

BCR-ABL Induces the Expression of Skp2 through the PI3K Pathway to Promote p27^{Kip1} Degradation and Proliferation of Chronic Myelogenous Leukemia Cells

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Abstract

Chronic myelogenous leukemia (CML) is characterized by the expression of the BCR-ABL tyrosine kinase, which results in increased cell proliferation and inhibition of apoptosis. In this study, we show in both BCR-ABL cells (Mo7e-p210 and BaF/3-p210) and primary CML CD34+ cells that STI571 inhibition of BCR-ABL tyrosine kinase activity results in a G₁ cell cycle arrest mediated by the PI3K pathway. This arrest is associated with a nuclear accumulation of p27^{Kip1} and down-regulation of cyclins D and E. As a result, there is a reduction of the cyclin E/Cdk2 kinase activity and of the retinoblastoma protein phosphorylation. By quantitative reverse transcription-PCR we show that BCR-ABL/PI3K regulates the expression of p27^{Kip1} at the level of transcription. We further show that BCR-ABL also regulates p27^{Kip1} protein levels by increasing its degradation by the proteasome. This degradation depends on the ubiquitinylation of p27^{Kip1} by Skp2-containing SFC complexes: silencing the expression of Skp2 with a small interfering RNA results in the accumulation of p27^{Kip1}. We also demonstrate that BCR-ABL cells show transcriptional up-regulation of Skp2. Finally, expression of a p27^{Kip1} mutant unable of being recognized by Skp2 results in inhibition of proliferation of BCR-ABL cells, indicating that the degradation of p27^{Kip1} contributes to the pathogenesis of CML. In conclusion, these results suggest that BCR-ABL regulates cell cycle in CML cells at least in part by inducing proteasome-mediated degradation of the cell cycle inhibitor p27^{Kip1} and provide a rationale for the use of inhibitors of the proteasome in patients with BCR-ABL leukemias. (Cancer Res 2005; 65(8): 3264-72)

Introduction

Chronic myeloid leukemia (CML) is a hematopoietic stem cell disease characterized at the molecular level by the expression of the Philadelphia chromosome translocation that results in the rearrangement between the breakpoint-cluster region (*BCR*) on chromosome 22 and the Abelson leukemia gene (*ABL*) on

chromosome 9 (1–3). *BCR-ABL* encodes a chimeric protein with a constitutive tyrosine kinase activity. It has been clearly shown that expression of p210^{BCR-ABL} in hematopoietic cells is essential and sufficient for malignant transformation (4). BCR-ABL-dependent kinase activity results in the deregulation of a number of signal transduction pathways that participate in cell proliferation, differentiation, migration, adhesion, and apoptosis (3, 5–9).

Expression of BCR-ABL in hematopoietic cells results in growth factor independence and progression through the G₁-S phase of the cell cycle (10). The mitogenic effects of BCR-ABL require the activation of the PI3K/Akt pathway that promotes cell survival by phosphorylation and inactivation of Bad, thus preventing apoptosis (11). The Ras/Raf/MEK/extracellular signal-regulated kinase pathway, which is involved in cell cycle regulation, is also altered in CML (12). Furthermore, we have previously shown that BCR-ABL also promotes cell survival through STAT5-mediated up-regulation of Bcl-x_L (13).

The development of the BCR-ABL inhibitor STI571 has had an impact in the treatment of CML patients but has also provided a new tool for studying the effect of inhibition of the BCR-ABL kinase activity in cells harboring the endogenous *BCR-ABL* gene (14). Initial clinical results in patients with chronic phase CML have been extremely encouraging with complete cytogenetic remissions in >40% of patients resistant or refractory to IFN (15). Results in patients with CML in more advanced phases have not produced durable responses (16). Newer combinations of STI571 with other drugs as well as development of other kinase inhibitors are currently being tested as treatment for CML and Philadelphia positive acute lymphoblastic leukemia (17).

The cyclin-dependent kinase inhibitor p27^{Kip1} is a key regulator of the cell cycle in mammalian cells (18, 19). It negatively regulates cell cycle progression by directly inhibiting cyclin/Cdk2 complexes. The activity of p27^{Kip1} is controlled by its distribution among the different cell compartments (nucleus and cytoplasm; ref. 20). Akt directly phosphorylates p27^{Kip1} at T157 promoting its translocation to the cytoplasm where it is inactivated (21–23). However, the key mechanism of regulation of p27^{Kip1} expression is proteolysis by the ubiquitin-proteasome pathway (24), mediated by Skp2 containing SCF complexes. The ubiquitinylation by Skp2 requires the previous phosphorylation of p27^{Kip1} at T187 by the same cyclin E/Cdk2 complexes that it inhibits. p27^{Kip1} is also regulated at the transcriptional level by transcription factors of the Forkhead/FoxO family, which are phosphorylated (and inhibited) by Akt (25).

We have previously shown that p27^{Kip1} plays a significant role in adhesion-mediated regulation of cell cycle in hematopoietic cells (26). Recent reports have also suggested a role for the cell cycle

Note: E.J. Andreu and E. Lledó contributed equally to this work and should be considered equal first authors.

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inhibitor *p27^{Kip1}* in the regulation of the cell cycle in BCR-ABL cells (27–30). In these studies, down-regulation of *p27^{Kip1}* has been shown in human and murine cell lines transfected with BCR-ABL. However, the mechanism by which BCR-ABL down-regulates *p27^{Kip1}* in primary CML cells or the role of *p27^{Kip1}* down-regulation in the pathogenesis of CML is unclear. Furthermore, analysis of CD34+ cells from patients with CML has suggested that BCR-ABL-mediated regulation of *p27^{Kip1}* depends on relocation of the cell cycle inhibitor from the nucleus to the cytoplasm in CML cells, thus preventing binding to cyclin E/Cdk2 complexes and promoting cell cycle progression (30).

In the current study, we have examined the role of *p27^{Kip1}* in the pathogenesis of CML. Our results show that BCR-ABL down-regulates *p27^{Kip1}* both by transcriptional regulation and by inducing proteasome-mediated degradation of the cell cycle inhibitor. The inhibition of BCR-ABL results in an accumulation of *p27^{Kip1}* in the nucleus and inhibition of cyclin E/Cdk2 kinase activity as well as a reduction of retinoblastoma phosphorylation. The enhanced degradation of *p27^{Kip1}* is associated with the transcriptional up-regulation of *Skp2* induced by BCR-ABL. Whereas overexpression of *p27^{Kip1}* has no effect on proliferation of BCR-ABL-positive cells, a T187 *p27^{Kip1}* mutant that cannot be recognized by *Skp2* induces a cell cycle arrest, providing evidence for the importance of *p27^{Kip1}* degradation in the pathogenesis of CML.

Materials and Methods

Cell lines and primary cells from chronic myeloid leukemia patients and normal donors. Human-derived Mo7e and Mo7e-p210 cells were grown in Iscove's modified Dulbecco's medium supplemented with 20% fetal bovine serum, L-glutamine, and penicillin-streptomycin with (Mo7e) or without (Mo7e-p210) 5 ng/mL of recombinant human interleukin-3 (IL-3). BaF/3 cells were grown in RPMI supplemented with 10% FCS, L-glutamine and penicillin-streptomycin, and 10% WEHI-conditioned medium was added to the culture as a source of murine IL-3 whereas growth of BaF/3-p210 was independent of growth factors. All media, serum and supplements were from Invitrogen Life Technologies (Paisley, United Kingdom), except for human IL-3 (R&D Systems, Minneapolis, MN).

Mobilized peripheral blood or bone marrow mononuclear cells were obtained from patients in chronic phase CML after informed consent. All patients were 100% Philadelphia chromosome-positive by direct cytogenetic analysis. CD34+ cells from bone marrow or mobilized peripheral blood were enriched using the MACS CD34+ isolation kit (Miltenyi Biotec, Cologne, Germany) and either the AutoMACS or the CliniMACS selection devices as previously described (13). CD34+ purity was always >90% after positive selection. CD34+ cells were cultured in Iscove's modified Dulbecco's medium supplemented with 20% FBS, IL-3 at a final concentration of 100 ng/mL was also added to the culture medium when the CD34+ cells were from healthy donors. When indicated, primary cells and/or cell lines were treated with the tyrosine kinase inhibitor STI571 at a concentration of 2 to 10 $\mu\text{mol/L}$ (generously provided by Dr. Elisabeth Buchdunger, Novartis, Basel, Switzerland). The PI3K inhibitor LY294002 (Calbiochem, San Diego, CA) was used at a concentration of 20 $\mu\text{mol/L}$, the MEK inhibitor PD98059 (Calbiochem) at 30 $\mu\text{mol/L}$ and lactacystin (AFFINITI Research Products, Ltd., Mamhead, Exeter, United Kingdom) at 10 $\mu\text{mol/L}$.

Flow cytometry analysis. For cell cycle analysis, 1 to 2 $\times 10^6$ cells were stained with propidium iodide as described (26). Briefly, the cells were washed twice in cold PBS and fixed in ice-cold 50% ethanol for 30 minutes. After two more washes in PBS, propidium iodide (Sigma Chemical Co., Poole, United Kingdom), and RNase A (Sigma) were added to a final concentration of 100 ng/mL each. After incubation for 1 hour at room temperature, the cells were kept at 4°C until analysis by flow cytometry (FACScan, Becton Dickinson, Temse, Belgium) using the CellQuest software.

Western blot analysis, immunoprecipitation, and kinase assays. For immunoblotting, cell lysates were prepared according to Kabarowski et al.

(31). Protein concentrations were determined with bicinchoninic acid protein assay (Pierce, Rockford, IL). Cellular proteins (50–100 μg) were resolved on SDS-PAGE, transferred to a nitrocellulose membrane (Bio-Rad, CA), detected with specific antibodies and visualized by enhanced chemiluminescence (Tropix, Foster City, CA). Antibodies used were from Santa Cruz Biotechnology (Santa Cruz, CA; ABL, *p27^{Kip1}*, cyclin E, Cdk2 and *Skp2*), BD Biosciences-PharMingen (San Jose, CA; cyclin D, *p21^{Cip1}*, and retinoblastoma) and Zymed (South San Francisco, CA; *Skp2*). Blots were stripped and reprobed with a β -tubulin antibody (Sigma) as a protein loading control. Subcellular fractionation of Mo7e-p210 cells cultured in the presence or absence of STI571 (2 $\mu\text{mol/L}$) or LY294002 (20 $\mu\text{mol/L}$) was done as previously described (32). Protein extracts were quantified with the Bio-Rad DC Protein Assay and 50 μg were used for the detection of *p27^{Kip1}* in each fraction by Western blot analysis. Lamin A (Cell Signaling, Beverly, MA) and β -tubulin (Sigma)-specific antibodies were used to assess the purity of the subcellular fractions. *Skp2* was immunoprecipitated with rabbit polyclonal antibodies (sc-7164, Santa Cruz Biotechnology) and True Blot anti-rabbit immunoglobulin IP beads (eBioscience, San Diego, CA), from 1 mg of protein extracts from BaF/3-p210 cells overexpressing *Skp2*. *Skp2* and *p27^{Kip1}* in the immunoprecipitates were detected with mouse monoclonal antibodies (Santa Cruz Biotechnology) and peroxidase conjugated anti-mouse IgG-Fc (Pierce).

For kinase assays, cyclin E- or Cdk2-containing complexes were immunoprecipitated from 150 μg of protein extracts with 1 μg of antibody and 30 μL of Protein A/G-plus Agarose (Santa Cruz Biotechnology). Kinase activity assays were done as described previously using histone H1 (Roche, Penzberg, Germany) as substrate (33). The intensity of the bands was quantified with the QuantityOne software (Bio-Rad).

Quantitative reverse transcription-PCR analysis. Mo7e-p210 cells were seeded at a density of 25×10^4 cells/mL and treated with 2 $\mu\text{mol/L}$ STI571 or 20 $\mu\text{mol/L}$ LY294002. Untreated controls were grown in parallel. At 24-hour intervals, total RNA was obtained with the RNeasy Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. After DNase I treatment (amplification grade, Invitrogen), 2 to 5 μg of total RNA were used as template for a reverse transcriptase reaction with the SuperScript First-Strand Synthesis System for reverse transcription-PCR (Invitrogen), using Oligo(dT) primers. For the relative quantification of the *p27^{Kip1}* and *Skp2* mRNA levels, this cDNA was used as template in PCR reactions in the presence of SYBR Green I (Molecular Probes, Leiden, The Netherlands) and using the Platinum Taq DNA polymerase (Invitrogen). The PCR reactions and their analysis were done in an iCycler machine and using the iCycler iQ Optical System software (Bio-Rad). Besides *p27^{Kip1}* and *Skp2*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was also amplified as an internal control. For the quantification, a standard curve was established using serial dilutions of a known amount of a plasmid harboring the human *GAPDH* cDNA as template for the PCR reactions. Three serial dilutions of the cDNA from each sample and four replicates of each dilution were used. The primers used were: *p27*, D 5'-GCACTGCAGAGACATGGAAG-3'; R 5'-AGAATCGTTCGGTTGCAGGTC-3'; *Skp2*, D 5'-GCTTCACGTGGGGATGGGAT-3'; R 5'-GCAGGCTTCCAGGCTTAGA-3'; *GAPDH*, D 5'-CATCACCATCTTC-CAGGAGC-3'; R 5'-CACGGAAGGCCATGCCAG-3'.

Pulse-chase experiment. To examine the turnover of *p27^{Kip1}*, BaF/3-p210 cells were incubated with 2 $\mu\text{mol/L}$ STI571 or vehicle for 24 hours and pulse-labeled for 2 hours with 120 $\mu\text{Ci/mL}$ [³⁵S]methionine in methionine-free MEM medium supplemented with 10% dialyzed FBS. Cells were then incubated in RPMI containing 10% FBS and excess methionine (chase medium) for different times. After washing with ice-cold PBS, cell lysates were prepared in radioimmunoprecipitation assay buffer (1 \times PBS, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L phenylmethylsulfonyl fluoride, 0.1 mmol/L leupeptin, 1 mmol/L sodium orthovanadate, and 10 mmol/L sodium fluoride). Cellular debris was removed by centrifugation and the supernatant was subjected to immunoprecipitation with agarose-conjugated anti-*p27^{Kip1}* monoclonal antibodies (Santa Cruz Biotechnology). Immune complexes were washed five times with radioimmunoprecipitation assay buffer and the eluted proteins were analyzed by SDS-gel electrophoresis on 12% acrylamide gels. The labeled *p27^{Kip1}* protein was detected by fluorography and quantified with the QuantityOne software

(Bio-Rad). p27^{Kip1} degradation rates were calculated from the slope of the logarithmic transformation of the densitometry data plotted against time, as previously described (34).

Retroviral infections. Wild-type p27^{Kip1} and two different mutants, p27P- (mutated at Thr¹⁸⁷ and therefore unable to be phosphorylated and degraded) and p27ck- (unable to interact with neither cyclin nor Cdk; ref. 35) were introduced in BaF/3-p210 cells using the LZRS-IRES-green fluorescent protein retroviral vector (36). Replication-defective retroviruses were produced by transfecting Bosc 23 producer cells with the recombinant DNAs. BaF/3-p210 cells (at a density of 2×10^6 cells/mL) were incubated for 3 hours with 4 mL of filtered supernatant from producer cells in the presence of 8 µg/mL polybrene (Sigma). The day after infection, cells were seeded at 400×10^3 cells/mL. Green fluorescent protein expression was used to detect infected cells by fluorescence microscopy.

Adenoviral infections. The AdEasy adenovirus system (37) was used to overexpress Skp2 in BaF/3-p210 cells. The Skp2 adenovirus was a kind gift from Dr. D. Wolf and was described previously (38). Silencing of Skp2 expression in BaF/3-p210 was achieved by infection with AdEasy-derived adenovirus harboring the H1 promoter from pSUPER (39) to generate small interfering RNA. The sequence of the Skp2 small interfering RNA was 5'-AAGGGAGTGACAAAGACTTTG-3' (40). Cells were infected as described (41), with a multiplicity of infection of 1,000. Briefly, adenovirus produced in 293 cells were mixed with 50×10^{-3} µg Superfect (Qiagen) for 10 minutes before their addition to 1×10^6 BaF/3-p210 cells. The cells were incubated with the adenovirus for 6 hours and then the medium was changed. Flow cytometry analysis of green fluorescent protein-positive cells gave an infection rate of 15% to 20%. Cell extracts were prepared 72 hours after the infection.

Results

STI571 induced cell cycle arrest in BCR-ABL cells is associated with an accumulation of the cell cycle inhibitor p27^{Kip1}. We initially examined the effect of STI571 on the proliferation of BCR-ABL cells. Incubation with STI571 inhibited the proliferation of Mo7e-p210 and BaF/3-p210 cells, but had no effect on the proliferation of wild-type Mo7e or BaF/3 cells (Fig. 1A, top). Mo7e-p210 and BaF/3-p210 cells express the protein BCR-ABL p210, whereas only ABL can be detected in their wild-type controls by Western blotting (Fig. 1A, bottom). Cell cycle analysis indicated that STI571 induces a G₁ arrest in BCR-ABL-positive cells, with a reduction in the proliferating population (S + G₂-M phases) from 35% to 5% in Mo7e-p210 cells and from 33% to 15% in BaF/3-p210 cells (Fig. 1B). Similar results were observed in CD34+ cells obtained from patients with CML in chronic phase (34 to 6% reduction in the percentage of proliferating cells), whereas no significant changes were found in control CD34+ cells (Fig. 1B). As we have previously described (13), STI571 induced an increase in apoptosis in BCR-ABL-positive cells (Fig. 1B).

To determine potential mechanisms involved in abnormal proliferation of BCR-ABL-positive cells we examined the expression of proteins that participate in the regulation of the cell cycle. Inhibition of BCR-ABL kinase activity was associated with an increase in the protein levels of the cell cycle inhibitor p27^{Kip1} and a decrease in p21^{Cip1}, cyclin E, and cyclin D protein levels in Mo7e-p210 cells, whereas there was no effect in the parental wild-type Mo7e cells (Fig. 1C). Similar changes were observed in BaF/3-p210 cells, although the reduction in the levels of the cyclins was less evident. When we examined the effect of STI571 on chronic phase CML and normal human CD34+ cells, we could show that similar to the Mo7e-p210 cells, inhibition of BCR-ABL was associated with an accumulation of p27^{Kip1} and a reduction of cyclin D and cyclin E proteins (Fig. 1D). We could not detect the expression of p21^{Cip1} in normal or CML CD34+ cells.

STI571 inhibition of BCR-ABL results in the underphosphorylation of retinoblastoma. The changes in the protein levels seen when we treated BCR-ABL-positive cells with STI571, specially the accumulation of p27^{Kip1}, suggests that the blockage of the cell cycle in the G₁ phase could be the result of an inhibition in the Cdk-dependent kinase activity responsible for the phosphorylation of retinoblastoma. To test this hypothesis, we analyzed the localization of p27^{Kip1} in Mo7e-p210 cells treated with STI571 for 9 hours. As shown in Fig. 2A, treatment with STI571 induced a marked increase in the amount of p27^{Kip1} in the nuclear fraction. This accumulation of p27^{Kip1} was associated with a 60% decrease in the cyclin E/Cdk2 kinase activity in the presence of STI571 in Mo7e-p210 in comparison with Mo7e-wt cells (Fig. 2B). Furthermore, treatment with STI571 results in a reduction of retinoblastoma phosphorylation in Mo7e-p210 and primary CML CD34+ cells but not in Mo7e-wt or normal CD34+ cells, as shown by Western blotting (Fig. 2C). These results suggest that STI571-mediated BCR-ABL-dependent cell cycle arrest is associated with a reduction in the phosphorylation status of retinoblastoma due to the inhibition of Cdk kinase activity by p27^{Kip1}.

The PI3K pathway mediates the effect of BCR-ABL on the cell cycle. BCR-ABL regulates the activity of two main signal transduction pathways involved in survival and proliferation, the PI3K/Akt and the Ras/Raf/MEK pathways. We wanted to know which of these two was the one by which the expression of p27^{Kip1} is regulated in BCR-ABL cells. We treated Mo7e-p210 and BaF/3-p210 cells with an inhibitor of PI3K (LY294002) or with an inhibitor of MEK (PD98059) and studied their effect on cell proliferation. As shown in Fig. 3A, inhibition of MEK with PD98059 had no effect on proliferation, whereas inhibition of PI3K with LY294002 blocked proliferation of both cell lines, suggesting that BCR-ABL-induced proliferation is mediated mainly by the PI3K/Akt pathway. Furthermore, inhibition of the PI3K pathway with LY294002 results in an accumulation of p27^{Kip1} in Mo7e-p210 and in BaF/3-p210 cells (Fig. 3B). Similar to the effect seen after inhibition of BCR-ABL kinase activity with STI571, the p27^{Kip1} protein accumulates mainly in the nuclear fraction of Mo7e-p210 cells (Fig. 3C).

BCR-ABL regulates the levels of p27^{Kip1} by transcription inhibition and induced degradation. We next studied the mechanisms by which BCR-ABL activity reduces the levels of p27^{Kip1}. p27^{Kip1} expression may be regulated both at the transcriptional level by the FoxO transcription factors or by proteasome-mediated degradation following ubiquitinylation. In order to determine which mechanism is altered in BCR-ABL cells, we did two types of experiments. Firstly, we treated Mo7e-p210 cells with STI571 or LY294002 and extracted the RNA at different time points. We analyzed the amount of p27^{Kip1} mRNA by quantitative reverse transcription-PCR using GAPDH as an internal control. As shown in Fig. 4, there was an increase in the levels of p27^{Kip1} mRNA when we treated the cells with either STI571 or LY294002, suggesting that BCR-ABL inhibits the transcription of the p27^{Kip1} gene through the PI3K pathway. Secondly, we did a pulse-chase experiment to determine the degradation rate of the p27^{Kip1} protein in BaF/3-p210 cells. Incubation of the cells with STI571 resulted in a time-dependent accumulation of labeled p27^{Kip1} in comparison with untreated BaF/3-p210 cells (Fig. 4B). Densitometric analysis of the bands (Fig. 4C) revealed that the degradation rate of p27^{Kip1}, calculated from the slope of the logarithmic transformation of the densitometry data plotted against time, is slower when BCR-ABL kinase activity is inhibited by STI571, meaning that BCR-ABL increases the degradation of p27^{Kip1}.

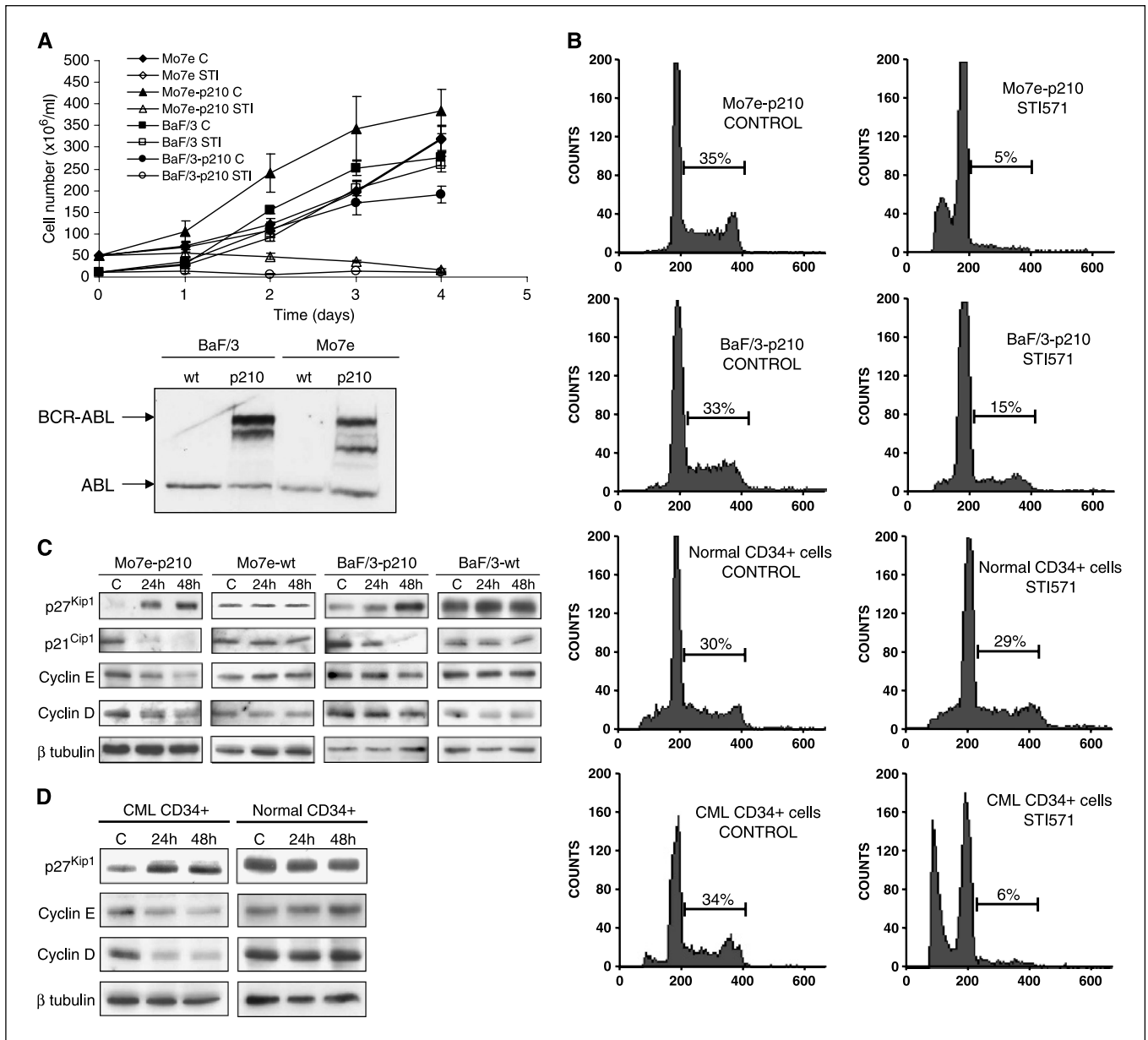


Figure 1. STI571 treatment blocks proliferation of BCR-ABL cells in G₁. **A**, Mo7e-wt, Mo7e-p210, BaF/3-wt, and BaF/3-p210 cells were treated with 2 μ mol/L STI571 (STI) or vehicle (C) and maintained in culture for 4 days. Viable cells (determined by trypan blue exclusion) were counted daily. The results represent the mean and SE of three independent experiments. Bottom, expression of BCR-ABL detected by Western blotting with an anti-ABL antibody in BaF/3-wt, BaF/3-p210, Mo7e-wt and Mo7e-p210 cell extracts; **B**, Mo7e-p210, BaF/3-p210, normal human CD34+ and chronic phase CML-derived CD34+ cells were incubated with 2 μ mol/L STI571 or vehicle (Control) for 24 hours and then the cell cycle profile was analyzed by flow cytometry as described in Materials and Methods. A representative example of three different experiments is shown. In each graph, the percentage of cells in the S + G₂-M phases is shown; **C**, Mo7e-p210, Mo7e-wt, BaF/3-p210, and BaF/3-wt cells were incubated with 2 μ mol/L STI571. Cells were harvested, washed and lysed at the times indicated as described in Materials and Methods. Protein extracts (50 μ g) were separated by SDS-PAGE and electrotransferred onto nitrocellulose membranes. Different blots with the same samples were probed with the indicated antibodies; **D**, CD34+ cells from patients with chronic phase CML or normal donors were incubated with 2 μ mol/L STI571. At the times indicated, cells were harvested, washed and lysed. A total of 50 μ g of protein extract were separated by SDS-PAGE and probed with the indicated antibodies. Representative examples of three independent experiments are shown.

Analysis of the data with a *t* test revealed that the slopes of the two lines (-0.0328 for the STI treatment and -0.0566 for control) are significantly different from each other ($P < 0.01$). The inhibition of the proteasome with 10 μ mol/L lactacystin also resulted in an increment of p27^{Kip1} stability (data not shown), confirming that the degradation of p27^{Kip1} depends on the proteasome activity.

Skp2 is involved in the degradation of p27^{Kip1} and its expression is induced by BCR-ABL. It has been shown that the

degradation of p27^{Kip1} in the proteasome requires its phosphorylation at T187 followed by its ubiquitinylation by the SCF^{Skp2} complex. We wanted to know whether Skp2 could also be the F-box protein involved in the degradation of p27^{Kip1} in our cell system. Silencing the expression of Skp2 with adenovirus-produced small interfering RNA resulted in an accumulation of p27^{Kip1} in BaF/3-p210 cells, whereas the overproduction of Skp2 resulted in a decrease of this protein (Fig. 5A, top). In Skp2

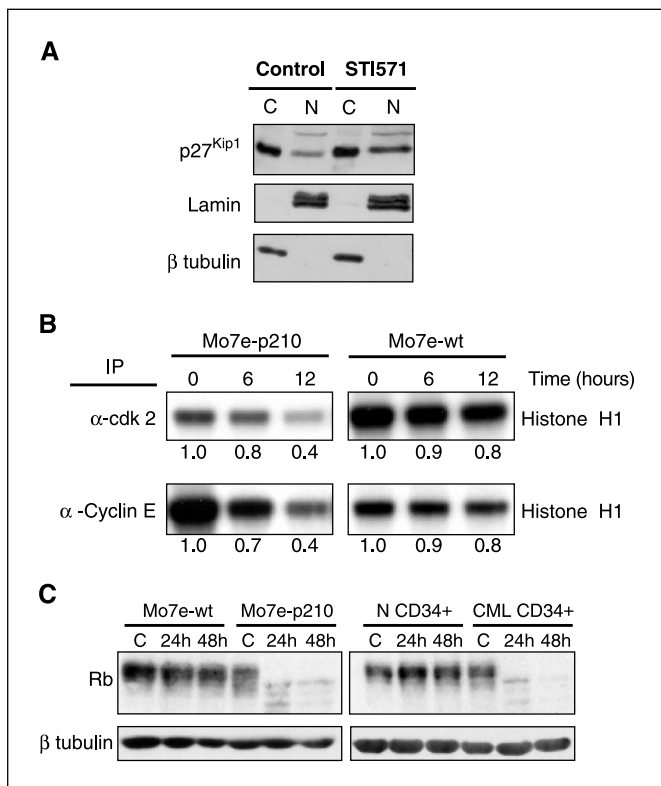


Figure 2. STI571-treated cells accumulate p27^{Kip1} in the nucleus and show a decrease in cyclin E/Cdk2 kinase activity. **A**, Mo7e-p210 cells were incubated with 2 μmol/L STI571 or vehicle (Control) for 9 hours and processed as described in Materials and Methods to separate the nuclear (N) and cytoplasmic (C) fractions. Extracts were separated by SDS-PAGE and probed with the indicated antibodies. Lamin and β-tubulin are markers for the nuclear and cytoplasmic fractions, respectively; **B**, Mo7e-p210 and Mo7e-wt cells were treated with 2 μmol/L STI571 for the indicated times. Protein extracts (150 μg) were incubated with antibodies against cyclin E or Cdk2 as indicated and kinase activity of the immunoprecipitated complexes was assayed as described in Materials and Methods. Reaction products were resolved by SDS-PAGE and the phosphorylated substrate (*histone H1*) was detected by exposing the dried gels to X-ray films. The numbers below each band represent the intensity of the bands relative to time 0; **C**, Mo7e-p210, Mo7e-wt, and CD34+ cells from normal donors and CML patients were treated with 2 μmol/L STI571 for 24 and 48 hours and 50 μg of cell lysates were separated by SDS-PAGE, electrotransferred, and incubated with antibodies against total retinoblastoma protein or β-tubulin as a loading control. The fast migrating bands represent the underphosphorylated form of retinoblastoma, whereas the slow migrating bands correspond to hyperphosphorylated retinoblastoma. A representative example of three independent experiments is shown.

overexpressing BaF/3-p210 cells, we could coimmunoprecipitate Skp2 and p27^{Kip1} (Fig. 5A, *bottom*), showing a direct interaction between these two proteins. We then explored whether the expression of Skp2 was dependent on BCR-ABL activity. We treated Mo7e-p210 and BaF/3-p210 cells with STI571 or LY294002 and analyzed the expression of Skp2 by Western blotting. As shown in Fig. 5B, both treatments resulted in a reduction of the Skp2 protein levels 24 hours after the treatment, suggesting that BCR-ABL signals through the PI3K pathway to induce the expression of Skp2. When we examined the effect of STI571 on chronic phase CML and normal human CD34+ cells, we could show that similar to the cell lines, inhibition of BCR-ABL was associated with a decrease in the expression of Skp2 (Fig. 5C). Moreover, analysis by quantitative reverse transcription-PCR showed that there is a reduction in the *Skp2* mRNA levels when Mo7e-p210 cells are treated with either STI571 or LY294002 (Fig. 5D). In

summary, these results indicate that the BCR-ABL/PI3K pathway regulates the expression of Skp2 at least at the level of transcription.

Expression of a T187V p27^{Kip1} mutant induces a cell cycle arrest in BCR-ABL-positive cells. Thus far, our results show that the inhibition of BCR-ABL induces a cell cycle arrest that is accompanied by an increase in the p27^{Kip1} protein levels that results from transcriptional induction and inhibition of degradation mediated by Skp2 activity. To determine to what extent p27^{Kip1} degradation is responsible for the increased proliferation of BCR-ABL-positive cells, we infected BaF/3-p210 cells with LZRS-IRES-green fluorescent protein-derived retroviruses expressing wild-type p27^{Kip1} or the T187V mutant, which cannot be phosphorylated by Cdk-containing complexes and therefore is not ubiquitinated by SCF^{Skp2} nor degraded at the proteasome (35). As shown in Fig. 6, cells expressing wild-type p27^{Kip1} kept proliferating as well as the uninfected cells, cells infected with the

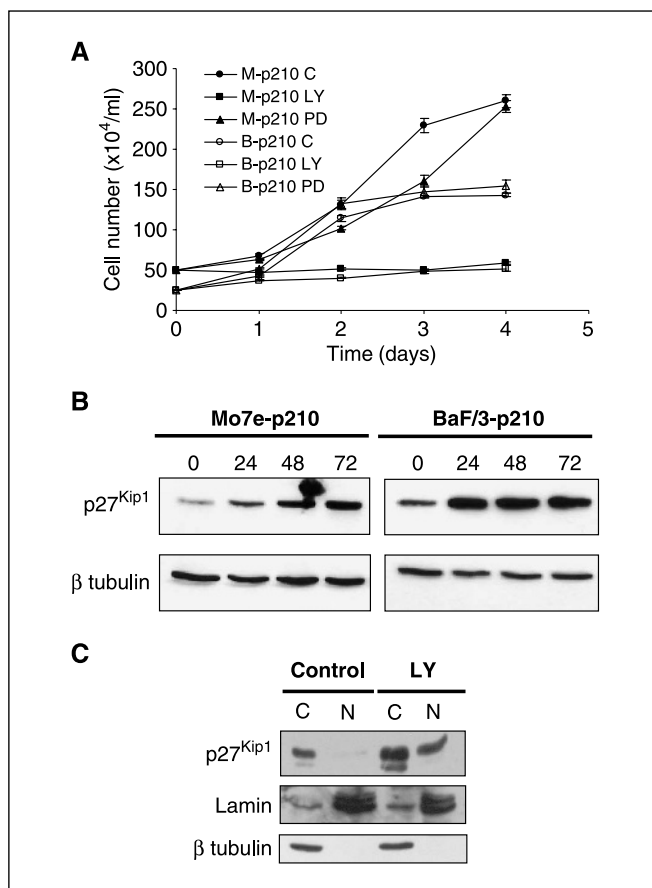


Figure 3. The PI3K pathway mediates the effect of BCR-ABL on cell proliferation and on p27^{Kip1} accumulation. **A**, Mo7e-p210 (M-p210) and BaF/3-p210 (B-p210) cells were treated with 20 μmol/L LY294002 (LY), 30 μmol/L PD98059 (PD) or vehicle (C) and maintained in culture for 4 days. Viable cells (determined by trypan blue exclusion) were counted daily. The results represent the mean and SE of three independent experiments; **B**, Mo7e-p210 and BaF/3-p210 cells were cultured in the presence of 20 μmol/L LY294002. At the times indicated, cells were harvested and cell extracts were prepared and analyzed by Western blotting as described in Materials and Methods. A representative example of three different experiments is shown; **C**, Mo7e-p210 cells were incubated with 20 μmol/L LY294002 (LY) or vehicle (Control) for 48 hours and processed as described in Materials and Methods to separate the nuclear (N) and cytoplasmic (C) fractions. Extracts were separated by SDS-PAGE and probed with the indicated antibodies. Lamin and β-tubulin are markers for the nuclear and cytoplasmic fractions, respectively.

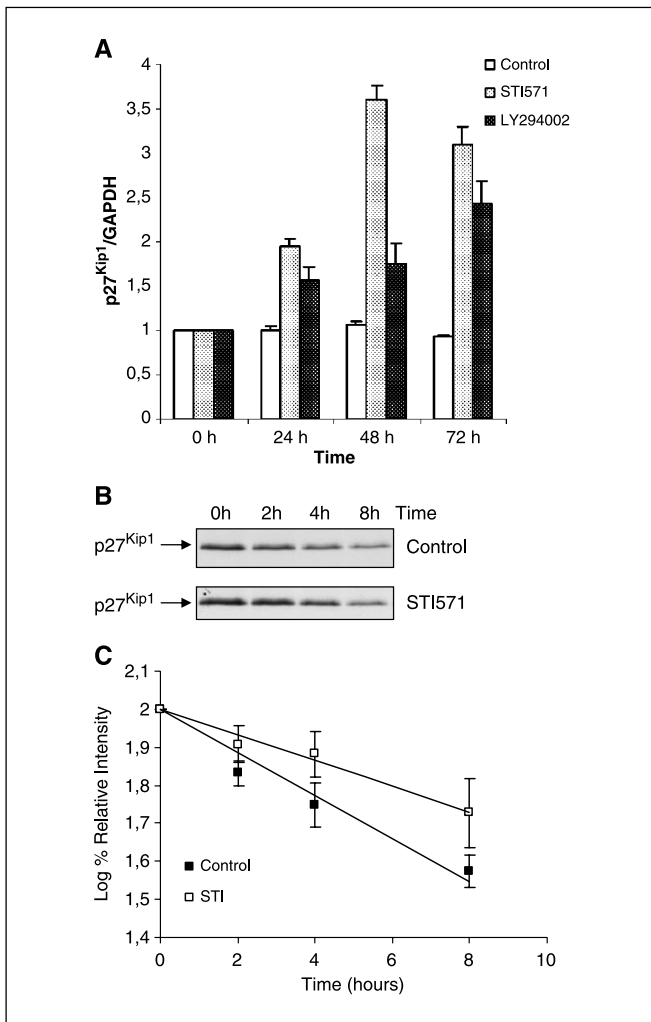


Figure 4. BCR-ABL regulates the transcription and the degradation of p27^{Kip1}. **A**, Mo7e-p210 cells were treated with 2 $\mu\text{mol/L}$ STI571, 20 $\mu\text{mol/L}$ LY294002 or untreated (Control). At the indicated times, total RNA was extracted and the amount of p27^{Kip1} mRNA was analyzed by quantitative reverse transcription-PCR as described in Materials and Methods. Columns, mean; bars, SE of the amount of p27^{Kip1} mRNA relative to that of GAPDH and referred to time 0 of three independent experiments; **B**, BaF/3-p210 cells were incubated in the absence (Control) or presence of 2 $\mu\text{mol/L}$ STI571 for 24 hours and metabolically labeled with [³⁵S]methionine and chased for the times indicated as described in Materials and Methods. Extracts from an equal number of cells were immunoprecipitated with agarose-conjugated anti-p27^{Kip1} antibodies and the radiolabeled immunoprecipitated p27^{Kip1} protein was resolved by SDS-PAGE and visualized by fluorography. Results from one representative experiment are shown; **C**, the intensity of the bands from four independent pulse-chase experiments done as described in (B) was quantified using the QuantityOne software (Bio-Rad) and the logarithmic transformation of the intensity relative to the zero time point \pm SD was plotted against time.

empty retrovirus (vector), or cells infected with a p27^{Kip1} mutant unable to interact with either cyclin or Cdk (p27 ck⁻). However, expression of the undegradable mutant of p27^{Kip1} (p27 P⁻) inhibited proliferation of BCR-ABL cells (Fig. 6). This result confirms that the degradation of p27^{Kip1} is necessary for the induction of cell proliferation by BCR-ABL.

Discussion

The main findings of our study pertain to the mechanism of BCR-ABL-mediated regulation of the cell cycle: by inhibiting the tyrosine kinase activity of the oncogene, we show that proliferation of CML

cells depends on BCR-ABL-mediated proteasome-dependent degradation of p27^{Kip1}, which is associated with increased levels of cyclins D and E and phosphorylation of retinoblastoma. Secondly, degradation of p27^{Kip1} is necessary for cell cycle progression in CML cells as shown by the fact that a nondegradable mutant of p27^{Kip1} blocks cell cycle proliferation on BCR-ABL-positive cells. There is an inverse regulation of the levels of p27^{Kip1} and Skp2 in BCR-ABL cells that is mediated by the PI3K pathway. More importantly, these results are observed not only on BCR-ABL cell lines but also on samples from patients with CML in chronic phase.

Recent studies have suggested a role for p27^{Kip1} in cell cycle regulation in BCR-ABL-positive cells (27–30). Most of these studies have used different models of BCR-ABL cell lines and have consistently suggested that inhibition of the BCR-ABL kinase activity is associated with cell cycle inhibition, increased p27^{Kip1} protein levels and decreased cyclin D expression (27–29). Our results have also extended these observations in both cell lines and primary CML cells demonstrating the mechanism involved in BCR-ABL-mediated regulation of p27^{Kip1} and their causal relation with cell cycle deregulation in CML.

Unlike p27^{Kip1} levels, inhibition of BCR-ABL was associated with a decreased expression of the cell cycle inhibitor p21^{Cip1}. Discordant expression of p27^{Kip1} and p21^{Cip1} has been previously described in other models of proliferating cells in which proliferation depends on the assembly between p21^{Cip1} and cyclin D-Cdk4 (42, 43), and BCR-ABL expression has also been associated with up-regulation of p21^{Cip1} (27).

Jiang et al. (30) have recently shown that levels of p27^{Kip1} are significantly higher in primary CML CD34⁺ cells versus normal CD34⁺ cells. However, p27^{Kip1} is not able to bind and inactivate Cdk2 kinase activity nor affect changes in cyclin E or cyclin D levels due to relocation of the protein to the cytoplasm instead of the nucleus. Differences in the *in vitro* models might explain some of the discrepancies between that work and our results. We compared the levels of p27^{Kip1} in CML cells before, 24, and 48 hours after inhibition of BCR-ABL activity with STI571, instead of normal versus CML cells. Furthermore, Jiang et al.'s studies were planned to assess the effect of β 1-integrin-mediated adhesion on cell cycle in CML cells in comparison with normal CD34⁺ cells, whereas our studies were always done using a nonadherent system. In any case, we have also observed that BCR-ABL induces an abnormal localization of p27^{Kip1} as treatment with STI571 induces relocation of p27^{Kip1} to the nucleus.

The decrease in the p27^{Kip1} protein levels that we see in BCR-ABL cells is also due to a regulation at the transcriptional level. p27^{Kip1} gene transcription is up-regulated by the Forkhead/FoxO transcription factors (25), which in turn are phosphorylated and inactivated by Akt (44). By activating the PI3K/Akt pathway, BCR-ABL is inhibiting transcription of the p27^{Kip1} gene, as we see in our quantitative reverse transcription-PCR experiments using inhibitors of both BCR-ABL and PI3K. This is in agreement with results from Komatsu et al. (45) showing that BCR-ABL controls the expression of p27^{Kip1} through the Akt-dependent phosphorylation of FKHR11/FoxO3a.

Several recent reports indicate that BCR-ABL participates in the regulation of the proteasome by increasing the proteasome-mediated degradation of a number of proteins (46–48). Indirect evidence also indicates that p27^{Kip1} is regulated by proteasome-mediated degradation in BCR-ABL-positive cells (27, 28). Our results show that BCR-ABL-mediated regulation of p27^{Kip1} is indeed dependent on proteasome degradation. The degradation of

p27^{Kip1} is specific and not due to an enhanced activity of the proteasome, because the levels of other cell cycle regulatory proteins (like cyclin E), which are also degraded by the proteasome, are reduced upon inhibition of BCR-ABL. p27^{Kip1} degradation is mediated by the F-box protein Skp2, a component of the E3 ubiquitin ligase SCF complex (49). It has been shown that the levels of Skp2 are up-regulated by PI3K (50). We have shown that BCR-ABL-induced degradation of p27^{Kip1} is associated with increased levels of the Skp2 protein related to activation of the PI3K pathway. This regulation is at the level of transcription, although

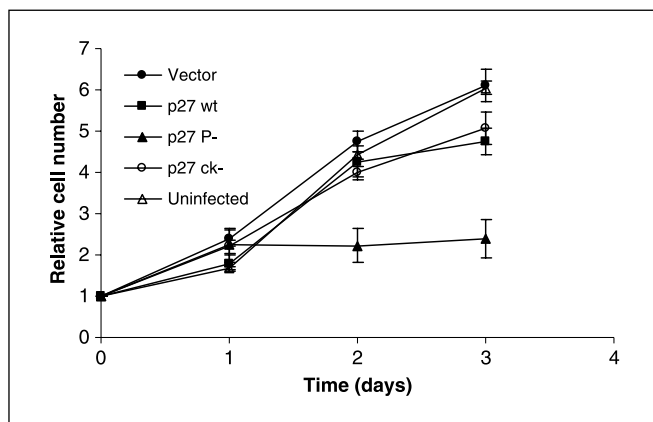
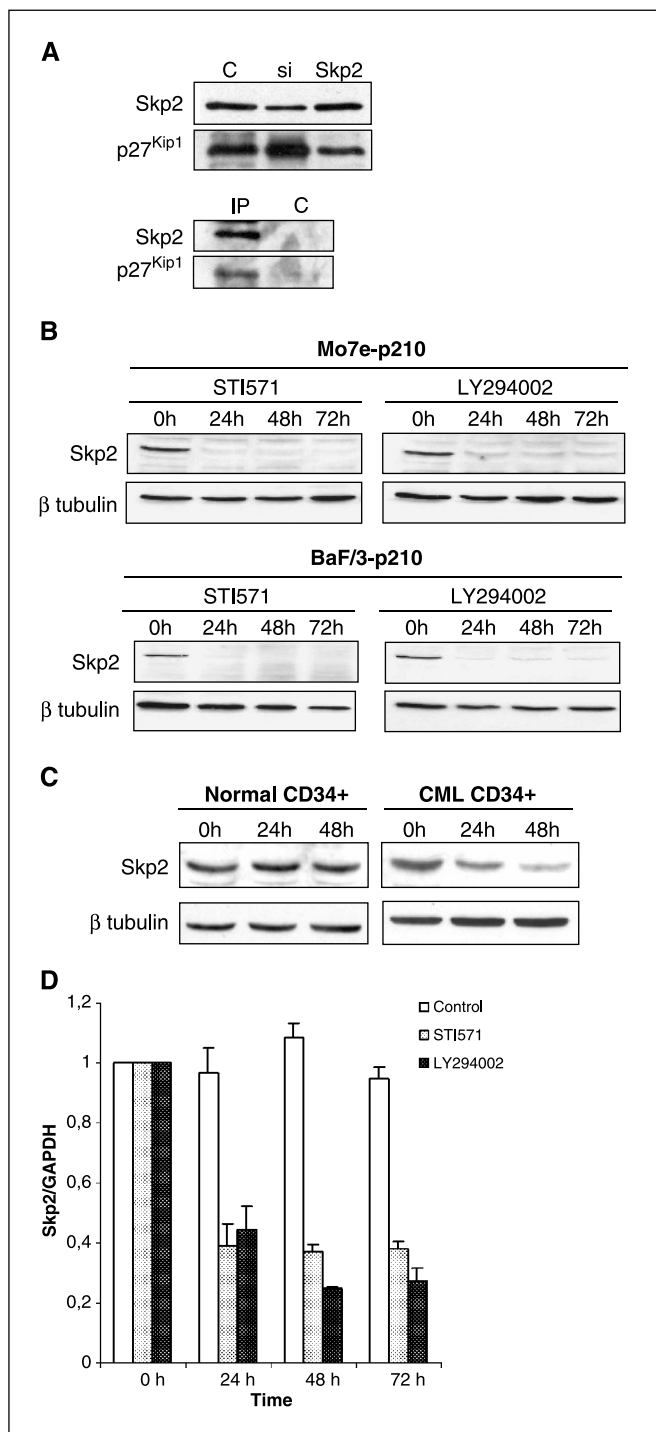


Figure 6. Expression of the T187V p27^{Kip1} mutant but not wild-type p27^{Kip1} blocks proliferation of BCR-ABL cells. BaF/3-p210 cells were infected with the empty LZRS-IRES-green fluorescent protein retrovirus (*Vector*) or derived retroviruses expressing either wild-type p27^{Kip1}, the T187V p27^{Kip1} mutant (*P-*) or an inactive p27^{Kip1} mutant (*ck-*). See text for an explanation of the different mutants. Two days after infection, cells were seeded at the same density (day 0) and infected cells (green fluorescent protein-positive cells) were counted using a fluorescence microscope for 3 more days. In each case, cell numbers were normalized using the number of green fluorescent protein-positive cells at day 0 as a reference. *Points*, mean; *bars*, SE of six independent experiments.

we still do not know which transcription factor may be involved. Based on these results as well as the fact that the expression of a p27^{Kip1} mutant, unable of being phosphorylated in T187 and therefore unable of being recognized by Skp2 and degraded, blocks cell cycle progression, proteasome inhibitors such as PS-341 or other new molecules may be useful drugs for treatment of BCR-ABL harboring diseases (51, 52). Furthermore, it has recently been shown that resistance to STI571 can arise as a consequence of point mutations in BCR-ABL that prevents STI571 from inhibiting the kinase activity of the oncoprotein. Inhibition of the proteasome or even of the PI3K pathway by inhibitors such as LY294002 could be a means to overcome these resistances (53).

Figure 5. Interaction between Skp2 and p27^{Kip1} and regulation of the expression of Skp2 by STI571 or LY294002 treatment. **A**, functional and physical interaction between Skp2 and p27^{Kip1} in BCR-ABL cells. BaF/3-p210 cells were infected with adenovirus producing a small interfering RNA (*si*) to silence the expression of Skp2, infected with adenovirus expressing Skp2 (*Skp2*), or left uninfected (*C*) and the expression of Skp2 and p27^{Kip1} was analyzed by Western blotting (*top*). Protein extracts from BaF/3-p210 cells infected with adenovirus expressing Skp2 were immunoprecipitated with polyclonal antibodies against Skp2 and anti-rabbit immunoglobulin IP beads (*IP*) or with anti-rabbit immunoglobulin IP beads alone (*C*) and the presence of Skp2 and p27^{Kip1} in the immunoprecipitates was analyzed by Western blotting with monoclonal antibodies (*bottom*); **B**, Mo7e-p210 (*top*) and BaF/3-p210 (*bottom*) cells were treated with 2 μmol/L STI571 or 20 μmol/L LY294002. At the indicated times, cells were harvested and lysed as described in Materials and Methods. Cell extracts (50 μg) were separated by SDS-PAGE, electrotransferred and probed with antibodies against Skp2 or β-tubulin as a loading control. A representative example of three independent experiments is shown; **C**, CD34+ cells from normal donors or CML patients were cultured in the presence of 2 μmol/L STI571. At the times indicated, 50 μg of cell lysates were separated by SDS-PAGE, electrotransferred, and incubated with antibodies against Skp2 or β-tubulin. A representative example of three independent experiments is shown; **D**, Mo7e-p210 cells were treated with 2 μmol/L STI571, 20 μmol/L LY294002, or untreated (*Control*). At the indicated times, total RNA was extracted and the amount of *Skp2* mRNA was analyzed by quantitative reverse transcription-PCR as described in Materials and Methods. *Columns*, mean; *bars*, SE of *Skp2* mRNA relative to that of *GAPDH* and referred to time 0 of three independent experiments.

Therefore, p27^{Kip1} is a major target of BCR-ABL to promote proliferation. There are three ways in which the levels and activity of p27^{Kip1} are regulated in CML cells: first, BCR-ABL induces a relocalization of p27^{Kip1} to the cytoplasm where it is inactive; second, BCR-ABL inhibits p27^{Kip1} gene transcription by affecting, presumably, the activity of the transcription factors of the Forkhead/FoxO family; and third, BCR-ABL induces p27^{Kip1} degradation by increasing the levels of Skp2 protein. All these effects are mediated by the PI3K pathway. Recently, it has been shown that this pathway regulates the localization of p27^{Kip1} and its transcription in HL60 leukemia cells (54), but ours is the first report in which we are able to show that BCR-ABL is affecting all three mechanisms and not only in cell lines but also in primary cells from CML patients.

In conclusion, our results provide new insights into the mechanism of BCR-ABL-mediated regulation of the cell cycle and pave the way for the use of new drugs such as proteasome

inhibitors in the treatment of patients with CML. Combinations of currently effective therapies such as STI571 with new compounds such as PS-341 may further improve the expectations of patients and indicate new alternatives in patients that have developed resistance to STI571.

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BCR-ABL Induces the Expression of Skp2 through the PI3K Pathway to Promote p27^{Kip1} Degradation and Proliferation of Chronic Myelogenous Leukemia Cells

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