested that PI3K is involved in Bcr-Abl-mediated cell growth (Skorski *et al*, 1995). At least 20 times more PI3K was found tyrosine phosphorylated in Bcr–Abl-expressing HL-60 cells compared to control cells, as detected by the lipid kinase assay in anti-Ptyr IPs (Figure 1B), confirming the participation of PI3K in down stream events initiated by Bcr–Abl in HL-60 cells. Since we did not observed any participation of PI3K activity in Bcr–Abl-mediated resistance to apoptosis, it is reasonable to propose that Bcr–Abl triggers two related events in the pathology of Ph^1-positive leukemia, namely cellular transformation and resistance to apoptosis, by biochemically distinct signaling pathways. It has been shown that Bcr–Abl can indeed trigger multiple alternative signals depending on the cell type (Cortez *et al*, 1995; Goga *et al*, 1995). Pendergast and colleagues (Cortez *et al*, 1995) showed that the IL-3-dependent cell line 32D was rescued from IL-3 deprivation- or γ-irradiation-induced apoptosis by expressing a mutant Bcr–Abl (Δ 176–427) that has an impaired transforming ability in these cells. Thus, Bcr–Abl-induced resistance to apoptosis can occur independently of cellular transformation.

Whether the anti-apoptotic effect or the transforming ability of Bcr–Abl, or a combination of both, are responsible for the myeloid expansion culminating in the blast crisis observed in CML patients remains to be determined. Understanding the molecular mechanisms by which Bcr–Abl generates its anti-apoptotic state will provide insights into the apoptotic machinery of the cell as well as help the development of new strategies for therapy of Ph1-positive leukemias.

Materials and Methods

Cell culture and reagents

HL-60.p185$^{bcr-abl}$ cells were obtained by retroviral transfection with pSRαMSVp185$^{bcr-abl}$ tkneo and described elsewhere (Amarante-Mendes *et al*, submitted). K562 cells were obtained from ATCC, BaF3 and BaF3.p185$^{bcr-abl}$ were kindly provided by Dr. Ann Marie Pendergast.

Etoposide (VP-16, Sigma), staurosporine (Sigma) and wortmannin (Sigma) were prepared as 100 mM, 1 mM and 10 mM stock solutions in DMSO, respectively. Cycloheximide (Sigma) was prepared in ethanol as a 50 mM stock solution.

Wortmannin treatment

Cells were washed three times in HBSS and resuspended in serum-free RPMI-1640, containing different concentrations of wortmannin (1×10^7 cells in 0.5 ml). After 45 min at 37°C, suspensions were diluted with RPMI-FCS to 1×10^6 cells ml^{-1} and then incubated with VP-16 or CHX for 5 h. At this point, additional wortmannin was added to maintain the same concentration. Activity of PI3K in the treated cells was determined in kinase assays on anti-p85 IPs.

Immunoprecipitations and Western-immunoblotting

A total of 1×10^7 cells were washed twice in PBS and solubilized in 0.5 ml of lysis buffer (20 mM Tris-Cl pH 7.4; 150 mM NaCl; 1% NP-40; 100 μg ml^{-1} soybean trypsin inhibitor; 10 μg ml^{-1} each leupeptin and aprotinin; 1 mM PMSF; 1 mM $NaVO_4$; 5 mM EDTA). Lysates were pre-cleared with protein G-agarose beads (Pharmacia) and immuno-

precipitated with either NRS, rabbit anti-p85 PI3K pAb (UBI, Lake Placid, NY), mouse anti-Bcr mAb (G6; Santa Cruz Laboratories) or mouse anti-phosphotyrosine (PTyr) pAb (4G10). Immunoprecipitates were resolved in 6% SDS–PAGE gels and transferred to PVDF membranes (Millipore). Filters were probed with suitable primary antibodies and the reactions were detected with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit Ab using an enhanced chemiluminescence system (ECL, Amersham)

PI3K assay

In vitro PI3K assays were performed as previously described (Fukui and Hanafusa, 1989; Von Willebrand *et al*, 1996). Briefly, immunoprecipitates were washed extensively to remove NP-40, and lipid kinase activity was assessed using [γ^{32}P]ATP and phosphatidylinositol as substrate. Reaction products were separated by thin-layer chromatography on silica-gel plates and detected by autoradiography. Relevant spots were quantitated by Cerenkov counting.

Determination of apoptosis

DNA fragmentation was estimated by cell cycle analysis of total DNA content as described elsewhere (Nicoletti *et al*, 1991), with slight modifications. A total of 2×10^5 cells were washed twice in PBS and resuspended in 0.5 ml of hypotonic fluorochrome solution (50 μg ml^{-1} propidium iodide and 0.1% Triton X-100 in 0.1% sodium citrate). After 30 min at 4°C in the dark, the fluorescence of individual nuclei was measured using a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA). The percentage of hypodiploid nuclei correlates with the extent of apoptosis in the sample.

The percentage of cell death was determined by analyzing changes in the light scattering properties due to reduction in cell size and increase in granularity, as well as permeability to propidium iodide (Martin *et al*, 1994; McGahon *et al*, 1995).

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