BCR/ABL Regulates Expression of the Cyclin-dependent Kinase Inhibitor p27\textsuperscript{Kip1} through the Phosphatidylinositol 3-Kinase/ AKT Pathway
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Deregulation of cell cycle checkpoints is an almost universal abnormality in human cancers and is most often due to loss-of-function mutations of tumor suppressor genes such as Rb, p53, or p16\textsuperscript{INK4a}. In this study, we demonstrate that BCR/ABL inhibits the expression of a key cell cycle inhibitor, p27\textsuperscript{Kip1}, by signaling through a pathway involving phosphatidylinositol 3-kinase (PI3K). p27\textsuperscript{Kip1} is a widely expressed inhibitor of cdk2, an essential cell cycle kinase regulating entry into S phase. We demonstrate that the decrease of p27\textsuperscript{Kip1} is directly due to BCR/ABL in hematopoietic cells by two different approaches. First, induction of BCR/ABL by a tetracycline-regulated promoter is associated with a reversible down-regulation of p27\textsuperscript{Kip1}. Second, inhibition of BCR/ABL kinase activity with the Abl tyrosine kinase inhibitor STI571 rapidly increases p27\textsuperscript{Kip1} levels. The PI3K inhibitor LY-294002 blocks the ability of BCR/ABL to induce p27\textsuperscript{Kip1} down-regulation and inhibits BCR/ABL-induced entry into S phase. The serine/threonine kinase AKT/protein kinase B is a known downstream target of PI3K. Transient expression of an activated mutant of AKT was found to decrease expression of p27\textsuperscript{Kip1}, even when PI3K was inhibited by LY-294002. The mechanism of p27\textsuperscript{Kip1} regulation is primarily related to protein stability, since inhibition of proteasome activity increased p27\textsuperscript{Kip1} levels in BCR/ABL-transformed cells, whereas very little change in p27 transcription was found. Overall, these data are consistent with a model in which BCR/ABL suppresses p27\textsuperscript{Kip1} protein levels through PI3K/AKT, leading to accelerated entry into S phase. This activity is likely to explain in part previous studies showing that activation of PI3K was required for optimum transformation of hematopoietic cells by BCR/ABL in vitro and in vivo.

Chronic myelogenous leukemia (CML)\textsuperscript{1} is a myeloproliferative disorder associated with expression of the Philadelphia chromosome (1), a translocation between chromosomes 9 and 22 that fuses the Bcr and Abl genes (2–4). Unlike many other leukemia oncogenes, BCR/ABL does not appear to alter differentiation of granulocyte lineage cells. In contrast, recent studies have suggested that the major cellular effects of BCR/ABL are related to increased mitogenic activity (5), reduced sensitivity to apoptosis (6), and altered adhesion and homing of CML progenitor cells (7).

The BCR/ABL oncogene is associated with both myeloproliferative disease and acute leukemias in human and murine models. There are three known breakpoints in the gene, resulting in three different protein products, p190, p210, and p230, which vary in the length of Bcr present in the fusion protein (8). Interestingly, the three proteins tend to be associated with different leukemias: ALL, CML, and chronic neutrophilic leukemia, respectively, for p190, p210, and p230\textsuperscript{BCR/ABL}. Each of the BCR/ABL proteins have elevated Abl tyrosine kinase activity (9), and this increased kinase activity is necessary for transformation (3). Although a number of substrates of the BCR/ABL tyrosine kinase have been identified, including CBL (10), CrkL (11), Dok (12), STAT5 (13, 14), SHP-2 (15), Shc (16), and Fak (17), the signaling pathways that result in dysregulated growth, viability, and adhesion are not yet well defined.

The mitogenic effects of BCR/ABL are likely to be important in the pathogenesis of CML. BCR/ABL reduces growth factor requirements of primary hematopoietic stem cells (18), converts IL-3-dependent murine hematopoietic cell lines to growth factor independence (19), and is mitogenic in fibroblasts (20). When compared with normal progenitor cells, CML progenitor cells are more likely to be in S phase, both in the marrow and blood, and the fraction of cells in G0 is reduced (21). Thus, BCR/ABL is likely to deregulate checkpoints at one or more sites within the cell cycle. Previous studies have shown that several immediate-early genes are induced by BCR/ABL, including myc (22), fos, and jun (23). The rapid induction of these genes correlates with an enhanced rate of transition from G0 to G1. The increased fraction of cells in S phase suggests that G0/S transition checkpoints are also suppressed.

A number of molecules play a key role in regulating cell cycle progression from G1 to S, including the G1 cyclins, cyclin-dependent kinases (CDks) and cyclin-dependent kinase inhibitors (CKIs). CKIs can be grouped in two categories based on similarities of sequence and actions: the INK4 family \(\text{(p16}^{\text{INK4a}}, \text{p15}^{\text{INK4b}}, \text{p16}^{\text{INK4c}}, \text{and p19}^{\text{INK4d}})\), and the CIP/KIP family \(\text{(p21}^{\text{WAF1/CIP1}}, \text{p27}^{\text{Kip1}}, \text{and p57}^{\text{Kip2}})\), reviewed in Sherr and Roberts (24). INK4 family members specifically inhibit the type.

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\textsuperscript{3} The abbreviations used are: CML, chronic myelogenous leukemia; ALL, acute lymphoblastic leukemia; IL, interleukin; CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; P13K, phosphatidylinositol 3-kinase; HA, hemagglutinin; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; TBS, Tris-buffered saline; TBS-T, TBS with 0.5% Tween; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; GFP, green fluorescent protein; WT, wild.

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activity of cdk4 and 6, whereas the CIP/KIP family members have a broader action. Overexpression of each of these CKI have been shown to induce a G1 arrest. p21CIP1 has recently been directly shown to be important for regulating hematopoiesis in vivo in mice (25, 26).

Although PI3K and AKT have previously been reported to play essential roles in BCR/ABL transformation (27), the mechanisms and downstream signaling targets have been unclear. PI3K and AKT have been linked to enhanced cell survival through the phosphorylation and subsequent inhibition of the pro-apoptotic molecule Bad (28). However, it has been difficult to demonstrate phosphorylation of Bad in some cell types transformed by BCR/ABL, so identification of other downstream targets is of interest. In the present study we demonstrate that BCR/ABL regulates the expression of p27<sub>Kip1</sub> in a proteasome-dependent manner and through activation of PI3K and AKT.

**MATERIALS AND METHODS**

**Reagents**

Anti-Abl monoclonal antibody 3F12 was a gift from R. Salgia (Dana Farber Cancer Institute). Monoclonal antibodies against p27<sub>Kip1</sub> (K25020 and RB100/1A) were purchased from Transduction Laboratories, (San Diego, CA). Anti-p85 antiserum (96–195) was obtained from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-HA monoclonal was purchased from Babco (Richmond, CA). AKT constructs, inserted in a pCDNA3.1 backbone, were described previously (29). RNase A, lactacystin, and N-acetyl-leucyl-leucyl-norleucinal (LNNle) were purchased from Sigma. E64 and calpain inhibitors were purchased from Calbiochem.

**Cell Lines and Culture Conditions**

The IL-3-dependent Ba/F3 cell line was maintained in RPMI 1640 (Mediatech Cellgro, Herndon, VA) supplemented with 10% fetal calf serum, 1 mg/ml l-glutamine, penicillin-streptomycin, and 10% WEHI-3B conditioned medium (WEHI-3B-CM) as a source of IL-3. Ba/F3 is commonly used as a model for BCR/ABL signaling because it is non-leukemic and factor-dependent in the absence of BCR/ABL transformation but becomes leukemic in syngeneic mice and factor-independent after transformation by BCR/ABL. p210<sub>BCR/ABL</sub>-transformed Ba/F3 cells (Ba/F3-p210) are maintained in culture in the medium described above, except without IL-3. All cells were maintained at 37 °C in a 5% CO<sub>2</sub> humidified incubator. Ba/F3 cells expressing the reverse tetracistronic reporter pUHD172–1 (Ton.B.1) and Ton.B.210 cells in which p210<sub>BCR/ABL</sub> expression is induced by the addition of doxycycline were obtained from G. Daley (Whitehead institute, Cambridge, MA) and grown as described previously (30).

**Transfections and Cell Sorting**

In experiments using transiently transfected cells, 1 × 10<sup>6</sup> cells were transfected by electroporation (Gene-Pulser Bio-Rad, 960 microfarads, 350V). 40 µg of the indicated plasmids were cotransfected with 10 µg of a pEGP plasmid (CLONTECH, Palo Alto, CA). 24 h post-transfection, the green fluorescent protein-expressing cells were sorted on a high speed cell sorter (Coulter Electronics, Miami, FL). After sorting, cells were pelleted, resuspended in culture medium, and kept in culture for 24 h with or without treatment as indicated.

**Antibodies and Protein Analysis**

For protein analysis, cells were harvested, washed in PBS and lysed at 5 × 10<sup>6</sup> cells/ml in ice cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaVO<sub>3</sub>, 1 µg/ml each leupeptin and aprotinin) for 30 min. Lysates were clarified by centrifugation at 15,000 × g for 20 min at 4 °C. The protein concentration was determined by Bradford assay, and equivalent amounts of proteins were separated by gel electrophoresis and transferred to a PVDF membrane (Millipore, Bedford, MA). Filters were blocked for 2 h at room temperature with either 5% nonfat dry milk or 3% bovine serum albumin in Tris-buffered saline (TBS), 0.5% Tween (TBS-T). Filters were washed three times in TBS-T and incubated for 1 h with optimal concentrations of primary antibodies diluted in TBS, 0.1% Tween. After four additional washes in TBS-T, filters were further incubated 45 min with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech). Visualization was performed using PerkinElmer Life Sciences, Renaissance system and Kodak X-Omat blue film (Eastman Kodak Co.).

**Cycloheximide Treatment**

Ton.B.210 cells were either left untreated or treated with 1 µg/ml doxycycline for at least 24 h before cycloheximide treatment in RPMI 1640 supplemented with 10% fetal calf serum and 10% WEHI-CM. 8 h before treatment, the cells were harvested, washed twice in 1× PBS, and resuspended in RPMI 1640 supplemented with 1% bovine serum albumin at a cell density of 1 × 10<sup>6</sup> cells/ml with or without doxycycline. After 8 h of IL-3 deprivation, 10 µg cycloheximide was added to the culture, and an aliquot of the cells was harvested at the indicated times. Cells were lysed as described above.

**Cell Cycle Analysis**

For cell cycle analysis, cells were treated as specified. 1–2 × 10<sup>6</sup> cells were harvested, washed once in 4 ml of PBS, and fixed in 1 ml of 70% ethanol solution. Fixed cells were kept at −20 °C and stained just before analysis. For staining, fixed cells were pelleted, washed once in PBS, and resuspended in 1 ml of propidium iodide-staining solution (PBS, 0.1% Triton X-100, 20 µg/ml propidium iodide, and 100 units/ml RNase A added contemporaneously). Cells were left in propidium iodide staining solution for 30 min at room temperature and analyzed immediately. DNA content and hence the cell cycle distribution was determined by flow cytometry. Repartition of the cells in the various stages of cell cycle was determined with cell cycle analysis software.

**Real Time Quantitative Polymerase Chain Reaction**

**Reverse Transcription (RT)—**For cDNA synthesis, 1 µg of total RNA was reverse-transcribed in a 20 µl reaction mixture containing 250 µM each dNTP, 20 units of RNase inhibitor, 50 units of murine leukemia virus reverse transcriptase, 2.5 µM random hexamers, and 1× buffer (1.5 mM MgCl<sub>2</sub>) (all reagents were purchased from PE Applied Biosystems, Foster City, CA). The reaction mix was incubated at 42 °C for 45 min and then denatured at 95 °C for 5 min. For each sample, a standard reaction not containing the reverse transcriptase enzyme was also performed.

**Real Time PCR—**Specific primers and probe for p27 (forward: 5'-GGTGGACCAATTGCCTCAGT-3'; reverse: 5'-GCCCTTTGTTTTGGAGGA-3'; probe: 5'-AATCTTCTGCGCGAGTCTTCC-3') were designed from sequences in the GenBank<sup>TM</sup> data base using the Primer Express 1.0 Software (PE Applied Biosystems). The hybridization probe contains a block of 15 nucleotides to prevent annealing to genomic DNA. The gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control to standardize the amount of RNA in each reaction (Taqman Rodent GAPDH control reagents). All primers and probes were synthesized by PE Applied Biosystems. PCR was performed on the cDNA samples using an ABI PRISM 7700 sequence detector (PE Applied Biosystems). The Taqman<sup>®</sup> PCR Core reagent kit (PE Applied Biosystems) was used according to the manufacturer's protocol with the modification that dUTP was replaced by dTTP, and incubation with AmpErase was omitted. For each sample tested, PCR reaction was carried out in a 50-µl volume containing 1 µl of cDNA reaction (equivalent to 50 ng of template RNA) and 2.5 units of AmpliTaq Gold. Oligonucleotide primers and fluorogenic probe were added to a final concentration of 100 n mole each. The amplification step consisted of 60 cycles of 94 °C for 45 s, 58 °C for 45 s, and 65 °C for 1 min.

In each experiment, additional reactions with 7 serial 2-fold dilutions of Ton.B.210 cDNA, prepared from cells induced or not with doxycycline, as template were performed with each set of primers and probes on the same 96-well plate to generate standard curves, which related the threshold cycle (C<sub>T</sub>) to the log input amount of template. All samples were amplified in triplicate. The relative amount of p27 transcripts in each sample was determined by using the standard curve method and by normalizing for GAPDH mRNA expression levels, as described previously (ABI PRISM sequence detection system user bulletin No. 2 (PE Applied Biosystems and Ref. 31).

**RESULTS**

**BCR/ABL Promotes Cell Cycle Progression in Baf3 Cells in the Absence of IL-3—**The ability of BCR/ABL to promote survival and proliferation in the absence of growth factors has been well documented in certain hematopoietic-derived cell lines. The Ba/F3 cell line used in our studies is a pre-B cell fully...
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**Fig. 1.** BCR/ABL expression promotes progression into cell cycle. Parental or BCR/ABL-transformed Ba/F3 cell line (upper panels) were IL-3-deprived for 12 h. Cell cycle progression was assessed by propidium iodide staining as described under “Material and Methods.” Similar experiments were performed with the Ton.B.210 cell line (lower panels). These cells were maintained in the presence of IL-3 or deprived as indicated. BCR/ABL expression was induced by addition of doxycycline (Dox) where indicated.

Dependent on the presence of IL-3 for survival and proliferation. IL-3 withdrawal for 16 h induces a partial G1 arrest in parental Ba/F3 cells but not in Ba/F3 cells transformed by p210BCR/ABL (Fig. 1, upper panels). Similar results were obtained with the previously described Ton.B.210 cell line (Fig. 1, lower panels). This cell line, derived from Ba/F3 cells, expresses p210BCR/ABL in response to the addition of doxycycline in the culture medium. After withdrawal of IL-3 for 16 h, non-induced Ton.B.210 cells arrest at G0/G1, whereas BCR/ABL-expressing Ton.B.210 cells progress through G1 to S phase (Fig. 1, lower panels). These results demonstrate that BCR/ABL expression regulates cell cycle progression in hematopoietic cells in a manner similar to cytokine stimulation.

**Fig. 2.** BCR/ABL activity induces a decrease of p27kip1 protein expression. A. Ton.B.210 cells were IL-3-deprived for 8 h, and doxycycline (Dox) was added to the culture medium to induce BCR/ABL expression. Cells were harvested at the indicated times, washed, and lysed as described under “Materials and Methods.” A, BCR/ABL-transformed Ba/F3-p210 cells were kept in culture in the absence of IL-3. Cells were treated for 12 h with the indicated concentrations of the Abl kinase inhibitor STI-571. Cells were lysed, and lysates were separated as indicated above. The effect of STI-571 was assessed by anti-p27 and anti-Ab1 blotting.

**BCR/ABL Activity Induces Down-regulation of p27kip1 Protein Expression.—**Based on these results, we sought to identify cell cycle-related proteins that might be regulated by BCR/ABL activity. The Ton.B.210 cell line was used to assure that changes were specifically due to BCR/ABL and not due to unrelated mutations in the cultured cell lines. Cells were left untreated or stimulated with doxycycline for 24 h and IL-3-deprived for 16 h. Immunoblotting of p27 on lysates from non-treated or doxycycline-treated Ton.B.210 cells demonstrated that resting, non-induced cells expressed a high amount of the CKI p27kip1, whereas BCR/ABL-expressing cells displayed a very low amount of p27kip1 (Fig. 2A). In addition, p27kip1 expression levels were higher in proliferating cells (Fig. 2A, lower panel). Discordant expression levels of p21cip1 and p27kip1 have also been described in other proliferating cells (32). In some circumstances, CKIs can promote rather than inhibit the formation of active cyclin D-cdk4 complexes (33, 34). Our data would be consistent with a model in which the increased level of p21cip1 expression is enough to participate in the activation of cyclin D-cdk4 but is not high enough to inhibit cyclinE-cdk2. In contrast to p27kip1, there was no variation of expression of cyclins A, E, D1, or D3 (data not shown). These results suggest that regulation of p27kip1 expression by BCR/ABL might be an important mechanism in BCR/ABL-mediated proliferative signaling.

To confirm that p27kip1 down-regulation was directly due to BCR/ABL activity, doxycycline-induced Ton.B.210 cells were treated for 14 h with increasing concentrations of the small molecule Abl tyrosine kinase inhibitor STI571 (Fig. 2B). At an optimal concentration of 1 × 10−6 M STI571, BCR/ABL tyrosine kinase activity was inhibited, and this was accompanied by a substantial increase in p27kip1 expression. In these experiments, the level of expression of the 85-kDa subunit of PI3K was used as a control for equal loading of gel lanes.

**p27kip1 Expression and pRb Phosphorylation Are Regulated through a PI3K-dependent Pathway.—**The results described above indicated that p27kip1 might be an important target for BCR/ABL in deregulating cell cycle control mechanisms, and therefore, we next sought to identify the signaling pathway responsible for p27kip1 expression. As an initial screen, three drugs that inhibit different signaling pathways were studied: PD98059, LY-294002, and rapamycin, known to specifically inhibit mitogen-activated protein kinase, PI3K, and p70S6K pathways, respectively. Treatment of p210BCR/ABL-expressing Ba/F3 cells with carrier alone (Me2SO) or with PD98059 had no effect on cell cycle progression (Fig. 3A, upper panels), whereas treatment for 14 h with either LY-294002 or rapamycin induced a G0/G1 arrest, without any significant effect on cell viability (note the absence of a sub-G1 population in Fig. 3A, lower panels). The effects of these three drugs on BCR/ABL-induced expression of p27kip1 was then investigated (Fig. 3B). Treatment of Ba/F3-p210BCR/ABL with LY-294002 induced a dramatic increase of p27kip1 expression, whereas a treatment with rapamycin and PD98059 had no effect. Since rapamycin effectively induces a G1 arrest in these cells, it is unlikely that the p27kip1 up-regulation induced by LY-294002 is simply a consequence of cell cycle arrest. As p27kip1 is a known inhibitor of the Rb kinase cdk2, we also sought to determine if pRb was found in a hyperphosphorylated form in BCR/ABL-expressing cells and if this phosphorylation was regulated by PI3K. As shown in Fig. 3C, hyper- (ppRb) and hypo- (pRb) phosphorylated forms of Rb can be distinguished by an electrophoretic shift on a 6.5% SDS-PAGE. Ba/F3 p210 cells displayed almost exclusively a hyperphosphorylated form of Rb. Although
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p27Kip1 expression in BCR/ABL-transformed cells. Determined 12 h with solvent alone (MeSO), or inhibitor (PD98052, upper left), or FRAP/TOR inhibitor (Rapamycin, lower left), or PI3K inhibitor LY-294002, IL-3-deprived for 18 h and lysed. As shown in Fig. 4A, IL-3 deprivation of cells without BCR/ABL leads to a dramatic increase of p27Kip1 expression in cells transfected either with an empty vector or with a vector encoding for a wild-type form of AKT. Expression of an activated form of AKT or BCR/ABL, however, led to a significant and equivalent decrease of p27Kip1 expression. These results suggest that activation of AKT, a known consequence of BCR/ABL signaling, is sufficient in these cells to regulate p27Kip1 expression.

To determine if AKT functions downstream of PI3K in the regulation of p27Kip1 expression in Ba/F3 cells, activated AKT was expressed in BCR/ABL-transformed cells in which PI3K had been inhibited by LY-294002 (Fig. 4B). Ba/F3-p210 cells were co-transfected as above with plasmids encoding GFP and either a wild-type or the activated form of AKT. After isolation of GFP-positive cells by flow sorting, the positive cells were allowed to recover for an additional 18 h and then either left untreated or were treated with LY-294002 or rapamycin. The expression of the constitutively active form of AKT (HA-AKT-CAAX) completely inhibited the increase of p27Kip1 expression induced by LY-294002, whereas the expression of a wild type form (HA-AKT-WT) had no detectable effect. These results indicate that AKT can regulate p27Kip1 expression in hematopoietic cells and that it is likely downstream of PI3K activity, because an activated AKT mutant can override the effect of PI3K inhibition on p27Kip1 expression.

Molecular Mechanisms of p27Kip1 Down-regulation in BCR/ABL-transformed Cells—In other cell systems, p27Kip1 regulation of expression has been shown to occur at both transcriptional and posttranscriptional levels (35). In some cells, p27Kip1 has been shown to be ubiquitinated and thereby targeted for proteasome-mediated degradation (36–38). To determine if BCR/ABL-induced p27Kip1 down-regulation is mediated by a protein degradation pathway, we studied several protease inhibitors, lactacystine and N-acetyl-leucyl-leucine norleucine. As shown in Fig. 5A, after a 5-h treatment, both proteasome inhibitors specifically induced an increase of p27Kip1 expression in Ba/F3-p210 cells, whereas the protease inhibitors had little or no effect. To further investigate possible degradation of p27Kip1, Ton.B.210 cells, induced or not with doxycycline, were treated with 10 μM ribosomal complex inhibitor cycloheximide. By blocking translation with cycloheximide, the role of post-translational events such as degradation in regulating protein levels can be specif-
Fig. 4. AKT/protein kinase B activity controls p27\textsuperscript{Kip1} protein expression level. A. Tö-B.210 cells were transiently cotransfected with a GFP-expressing vector and with the indicated vectors expressing either WT AKT (HA-AKT-WT) or a constitutively active form of AKT by addition of a CAAX box (HA-AKT-CAAX). After transfection, the cells were either left untreated or treated with doxycycline (Dox) for 24 h in the presence of IL-3. The cells were then elutriated on the basis of the GFP expression and then IL-3-deprived for 16 additional h. Cells were then harvested and lysed, and equivalent amounts of protein were separated by gel electrophoreses and transferred to PVDF filter. B, Ba/F3 cells expressing BCR/ABL were transiently cotransfected with a GFP-expressing vector and with HA-AKT-WT or HA-AKT-CAAX. 24 h post-transfection, cells were elutriated on the basis of the GFP expression. Cells were then treated for 12 h with the indicated drugs. After lysis, equivalent amounts of lysates were separated and transferred to a PVDF filter. The filter was probed with the indicated antibodies. DMSO, Me\textsubscript{3}SO.

Fig. 5. p27\textsuperscript{Kip1} protein expression level is regulated by proteasome activity. A, Ba/F3-p210 cells were treated for 5 h with either Me\textsubscript{3}SO (DMSO) (1/2000), E64 (50 μM), N-acetyl-leucyl-leucyl-methionyl (100 μM), lactacystine (50 μM), N-acetyl-leucyl-leucyl-norleucinal (50 μM), or ethanol (EtOH) (1/1000). After treatment, cells were washed and lysed as described under “Materials and Method.” Lysates were resolved by SDS-PAGE, transferred to PVDF filters, and probed with the indicated antibodies. Abl and p85 blots were used as equal loading controls. B, Tö-B.210 cells were left untreated or induced with 1 μg/ml doxycycline (Dox) 24 h before cycloheximide (Chx) treatment, as indicated under “Materials and Method.” At t = 0, cycloheximide was added to the culture medium at a final concentration of 10 μg/ml. A fraction of the cells was harvested at the indicated time. The cells were lysed as indicated under “Materials and Methods,” and an equivalent amount of protein was loaded on 12% SDS-polyacrylamide electrophoresis gel. After electro-transfer to a PVDF filter, the proteins were probed with the indicated antibody. The equal loading was assessed by β-actin blot. Because of the higher expression of p27\textsuperscript{Kip1} in non-induced cells and in order to better demonstrate the difference of half-life, two different exposures (shorter exposure on upper panel for non-induced cells) of the same filter are shown.

In other cells, p27\textsuperscript{Kip1} has also been shown to be regulated at the level of transcription (35, 39). Therefore, p27\textsuperscript{Kip1} RNA levels were compared before and after induction of BCR/ABL in Tö-B.210 cells using semiquantitative real time PCR, as described under “Materials and Methods.” Treatment of Tö-B.210 cells with doxycycline in presence or in absence of LY-294002 resulted in a less than 1.5-fold change in p27\textsuperscript{Kip1} RNA levels in two independent experiments in (Fig. 6). These results suggest that transcriptional regulation is likely to play a minimal role in this system.

DISCUSSION

The BCR/ABL oncogene encodes an activated tyrosine kinase (9) that is located in the cytoplasm of hematopoietic cells (40). The kinase phosphorylates a number of well known signaling proteins, including Shc, SHP2, GAB2, DOK, CBL, and CRKL. Also, BCR/ABL is phosphorylated itself, and it is likely that complexes of activated signaling intermediates accumulate on BCR/ABL, resulting in constitutive activation of several signaling pathways normally tightly regulated by growth factors, extracellular matrix proteins, or other external signals.

The biological consequences of activation of these signaling pathways have been controversial, particularly for p210\textsuperscript{BCR/ABL}. Currently, there is reasonable consensus that p210\textsuperscript{BCR/ABL} is mitogenic (5), prolongs viability (6), and alters adhesion and homing of myeloid lineage cells (7, 41). p210\textsuperscript{BCR/ABL} is also believed to cause genomic instability, although the mechanism and type of new DNA damage in CML cells has not been clearly defined (42, 43).
The mitogenic effects of BCR/ABL are of particular interest because a hyperproliferative state of myeloid cells in CML is the most striking feature of the disease. BCR/ABL is known to activate several signaling pathways associated with proliferation, including p21CIP1 (23) and p38 (27). The nuclear events associated with enhanced proliferation have not been well studied. Both c-myc and cyclin D1 have been shown to be up-regulated by BCR/ABL (22, 44) and are likely to be important. The signals leading to activation of these two nuclear factors have not been elucidated, although up-regulation of c-myc is believed to require the SH2 domain of ABL (45). The significance of cyclin D1 in human myeloproliferative disease needs clarification, since cyclin D1 is not normally expressed in human hematopoietic cells (46, 47), and mice with cyclin D1 knockout do not display clear defects in hematopoiesis (48). Cyclins D2 and D3 are expressed in human hematopoietic cells, however, and are good candidates as BCR/ABL target molecules.

The mitogenic signals from BCR/ABL are likely to ultimately deregulate cell cycle control in some way, but few direct effects of BCR/ABL on cell cycle regulatory proteins have previously been identified. In hematopoietic cells, both the transition from G0 to G1 and the transition from G1 to S phase are believed to be key steps in controlling the cell cycle, and most of the known external influences on cell proliferation are thought to act at one or both of these sites. Deregreation of G0/G1 and G1/S checkpoints is an almost universal abnormality in human cancers. Mutations of cell cycle regulators such as the retinoblastoma protein, Rb, or cdk inhibitors such as p16INK4A, p21WAF1, or p27Kip1 are common in stable cell lines (49). Thus, BCR/ABL is likely to bypass normal controls on the proliferation of hematopoietic cells by altering expression of cell cycle regulatory proteins through aberrant activation of upstream signaling pathways.

In this study, we demonstrate that BCR/ABL regulates expression of one of the key cell cycle inhibitors, p27Kip1, and that this occurs through the PI3K/AKT pathway. p27Kip1 is a member of a family of cell cycle regulatory proteins that include p21CIP1 and p57Kip2. p27Kip1 is a widely expressed inhibitor of the essential cell cycle kinase regulating entry into S phase, a member of the cdk2/cyclin E complex and prevent phosphorylation of critical target molecules necessary for initiation of S phase, including Rb. Phosphorylation of Rb is necessary for release of sequenced transcription factors of the E2F family and induction of E2F-dependent gene expression (50, 51). In normal cells, progression through G1/S phase requires that p27Kip1 be displaced from cdk2, either by sequestration in cyclin D-cdk4 complexes, which are not inhibited by p27Kip1, or by down-regulation of the protein through multiple mechanisms. In cancer cells, the Rb checkpoint can be bypassed by loss of Rb, loss of p27Kip1, overexpression of cyclin E or other cyclins, and likely by other mechanisms as well.

In the studies reported here, expression of BCR/ABL was shown to specifically and rapidly decrease expression of p27Kip1, coincident with progression from G1 to S phase. The down-regulation of p27Kip1 was shown to be directly due to BCR/ABL activity by two different approaches. First, induction of BCR/ABL by a tetracycline-regulated promoter was associated with a reversible down-regulation of p27Kip1. Second, inhibition of BCR/ABL kinase activity with the ABL tyrosine kinase inhibitor STI571 specifically increased p27Kip1 levels. The STI571 is a 2-phenylaminopyrimidine derivative that was reported to selectively inhibit the tyrosine kinase activities of ABL, BCR/ABL, c-KIT, and the platelet-derived growth factor receptor-β (52) and was shown to selectively suppress the growth of BCR/ABL-positive cell lines (53). Thus, BCR/ABL activates one or more signaling pathways involving tyrosine phosphorylation that leads to p27Kip1 regulation. To identify intermediate signaling pathways, several additional inhibitors were used, including chemical inhibitors of the mitogen-activated protein kinase pathway (PD98059), PI3K pathway (LY294002), and the PI3K pathway (rapamycin). The PI3K inhibitor blocked the ability of p21(C/ABL) to inhibit p27Kip1 and inhibited BCR/ABL-induced entry into S phase. These results suggested that activation of the PI3K pathway might be necessary for the cell cycle effects of BCR/ABL through p27Kip1.

The PI3K enzyme is a heterodimer composed by a 110-kDa catalytic subunit and an 85-kDa regulatory subunit containing 2 SH2 domains and 1 SH3 domain (54). Activation of PI3K by many growth factor receptors involves recruitment of the enzyme to the membrane through binding of one or both of the SH2 domains to specific pYXXM (pY, phosphorylated tyrosine) motifs in the receptor or in phosphorylated adapter molecules. PI3K is known to be activated by v-abl and BCR/ABL, although the mechanism is unclear since both oncogenes lack motifs for binding p85 PI3K (55). There is abundant evidence, however, that activation of PI3K is important for transformation. Skorski et al. (27) used antisense oligonucleotides and the PI3K inhibitor wortmannin to show that PI3K is required for the growth and survival of BCR/ABL-transformed cells in vitro. Furthermore, they showed that one of the targets of PI3K, the AKT kinase, was likely to be involved in BCR/ABL transformation, since dominant negative mutants of AKT also inhibited BCR/ABL-induced transformation in vitro and in vivo. Finally, c-myc was identified as a potential target of AKT and PI3K in these studies. It is clear, however, that PI3K has a number of downstream targets, and it is likely that the significance of various targets is cell type-specific.

Since AKT is known to mediate a number of PI3K actions, we examined the role of AKT in the signaling of BCR/ABL to p27Kip1. An activated mutant of AKT (AKT fused to a membrane-targeting sequence, HA-AKT-CAAX) was shown to increase p27Kip1 levels, whereas overexpression of a non-activated AKT (HA-AKT) had no effect. These results showed that activation of AKT by itself was capable of regulating p27Kip1 levels and were consistent with previous studies linking AKT to p27Kip1 in T cells (56), mouse embryonic fibroblasts (57), and glioblastoma cells (58). We next asked if AKT functioned up-

![Graph showing the effects of BCR/ABL on p27Kip1 mRNA levels.](Image 85x589 to 261x729)
stream or downstream of PI3K in the regulation of p27Kip1 in Ba/F3 cells. Expression of the activated AKT mutant, but not wild-type AKT, down-regulated expression of p27Kip1 in cells exposed to LY-294002 to block PI3K, indicating that AKT function downstream of PI3K. Thus, taking these results together, a model is proposed in which BCR/ABL activates AKT through PI3K, resulting in a significant down-regulation of p27Kip1 and accelerated entry into S phase.

The mechanism of down-regulation of p27Kip1 by PI3K/AKT is likely to be of interest. p27Kip1 protein expression level is likely to be of interest. p27Kip1 protein expression level is monophosphorylated by activated cdk2, ubiquitinated, and then degraded in the proteasome (60). Furthermore, degradation of p27Kip1 can be mediated by other types of proteolysis (61). However, this level of regulation requires activation of cdk2. p27Kip1 can also be regulated by transcription, and recent studies suggest that Forkhead transcription factors may be important (39). The Forkhead transcription factors AFX (62), FKHR (63), and FKHR-L1 (64) are orthologues of DAF-16 of Caenorhabditis elegans and have previously been shown to be involved in regulating viability and G1 to S progression (65). In addition, Medema et al. (39) show that AFX up-regulates p27Kip1 promoter activity. The Forkhead factors are exported from the nucleus in response to an AKT-dependent phosphorylation event, thus suggesting the model that transcription of p27Kip1 is decreased when AKT or a kinase regulated by AKT phosphorylates one or more Forkhead transcription factor. However, semi-quantitative PCR, real time PCR, p27 promoter luciferase assay, or Northern blot did not suggest that there was any significant level of transcriptional regulation of p27Kip1 in BCR/ABL-transformed cells.

In contrast, our results indicate that p27Kip1 expression is regulated by BCR/ABL through a proteasome-dependent degradation pathway. A direct consequence of the dramatic decrease of p27Kip1 expression observed in BCR/ABL-transformed cells is likely to be deregulation of the cyclinE-cdk2 complex and resultant constitutive phosphorylation of Rb. Strengthening this model, the increase of p27Kip1 expression due to a PI 3-kinase inhibition by the inhibitor LY-294002 is also accompanied by a decrease of Rb phosphorylation in p210BCRABL-transformed cells. Our results show that a key event of the progression of BCR/ABL-transformed cells through cell cycle is regulated by p27Kip1, which in turn regulates the phosphorylation of endogenous Rb through cdk2. Kramer et al. (66) recently suggested that adhesion to fibronectin of BCR/ABL-transformed murine hematopoietic cells induces proliferation through a decrease of p27Kip1 expression. All our data were performed in the murine pre-B cell line Ba/F3 and were strictly reproducible in the murine myeloid progenitor 32D used by Kramer et al. (Ref. 66; data not shown). However, p27Kip1 down-regulation described here was not dependent on fibronectin adhesion and was found to be directly due to BCR/ABL signaling. Proteasome-dependent degradation of other proteins has also been shown to be important for BCR/ABL transformation. Pendergast and co-workers (67) recently demonstrated proteasome-dependent degradation of the inhibitory molecules ABL-1 and ABL-2.

Overall, the studies reported here describe a new signaling pathway for BCR/ABL. Although BCR/ABL is known to have prominent growth promoting effects, the mechanisms used by this oncogene to bypass normal cell cycle checkpoints have been unclear. The down-regulation of p27Kip1 through PI3K and AKT is likely to be a significant component of this activity.
BCR/ABL Regulates Expression of p27Kip1

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