

Direct Relation between BCR-ABL Tyrosine Kinase Activity and Cyclin D2 Expression in Lymphoblasts¹

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ABSTRACT

Leukemia cells bearing the Philadelphia (Ph) chromosome express a Bcr-Abl fusion protein with deregulated protein tyrosine kinase (PTK) activity, which plays a central role in the malignant transformation. Many different signal transduction pathways are activated by Bcr-Abl, but little is known about their downstream targets in specific cell lineages. We show here that Ph-positive cell lines as well as primary cells derived from chronic myeloid leukemia (CML) in lymphoid blast crisis or from acute lymphoblastic leukemia (ALL) consistently express high levels of cyclin D2, whereas expression of this protein is low or absent in comparable Ph-negative lines and Ph-positive myeloid lines. Inhibition of Bcr-Abl with STI571 resulted in down-regulation of cyclin D2 and reduction of the number of cells in S phase, although complete G₁ arrest was not induced. The expression of cyclin D2 in Ph-positive lymphoblasts was mediated via the phosphatidylinositol-3 kinase pathway. Analogous results were seen in murine BaF/3 cells transfected with a *BCR-ABL* expression vector. In contrast to the human cell lines, murine BaF/*BCR-ABL* cells exposed to STI571 inhibitor were all arrested in G₁. This arrest could be abrogated by exogenous expression of cyclin D2 from a transfected cDNA construct. We conclude that a direct connection exists between Bcr-Abl PTK activity and cell cycle progression in which cyclin D2 plays a critical role. However, cell cycle progression in human Ph-positive lymphoid cells is not entirely dependent on Bcr-Abl PTK, and additional genetic lesions must be present.

INTRODUCTION

Nearly all cases of CML³ (1) and about one-third of cases of ALL in adults (2) are characterized by the t(9;22)(q34;q11) reciprocal translocation. As a result, a *BCR-ABL* fusion gene is formed on the derivative 22q-, or Ph chromosome that gives rise to a chimeric protein with deregulated PTK activity compared with normal Abl. The Bcr-Abl protein causes extensive tyrosine phosphorylation of many cellular proteins and activation of multiple signaling pathways that eventually lead to cellular transformation (3, 4).

Basic mechanisms thought to be relevant in the pathogenesis of Ph-positive leukemias are activation of mitogenic signaling (5), inhibition of apoptosis (6), and altered adhesion to stroma (7). However,

it is not clear which downstream effectors of Bcr-Abl are functional in eliciting the respective cellular responses.

Mitogenic signals from different types of growth factor receptors eventually converge in the early G₁ phase of the cell cycle before the restriction point (8, 9). If the balance between D cyclins, cdk, and cdk inhibitors like p16^{INK4A} and p27^{KIP1} is shifted toward cdk activity, the Rb is phosphorylated. Transcription factors like E2F that are tethered to hypophosphorylated Rb are released, S phase-specific genes activated, and the restriction point passed (10). Many human cancer cells carry lesions that perturb this system (11). Homozygous deletions of p16 have been demonstrated in various human cancers including leukemia/lymphoma (12, 13) and are thought to promote cell proliferation. Cyclin D1 is overexpressed in a variety of human malignancies and through a variety of mechanisms. In mantle cell lymphoma, the cyclin D1 locus comes under the control of the IgH enhancer as a consequence of the t(11;14) translocation (14). Amplification of the cyclin D1 gene is observed in breast and squamous cell carcinoma (15), whereas the loss of transcriptional repression appears to be critical in large bowel tumors (16). By contrast, much less is known about the role of other D cyclins in oncogenesis. Increased expression of cyclin D2 is a consistent feature of male germ cell tumors (17) as well as chronic lymphocytic leukemia (18) and identifies poor-risk gastric cancer (19), but the mechanisms underlying the overexpression are not known. Cyclin D3 has until now not been linked to malignancy.

Cooperation between cyclin D1 and activated Abl (*v-abl* or Bcr-Abl) in the transformation of fibroblasts and murine B-lymphocytes has been reported (20). Although these data clearly show that *BCR-ABL* and cyclin D1 are able to synergize on transfection into the same cell, it is not clear how this relates to cells in which the physiological regulation of the two genes is intact.

Recently, a chemical agent STI571 (formerly CGP57418B) has become available that acts as a specific inhibitor of Bcr-Abl PTK (21, 22). Thus, the consequences of inhibition of *BCR-ABL* tyrosine kinase can be studied in cells that carry an endogenous *BCR-ABL* gene. Using differential display to study alterations of gene expression in BV173, a Ph-positive B-lymphoid cell line, we found that cyclin D2 was strongly down-regulated on exposure of these cells to STI571. Identical or similar results were obtained in other human cells lines as well as in murine cells transfected with *BCR-ABL*. We provide evidence that the PI-3 kinase pathway is involved in regulating cyclin D2 expression, and that cyclin D2 is required for cell cycle progression. Our data demonstrate a connection between Bcr-Abl and cyclin D2 and strongly suggest a role for cyclin D2 in the pathogenesis of Ph-positive lymphoblastic leukemias.

MATERIALS AND METHODS

Cell Culture. All of the cell lines were grown in RPMI 1640 (Life Technologies, Inc., Paisley, United Kingdom) supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin. In the case of BaF/3 cells, 10% WEHI-conditioned medium was added to the culture as a source of

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³ The abbreviations used are: CML, chronic myeloid leukemia; ALL, acute lymphoblastic leukemia; Ph, Philadelphia; PTK, protein tyrosine kinase; cdk, cyclin-dependent kinase; Rb, retinoblastoma protein; PI-3, phosphatidylinositol-3; AML, acute myeloblastic leukemia; PI, propidium iodide; MNC, mononuclear cell; CP, chronic phase; IL, interleukin; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAP, mitogen-activated protein; MEK, MAP kinase; PCNA, proliferating cell nuclear antigen.

murine IL-3. All of the experiments were performed on exponentially growing cells kept at low cell concentrations ($<1 \times 10^6$ /ml) unless indicated otherwise. STI571 (kindly provided by Dr Elisabeth Buchdunger, NOVARTIS, Basel, Switzerland) was added to the media at the appropriate concentration from a 10-mmol/liter stock solution in distilled water. LY294002, a PI-3 kinase inhibitor, and U0126, a dual MEK1 and MEK2 inhibitor (23), were purchased from Calbiochem (Nottingham, United Kingdom) and Promega (Southampton, United Kingdom), respectively, and added to the cultures at a final 25- μ mol/liter concentration. The cell lines used in this study were purchased from cell repository banks (American Tissue Culture Collection; European Collection of Cell Cultures, Winchester, United Kingdom; or German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), or were kindly donated by the originators.

Primary Cells. MNCs from the bone marrow or peripheral blood of patients with CML in various stages or with Ph-positive ALL or AML were isolated by density gradient centrifugation over Lymphoprep (Nycomed, Oslo, Norway). Informed consent was obtained in all cases.

Transfection of BaF/3 Cells. BaF/3 cells were transfected by standard electroporation (250 V and 960 μ F) with a P210^{BCR-ABL} expression vector (Ref. 24; kindly provided by Dr. George Daley, Whitehead Institute, Cambridge, MA), a temperature-sensitive (ts) P210^{BCR-ABL} (Ref. 25; kindly provided by Dr Leanne Wiedemann, Institute for Cancer Research, London, United Kingdom), a pBabe-Puro vector expressing murine cyclin D2 (26) constructed by inserting the full-length mouse cyclin D2 cDNA into the *Bam*HI site of the pBabe-Puro vector (27), and the respective "empty" vectors.

Flow Cytometric Analyses. For cell cycle analysis, $1-2 \times 10^6$ cells were stained with PI. Briefly, the cells were washed twice in cold PBS and fixed in ice-cold 50% ethanol for 30 min. After two more washes in PBS, PI (Sigma Chemical Co.), and RNaseA (Sigma Chemical Co.) were added to a final concentration of 100 μ g/ml each. After 1-h incubation at room temperature, the cells were kept at 4°C until analyzed by flow cytometry (FACScan, Becton Dickinson) using the CELLQUEST software.

Assessment of apoptosis was carried out by annexin V staining as recommended by the manufacturer (R&D Systems Europe Ltd, Oxon, United Kingdom). Briefly, centrifuged cells were resuspended in binding buffer [100 mmol/liter HEPES (pH 7.4), 1.5 mol/liter NaCl, 50 mmol/liter KCl, 10 mmol/liter MgCl₂, and 18 mmol/liter CaCl₂] and incubated with 0.5 μ g/ml fluorescein-conjugated annexin V and 20 μ g/ml PI for 30 min at room temperature prior to fluorescence-activated cell sorting (FACS) analysis.

Northern Blot Analysis. RNA was extracted by the acid guanidinium thiocyanate method (28). Fifteen μ g/lane were resolved on a 0.8% agarose gel and transferred to nylon membranes (HYBOND^N, Amersham, Little Chalfont, United Kingdom). The cyclin D2 probe was the *NotI-HindIII* cDNA insert from the I.M.A.G.E. clone no. 48206 (GenBank accession no. H11125; provided by the Medical Research Council Human Genome Mapping Project Biological Resources, Hinxton, Cambridge, United Kingdom). A human β -actin cDNA probe (kindly provided by Dr. Philip Mason, Imperial College School of Medicine, London, United Kingdom) was used as control for RNA loading. The filters were prehybridized at 42°C for 3 h in 50% formamide, 1 M NaCl, 10% dextran sulfate (Pharmacia, Uppsala, Sweden), 1% SDS and 0.2 mg/ml carrier DNA. Probes were labeled with the Megaprime system (Amersham) and allowed to hybridize overnight. Filters were washed at 42°C (15 min in $3 \times$ SSC + 1% SDS; twice for 30 min in $2 \times$ SSC + 1% SDS) and at 55°C (15 min in $0.2 \times$ SSC + 1% SDS) and exposed to autoradiography film for 12–48 h. Densitometric analysis of the autoradiographs for semiquantification of fold-regulation was performed using the GelBlotPro software (UV Products, Cambridge, United Kingdom).

Western Blot Analysis. For immunoblotting, protein lysates were prepared according to Kabarowski *et al.* (25). Protein concentrations were determined with the Bradford method (D_c Protein Assay; BIO-RAD, Hercules, CA), and 50–100 μ g were resolved on SDS-PAGE gels. After transfer to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA) on a semi-dry electrophoretic transfer system, the proteins were detected with the respective antibody and visualized by enhanced chemiluminescence (Amersham). Antibodies were from Santa Cruz Biotechnology [cyclin D2 (sc-593), cyclin D3 (sc-182), cdk4 (sc-601), cdk6 (sc-177), pRb (sc-4112), phosphotyrosine (sc-7020), p27 (sc-528)] or from Calbiochem [Abl (OP20)]. The cyclin D2 monoclonal antibodies DCS 3.1 and DCS 5.2 (29) were a gift from Jiri Bartek, Danish Cancer Society, Copenhagen. To control for protein loading, the blots

were stripped and restained with an actin (A-2066; Sigma Chemical Co., Poole, United Kingdom) or a PCNA (PC10; Santa Cruz Biotechnology) antibody.

Immune Complex Kinase Assay for Cyclin D2-associated Kinase Activity. Protein lysates were prepared as described by Sweeney *et al.* (30). The samples were precleared with 20 μ l of protein A beads (Santa Cruz Biotechnology) and 2.5 μ l of normal mouse IgG (Santa Cruz Biotechnology) for 30 min at 4°C under constant agitation. Cyclin D2 was immunoprecipitated from 500 μ g of protein with 5 μ l of DCS 5.2 and with 7.5 μ l of control antibody (mouse IgG2b, Life Technologies, Inc.), respectively. The immunocomplex kinase assay was performed with GST-RB (Santa Cruz Biotechnology) as the substrate.

RT-PCR. RNA was isolated as for Northern blot analysis. Reverse transcription and PCR amplification of *BCR-ABL* and *ABL* as a control gene were done as described previously (31, 32). Cyclin D2 transcripts were measured by TaqMan PCR using a ready-to-use PCR assay (Roboscreen Gesellschaft für molekulare Biotechnologie mbH, Leipzig, Germany), according to previously published protocols (33). Primers corresponded to nucleotides (nt) 786–806 (on exon 3) and nt 847–866 (on exon 4), and the TaqMan probe to nt 816–840 (exon 3) of the published cyclin D2 sequence (GenBank accession no. D13639). The amplification was performed in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany) for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 59°C. Duplicate aliquots from the same cDNA were tested, and the values normalized for GAPDH expression.

Statistics. The difference in cyclin D2 expression between different types of primary cells was compared by the Mann-Whitney *U* test using the Statistical Package for the Social Sciences software.

RESULTS

Down-Regulation of Cyclin D2 on Inhibition of BCR-ABL PTK in Ph-positive Lymphoid Cells. We previously used differential display to compare the mRNA expression of BV173 cells treated with STI571 with that of untreated control cells (34). The band corresponding to cyclin D2 was found to be strongly down-regulated in BV173 cells incubated with 1 μ M STI571 for 24 h. On the basis of this result, we decided to examine a panel of both lymphoid and myeloid cell lines (Fig. 1). The Ph-status of the lines was confirmed by RT-PCR prior to the experiments (not shown). Northern blot analysis showed that moderate-to-high levels of cyclin D2 were detectable in all of the Ph-positive lymphoid lines (Table 1). Ph-negative lymphoid lines expressed cyclin D2 only if they were EBV-transformed. With the exception of KG1, cyclin D2 levels in myeloid lines were low or undetectable, regardless of their Ph status. More importantly, on inhibition of Bcr-Abl PTK with STI571, cyclin D2 was down-regulated in all of the Ph-positive lymphoid lines except SD1. This down-regulation was almost complete in some lines, whereas there was residual message in others, possibly reflecting higher starting levels; the discordant behavior of SD1 cells is probably attributable to their EBV transformation. In contrast to cyclin D2, there was no relation between the expression of cyclin D3 and the phenotype and

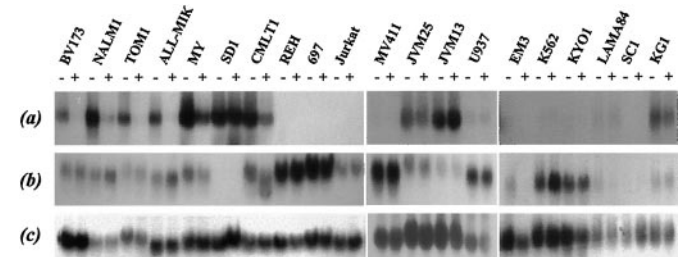


Fig. 1. Northern blot analysis of (a) cyclin D2, (b) cyclin D3, and (c) β -actin (loading control) expression in cell lines exposed (+) and not exposed (-) to 1 μ mol/liter STI571 for 24 h

Table 1 Phenotype, BCR-ABL status, and cyclin D2 expression in the cell lines used in this study

Cell line	Phenotype	p ^{BCR-ABL}	Cyclin D2 ^a
BV173	Pre-B cell (CML)	210	++
NALM1	Pre-B cell (CML)	210	+++
TOM1	Pre-B cell (ALL)	190	++
ALL/MIK	Pre-B cell (ALL)	190	++
MY	Biphenotypic (myeloid/B-lymphoid)	180	+++
SD1	B-lymphoblastoid (ALL) ^b	190	+++
CML-T1	T cell (CML)	210	+++
697	Pre-B cell (ALL)	-	-
REH	Pre-B cell (ALL)	-	-
MV411	Monocytic (AML)	-	+
Jurkat	T cell (ALL)	-	++
JVM13	B-lymphoblastoid (PLL) ^{b,c}	-	+++
JVM25	B-lymphoblastoid (normal) ^b	-	++
U937	Histiocytic (lymphoma)	-	-
KYO1	Myeloid (CML)	210	-
K562	Myeloid (CML)	210	-
EM3	Myeloid (CML)	210	-
LAMA84	Myeloid (CML)	210	+
KG1	Myeloid (AML)	-	++
SC-1	B cell (lymphoma)	-	-

^a Signal intensity on Northern blot: -, not detectable; +, weak; ++, moderate; +++, strong.

^b EBV-positive cell lines.

^c PLL, prolymphocytic leukemia.

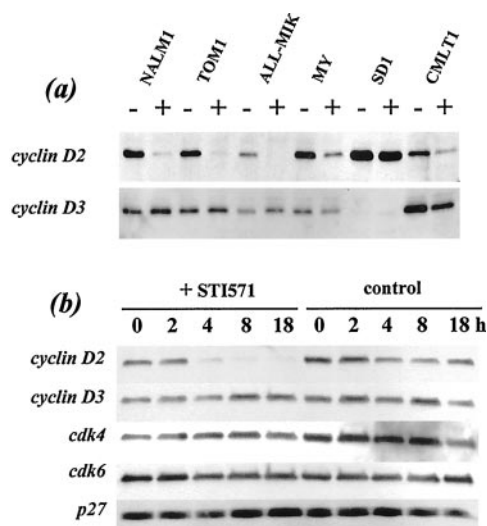


Fig. 2. Western blot analysis. *a*, expression of cyclin D2 and D3 in Ph-positive lymphoblastic cell lines treated (+) and not treated (-) with 1 $\mu\text{mol/liter}$ STI571 for 24 h. *b*, expression of cell-cycle related genes in BV173 cells treated with 1 $\mu\text{mol/liter}$ STI571 for the indicated times.

Ph status of the cell lines. The consistent results in the Ph-positive lymphoid lines prompted us to investigate them in more detail.

The Effect of STI571 Is Specific for Cyclin D2. Down-regulation of cyclin D2 in the Ph-positive lymphoid lines was confirmed at the protein level (Fig. 2*a*). Time course experiments in BV173 cells showed that the down-regulation of cyclin D2 protein occurred within 4 h (Fig. 2*b*). In contrast to cyclin D2, the levels of cyclin D3, cdk4/6, and p27 remained constant (Fig. 2*b*). Taken together, these results indicated that the effect of STI571 was specific for cyclin D2.

BCR-ABL Tyrosine Kinase Activity Is Required for Cyclin D2 Expression. We then asked whether cyclin D2 expression directly correlated with Bcr-Abl tyrosine kinase activity. Because BCR-ABL is known to autophosphorylate on tyrosine (35), its tyrosine kinase activity can be quantified by measuring the amount of phosphorylated Bcr-Abl protein in total cellular lysates. BV173 cells were incubated with graded concentrations of STI571 for 90 min, phosphotyrosine

blots were prepared, and the Bcr-Abl phosphorylation was quantified by densitometry (Fig. 3, *a* and *b*). Bcr-Abl autophosphorylation decreased in a dose-dependent manner, with an IC₅₀ of $\sim 0.25 \mu\text{mol/liter}$, in line with previously published data (21). The phosphorylation of several other proteins dropped sharply between 0.1 and 0.5 $\mu\text{mol/liter}$ STI571, in parallel to the decrease of cyclin D2 mRNA and protein (Fig. 3*c*). More detailed dose-response analysis showed that 0.4 $\mu\text{mol/liter}$ STI571 was required to induce maximal down-regulation of cyclin D2 (not shown). The IC₅₀s for Bcr-Abl tyrosine phosphorylation in the other Ph-positive lymphoid cell lines were also determined. They ranged between 0.25 and 1 $\mu\text{mol/liter}$ STI571 for all cell lines except CMLT1, for which no significant inhibition was seen (Table 2). Altogether these results show that Bcr-Abl PTK activity is required to maintain cyclin D2 expression in these cells, presumably via the phosphorylation of one or more intermediates.

STI571 Reduces the Proportion of Cells in S Phase. We next examined the effect of STI571 on the cell cycle kinetics of BV173 cells. Incubation of unsynchronized BV173 cells with graded concentrations of STI571 resulted in a dose-dependent inhibition of proliferation (Fig. 4*a*). At a concentration of 1 $\mu\text{mol/liter}$ STI571, cells in S phase decreased from 17.9 ± 2 to $8.8 \pm 0.8\%$ after 24 h and from 25.6 ± 0.6 to $11.7 \pm 2.6\%$ after 48 h (Fig. 4*b*). There was a slight

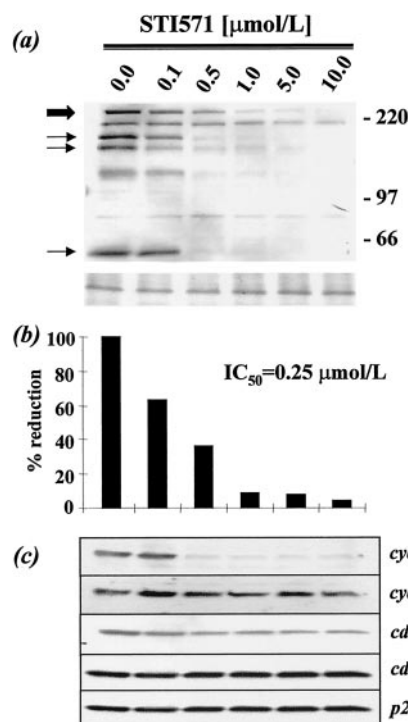


Fig. 3. BV173 cells treated with graded concentrations of STI571. *a*, protein tyrosine phosphorylation in total cellular lysates (90-min exposure to STI571). *Thick arrow*, P210^{BCR-ABL}; *thin arrows*, prominent phosphoprotein bands; *bottom*, a Coomassie Blue staining to control for equal protein loading. *b*, densitometric analysis of P210^{BCR-ABL}. *c*, Western blot analysis of cell-cycle related genes.

Table 2 IC₅₀s for inhibition of BCR-ABL phosphorylation by STI571

Cell line	IC ₅₀ ($\mu\text{mol/liter}$ STI571)
BV173	0.25
NALM1	0.5
TOM1	0.5
ALL/MIK	0.3
SD1	0.5
CMLT1	>10
Baf/BCR-ABL	1.0

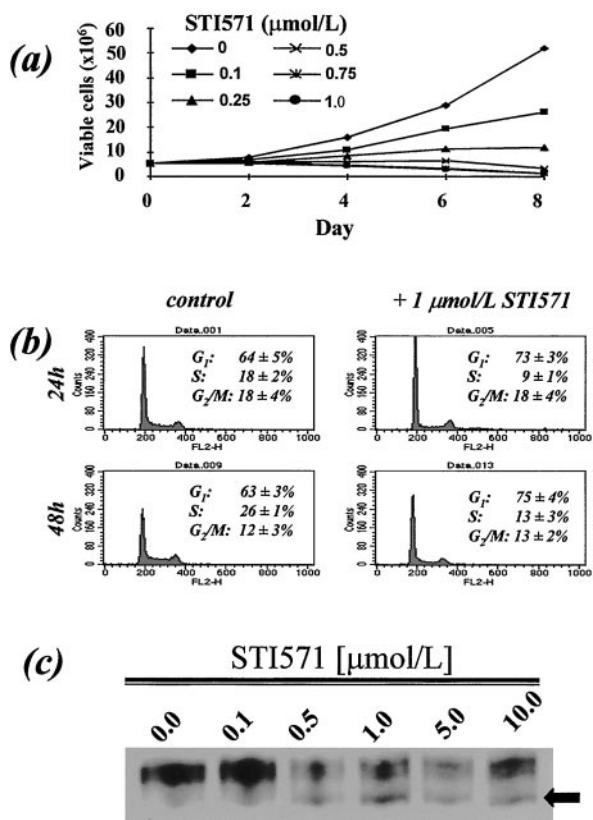


Fig. 4. *a*, proliferation of BV173 cells grown in graded concentrations of STI571. *b*, cell cycle analysis of BV173 cells treated with 1 $\mu\text{mol/liter}$ STI571 for 24 and 48 h. *c*, Western blot analysis of RB protein. Arrow, the faster migrating hypophosphorylated form. *d*, cyclin D2-associated kinase activity in BV173 cells cultured with and without 1 μM STI571.

accumulation of cells in G₁ (from 64.1 ± 4.5 to $72.8 \pm 3.3\%$ after 24 h, and from 62.7 ± 2.5 to $74.6 \pm 3.7\%$ after 48 h), but no G₁ arrest could be induced, even at concentrations up to 10 $\mu\text{mol/liter}$ STI571 (not shown). In line with the cell cycle analysis, Rb phosphorylation was slightly reduced in BV173 cells treated with STI571 (Fig. 4c), as was cyclin D2-associated kinase activity (Fig. 4d). Altogether, these data indicate that down-regulation of cyclin D2 delays entry into S phase but is not sufficient to abrogate the G₁-S transition completely.

We then asked whether STI571 might affect the progression of BV173 cells synchronized in G₀-G₁. None of the human cell lines could be arrested by serum starvation, but BV173 cells grown to high cell densities ($>3 \times 10^6/\text{ml}$) clearly accumulated in G₀-G₁. Twenty-four to 36 h after replating at $0.5 \times 10^6/\text{ml}$, an increase in the S phase fraction of cells became apparent, which was abrogated by 1.0 $\mu\text{mol/liter}$ STI571 (Fig. 5a). Cyclin D2 levels dropped in the presence of STI571. In contrast to exponentially growing cells, there was an accumulation of p27 and cyclin D3 in the treated cells (Fig. 5b), which may be relevant to the almost complete G₁-G₀ arrest seen in this situation. Taken together, these results provide evidence for a link between Bcr-Abl, cyclin D2, and cell cycle progression.

BaF/3 Cells Transfected with BCR-ABL Arrest in G₁ on Exposure to STI571. To substantiate and extend these findings, murine pre-B cells (BaF/3) cells were transfected with a P210^{BCR-ABL} expression vector (24) and exposed to graded concentration of STI571. Phosphotyrosine blots indicated that higher concentrations of STI571 were required to inhibit the phosphorylation of P210^{BCR-ABL} and other proteins; therefore, 10 μmol of the compound/liter were chosen for these experiments. BaF/BCR-ABL cells incubated with 10 $\mu\text{mol/liter}$ STI571 showed a decrease of cyclin D2 levels (Fig. 6a). However, the

effect was less rapid than in BV173 cells. The expression of cyclin D3 also decreased markedly after 4 h but returned to baseline levels after 16 h. Levels of cdk4 and cdk6 remained practically constant, whereas the expression of p27 increased strongly. Cell cycle analysis (Fig. 6b) showed a complete G₁ arrest at 16 h, the time when cyclin D2 levels were minimal, cyclin D3 levels back to baseline, and p27 levels maximal. At this time, Rb was mostly dephosphorylated (Fig. 6c), and a slight decrease of cyclin D2-associated kinase activity was demonstrable. After 24 h, the cells had undergone apoptosis (not shown). All effects of STI571 were abrogated by murine IL-3 (WEHI-conditioned medium). To substantiate the specificity of the observations further, BaF/3 cells were transfected with a temperature-sensitive mutant of P210^{BCR-ABL} (Baf/tsBCR-ABL; Ref. 25). The functionality of the temperature shift in terms of kinase activity was assessed on phosphotyrosine blots (Fig. 7a). Shift to the nonpermissive temperature (39°C) or incubation with STI571 resulted in strong down-regulation of cyclin D2, which was abrogated by murine IL-3 in both cases (Fig. 7b). Altogether, these data show that, by analogy to the human lymphoid cell lines, down-regulation of cyclin D2 in BCR-ABL-transfected BaF/3 cells is a consequence of reduced Bcr-Abl tyrosine kinase activity and is not attributable to nonspecific effects of STI571.

Cyclin D2 Overexpression Abolishes Cell Cycle Arrest by STI571 in BCR-ABL-positive Cells. To examine the significance of cyclin D2 down-regulation in the STI571-treated BCR-ABL-express-

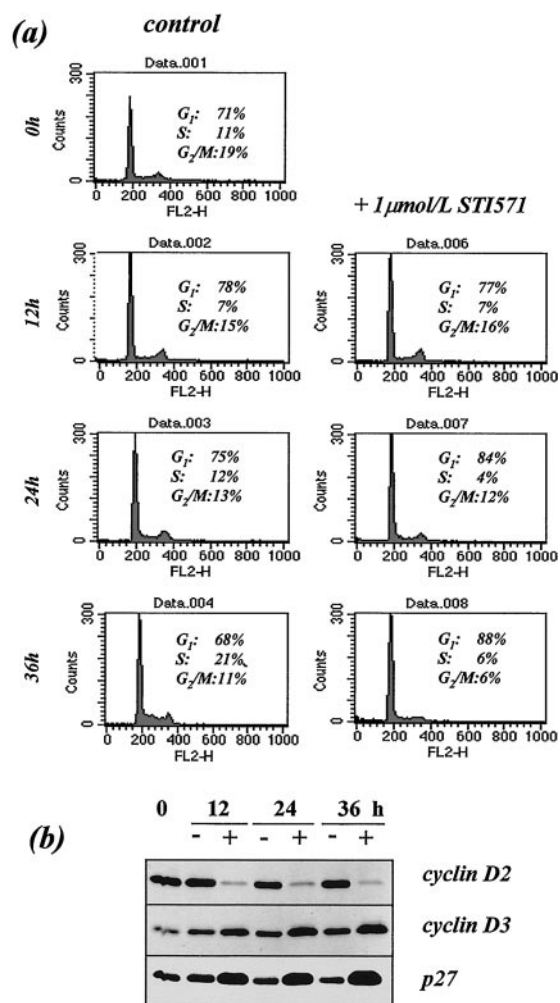


Fig. 5. *a*, cell cycle of BV173 cells released into cycle by replating after density arrest; *b*, Western blot analysis of BV173 cells released into cycle by replating after density arrest.

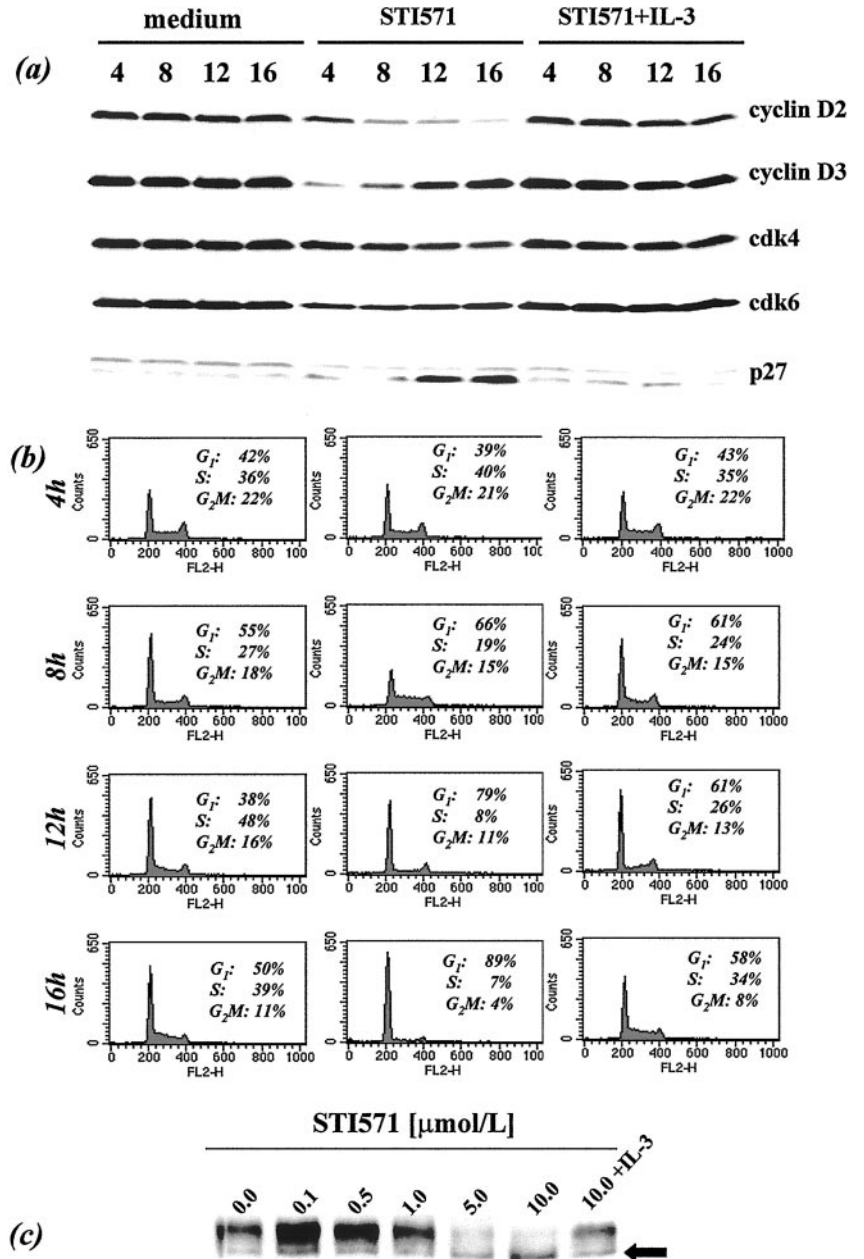


Fig. 6. *a*, Western blot analysis of cell cycle-related proteins in Baf/BCR-ABL cells treated with 10 $\mu\text{mol/liter}$ STI571. *b*, cell cycle analysis. *c*, Western blot analysis of RB protein. Arrow, the faster migrating hypophosphorylated form.

ing hematopoietic cells, we studied the effects of ectopic expression of cyclin D2 on Baf/BCR-ABL cells in response to STI571. To this end, we infected Baf/BCR-ABL with an expression vector encoding cyclin D2 or with an empty vector as control. The transfected cells were selected with puromycin for 2 weeks, and the resistant cells were used for cell cycle analysis. Similar attempts at transfecting BV173 cells were not successful because the transduction efficiency was low, and no stable transfectants could be established.

To confirm stable expression of cyclin D2 in the transfected cells, we performed Western blot analysis and confirmed that the level of cyclin D2 expression was higher in the Baf/BCR-ABL/cyclin D2 cells than in the control parental line. After 24 h of STI571 treatment, the expression of cyclin D2 was significantly down-regulated in the control, whereas cyclin D2 expression was maintained at a high level in the Baf/BCR-ABL/cyclin D2 cells (Fig. 8*a*).

Cell cycle analysis by PI staining revealed that the majority of the control Baf/BCR-ABL cells arrested at the G_1 phase of the cell cycle after 24 h of STI571 treatment before undergoing apoptosis (as

revealed by their $<G_1$ DNA content), whereas most of the Baf/BCR-ABL cells that overexpressed cyclin D2 proliferated normally in the presence of STI571 (Fig. 8*b*). In addition, overexpression of cyclin D2 also partially abrogated apoptosis induced by STI571 in these cells. To verify this phenomenon further, we performed annexin V staining on the Baf/BCR-ABL/cyclin D2 and the control cells after STI571 treatment (Fig. 9). The result confirmed the findings obtained with PI staining, which indicated that ectopic expression of cyclin D2 prevents, at least partially, the cell cycle arrest and apoptosis induced by STI571 in Baf/BCR-ABL cells. It is notable that the level of apoptotic cells detected by annexin V staining is higher than that revealed by PI staining. This discrepancy probably reflects the fact that annexin V is a more sensitive and earlier marker for apoptotic cells than for sub- G_1 DNA content.

Bcr-Abl Regulates Cyclin D2 Expression through the PI-3 Kinase Pathway. Having established that cyclin D2 is both a consistent and relevant downstream effector of Bcr-Abl, we asked which pathway might be functional in transducing the signal between Bcr-Abl

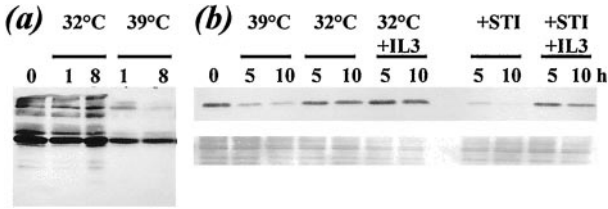


Figure 7. *Baf/tsBCR-ABL* cells. *a*, protein tyrosine phosphorylation on shift from the permissive (32°C) to the restrictive temperature (39°C). *b*, Western blot analysis of cyclin D2 on temperature shift or exposure to STI571 in the presence or absence of murine IL-3.

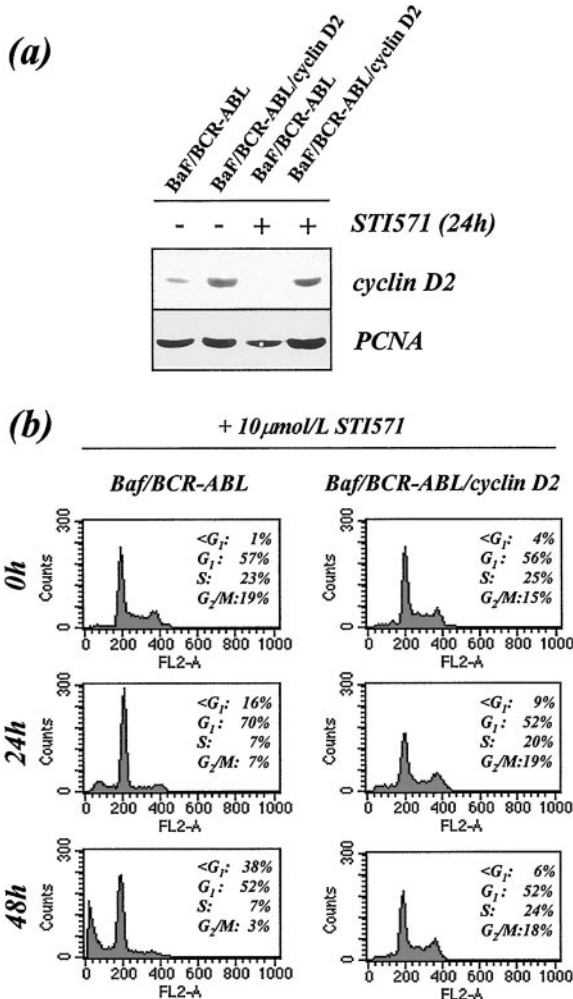


Figure 8. *a*, Western blot analysis of cyclin D2 and PCNA (loading control) expression in both *Baf/BCR-ABL/cyclin D2* and control cells in the presence or absence of 10 $\mu\text{mol/liter}$ STI571 treatment. *b*, cell cycle analysis of *Baf/BCR-ABL* cells stably expressing murine cyclin D2 (*Baf/BCR-ABL/cyclin D2*) treated with 10 $\mu\text{mol/liter}$ of STI571. *Baf/BCR-ABL*, harboring the empty expression vector, was also included as control.

PTK activity and cyclin D2. Both the MAP and PI-3 kinase pathways are known to be active in *BCR-ABL*-transformed cells (5, 36), and can be interrupted by specific chemical inhibitors. BV173 cells, grown in the presence of the PI-3 kinase blocker LY294002, showed a strong down-regulation of cyclin D2 mRNA (8-fold peak at 6 h) and protein (5-fold peak at 10 h; Fig. 10), with a time course very similar to the effects of STI571 (Fig. 2). In contrast, blocking the MAP kinase cascade at the level of MEK with U0126 had no significant effect on cyclin D2 expression (Fig. 10). Thus, the signal from Bcr-Abl to cyclin D2 is transmitted via the PI-3 kinase pathway.

Cyclin D2 Is Consistently Overexpressed in BCR-ABL-positive Primary Lymphoblasts. To investigate whether the results obtained in the cell lines could be confirmed in primary human cells, a quantitative RT-PCR assay for measurement of cyclin D2 transcripts was devised. Samples from patients with CML in CP ($n = 12$), myeloid blast crisis ($n = 10$), lymphoid blast crisis or Ph-positive ALL ($n = 6$), and Ph-negative AML ($n = 12$) were analyzed. Cyclin D2 levels were significantly higher in lymphoid blast crisis and Ph-positive ALL than in CP CML ($P < 0.001$), myeloid blast crisis ($P = 0.011$), or AML ($P = 0.001$; Fig. 11*a*). Furthermore, cells from a patient with relapsed Ph-positive ALL were exposed to graded concentrations of STI571 for 6 h. Cyclin D2 levels were dose-dependently reduced in the presence of STI571 (Fig. 11*b*). These results show that the relation between Bcr-Abl PTK activity and cyclin D2 expression is observed in primary leukemia cells and is not restricted to immortalized cell lines.

DISCUSSION

Our results demonstrate that the Bcr-Abl tyrosine kinase activity correlates with expression of cyclin D2 in Ph-positive lymphoblasts and strongly implicate cyclin D2 in the pathogenesis of lymphoid blast crisis of CML as well as of Ph-positive ALL. Several lines of evidence support this notion: (a) moderate to high levels of cyclin D2 are consistently expressed in all of the Ph-positive lymphoblastic cell lines tested but not in the Ph-negative lines of similar phenotype; (b) cyclin D2 is consistently and specifically down-regulated in Ph-positive lymphoblastic lines treated with STI571; (c) analogous results are seen in *BCR-ABL*-transfected *Baf/3* cells; (d) cell cycle arrest on inhibition of Bcr-Abl PTK can be rescued by ectopic overexpression of cyclin D2; and (e) the phenomena are also detected in leukocytes from patients with *BCR-ABL*-positive leukemias.

Down-regulation of cyclin D2 in exponentially growing BV173 cells is associated with a 40–50% reduction of cells in S phase. Effects of STI571 on *Baf/BCR-ABL* cells are more pronounced, and a complete G_1 arrest is induced. Current thinking suggests that cyclin D2-associated kinase activity promotes cell cycle progression via the phosphorylation and inactivation of the Rb protein (29). Indeed, levels of hypophosphorylated Rb protein increased slightly in BV173 and

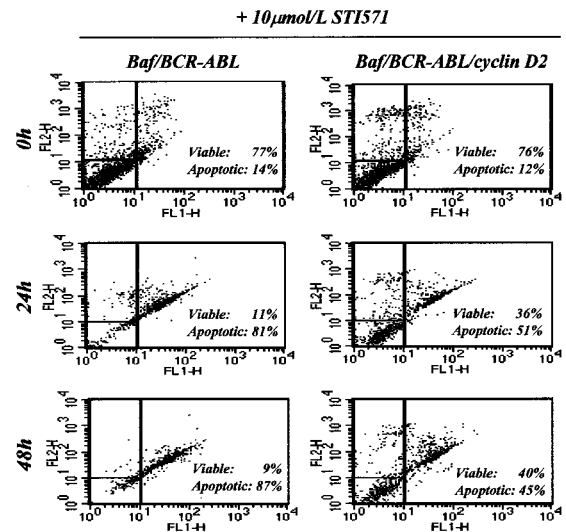


Figure 9. Apoptosis assay of *Baf/BCR-ABL/cyclin D2* and *Baf/BCR-ABL* cells exposed to 10 $\mu\text{mol/liter}$ STI571. The viability profile was expressed as the levels of PI against annexin V staining. Viable cells have low PI and annexin V staining, apoptotic cells have high levels of annexin V staining, and necrotic cells (% not shown) have high PI and low annexin V.

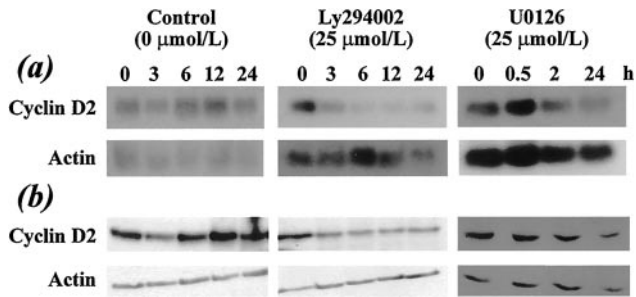


Fig. 10. (a) Northern and (b) Western blots of BV173 cells treated with LY294002 and U0126 for the indicated time-periods. Densitometric analysis of the cyclin D2:actin ratios at each time point showed a maximum 7.7-fold down-regulation of cyclin D2 at the mRNA and 5.3-fold at the protein levels at 6 h of exposure to LY294002, lasting up to at least 24 h. The same analysis of bands from cells treated with U0126 did not show any significant difference in cyclin D2 expression.

more profoundly in Baf/*BCR-ABL* cells treated with STI571. We also observed a moderate reduction of cyclin D2-associated kinase activity. However, given the strong down-regulation of cyclin D2 protein, a more pronounced reduction of cyclin D2-associated kinase activity would have been expected. One possible explanation for this discrepancy could be that the primary role of cyclin D2 in these cells is not cdk activation but the binding of cdk inhibitors such as p27. If this is the case, the primary consequence of cyclin D2 down-regulation may be the redistribution of p27 from cyclin D2/cdk4 complexes to cyclinE/cdk2 (37). The up-regulation of p27 that is seen in Baf/*BCR-ABL* cells would further contribute to a cell cycle block at this level. As a whole, our data are in line with recent work showing that Bcr-Abl promotes degradation of p27 in BaF/3 cells via the proteasome pathway (38). Interestingly, up-regulation of p27 in addition to down-regulation of cyclin D2 also occurred when density-arrested human Ph-positive lines were replated in the presence of STI571. These cells were unable to progress into S phase. Although the precise nature of the "high density growth arrest" is not known, it is reminiscent of contact inhibition (39). Thus, Bcr-Abl kinase activity is indispensable for the cells to resume proliferation, even if the growth restraint imposed by high cell density is no longer in place. Alternatively, the presence of functionally inactive Bcr-Abl protein may interfere with positive signals that regulate the release into cycle after the G_0 arrest. The increase in cyclin D3 levels under these conditions could reflect a situation similar to the accumulation noted in other growth-arrested cells (40, 41). In contrast to the human cell lines, cyclin D3 levels fell in Baf/*BCR-ABL* cells on treatment with STI571 but returned to baseline levels at the time of their G_1 arrest. This could reflect the dual functions in the G_1 -S phase transition and in the quiescence that has been ascribed to cyclin D3 (42).

The essential role of cyclin D2 for cell cycle progression is documented by the fact that Baf/*BCR-ABL* cells exposed to STI571 can be rescued for cell cycle progression by overexpression of cyclin D2. Using both PI and annexin V staining, we demonstrated that ectopic expression of cyclin D2 protects the Baf/*BCR-ABL* cells from undergoing the G_1 cell cycle arrest and subsequent cell death induced by STI571 treatment. This finding strongly suggests that cyclin D2 is targeted by STI571 via Bcr-Abl to mediate the G_1 cell cycle arrest and the associated apoptosis in *BCR-ABL* expressing hematopoietic cells. It also indicates that cyclin D2 has a role not only in regulating cell cycle progression but also in cell survival, and that it integrates the signals that regulate the balance between proliferation and apoptosis in these hematopoietic cells. This idea is supported by a recent report showing that forced expression of another D-type cyclin, cyclin D3, can overcome the proliferation arrest and apoptosis induced by activation of T-cell receptors in leukemic T cells (43).

What cannot be ruled out is the possibility that overexpression of cyclin D2 compensates for another activator of G_1 -S phase transition and cell survival. Because it was recently shown that cyclin D2 is upstream of p27 and cyclin E (44), this appears to be less likely. To test the hypothesis would require mimicking the down-regulation of cyclin D2 mRNA seen on exposure to STI571 of Baf/*BCR-ABL* and BV173 cells. Although this could, in theory, be achieved by an antisense strategy, this approach is fraught with a number of difficulties, particularly nonspecific effects (45, 46), and is, thus, not likely to yield reliable results.

Different external stimuli from growth-factor receptors converge at the level of cyclin D-cdk-Rb (8). Thus, it is likely that Bcr-Abl activity provides a signal analogous to a growth factor that, in the cellular background of lymphoblasts, specifically targets cyclin D2. This signal might be transduced through pathways that under physiological conditions mediate the response to B-cell mitogens. In fact, induction of cyclin D2 expression is an early event after stimulation with IL-4 and IgM (47-49). Conversely, growth arrest of immature murine B cells is accompanied by down-regulation of cyclin D2 (50). Our data show that the signal from Bcr-Abl to cyclin D2 is transduced via the PI-3 kinase pathway. In line with these results, PI-3 kinase has been implicated in cyclin D2 transcription during the activation of B cells (51) and is required for protein stabilization of D cyclins (52). In contrast, there is no evidence that the Ras/MAP kinase pathway is driving cyclin D2 expression in BV173 cells, because cyclin D2 levels are unaffected by the inhibition of MEK. This is somehow surprising, because it has been demonstrated that MEK activity alone is able to induce cyclin D1 expression in serum-starved fibroblasts in the absence of any other signals (37). The apparent discrepancy may be related to differences in signal transduction pathways between fibroblasts and B-lymphoblasts. This view derives further support from our experiments with the temperature-sensitive mutant of Bcr-Abl. Although it has been demonstrated that this mutant is not able to activate MEK (25), cyclin D2 expression in Ba/F3 cells that are transfected with this mutant is clearly dependent on Bcr-Abl kinase activity. This implies that the signal from Bcr-Abl to cyclin D2 must be transduced

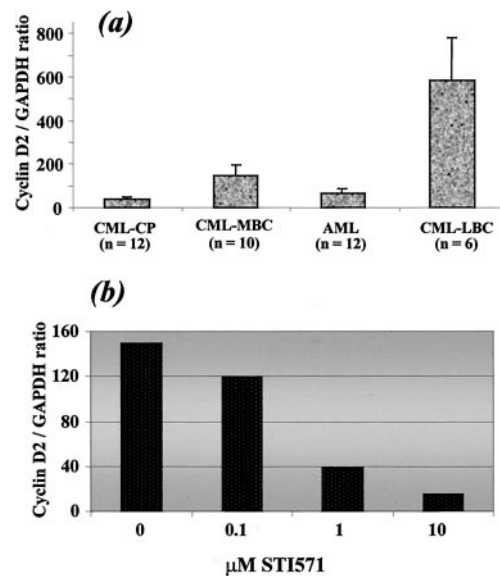


Fig. 11. a, expression of cyclin D2 in primary cells from Ph-positive leukemias and AML. Cyclin D2 levels were significantly higher in lymphoid blast crisis and Ph-positive ALL than in CP CML ($P < 0.001$), myeloid blast crisis ($P = 0.011$), and AML ($P = 0.001$; two-sided Mann-Whitney U test). b, expression of cyclin D2 in fresh MNCs isolated from a patient with Ph-positive ALL. The cells were exposed to STI571 for 6 h, without added cytokines. The results are expressed as attomoles cyclin D2/zeptomoles GAPDH.

by pathways other than MEK. However, the fact that the down-regulation of cyclin D2 in BV173 cells on the inhibition of PI-3 kinase was less complete than on the inhibition of Bcr-Abl indicates that additional pathways may be involved.

It is also important to bear in mind that the proliferative stimulus derived from Bcr-Abl alone is not sufficient; otherwise, one would expect some kind of B-cell proliferation during the CP of CML, during which at least part of the B cells are Ph positive (53). Thus, one could envisage a scenario in which Bcr-Abl "primes" the B cells (in analogy to IL-4 in the context of normal resting B cells) but is not capable of inducing long-term proliferation without a second transforming event. When the latter happens, the uncontrolled proliferation of lymphoid blast crisis takes place. Overall, the situation is reminiscent of CML CD34+ progenitor cells, in which Bcr-Abl alone appears to promote G₁-S phase progression in the absence of growth factors but does not completely abrogate growth-factor dependence (54).

The aberrant behavior of the SD1 cell line is probably attributable to the fact that it was transformed by EBV (55). Because the EBV proteins EBNA-2 and EBNA-LP activate transcription of cyclin D2 and promote G₀-G₁ transition in resting lymphocytes (56, 57), it is conceivable that the expression of cyclin D2 in SD1 cells is primarily dependent on EBV rather than *BCR-ABL*. Thus, cyclin D2 expression is unaffected by inhibition of Bcr-Abl PTK. By contrast, in CML-T1, a T-cell lineage line, there was no significant reduction of Bcr-Abl tyrosine phosphorylation on exposure to STI571. The mechanism responsible for this exception is unclear, but it may explain the relatively weak down-regulation of cyclin D2 in these cells.

Given the low level of expression in myeloid cell lines, it is unlikely that cyclin D2 plays a similar role in BCR-ABL-positive myeloblasts. Most likely, this reflects the fact that cyclin D2 is particularly important in the lymphoid series (29). Our observation implies that transformation by *BCR-ABL* occurs on a specific cellular background using pathways that would normally be used by physiological growth and survival signals. A cell cycle regulator other than cyclin D2 may be the target of Bcr-Abl PTK in myeloblasts and primary CML progenitor cells.

Evidence that our findings relate not only to cell lines but also to primary cells comes from the observation that cyclin D2 mRNA levels are much higher in Ph-positive ALL and lymphoid blast crisis than in CP CML, myeloid blast crisis, or AML. Moreover, down-regulation of cyclin D2 was demonstrable *in vitro* in MNCs from a patient with Ph-positive ALL. Thus, the data from cell lines and primary cells are concordant. It will be interesting to see whether cyclin D2 expression is reactivated in patients with Ph-positive ALL who become resistant to STI571.

In summary, our results strongly implicate cyclin D2 as a critical downstream target of Bcr-Abl in lymphoblasts, in line with the concept that mitogenic signaling is one of the pathogenetic principles in *BCR-ABL*-mediated malignant transformation.

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