

Critical Role for *Cyclin D2* in BCR/ABL-induced Proliferation of Hematopoietic Cells¹

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ABSTRACT

Chronic myeloid leukemia is caused by the tyrosine kinase oncoprotein BCR/ABL. Using oligonucleotide arrays to assay mRNAs at different phases of the cell cycle in BCR/ABL-transformed cells, we found that *cyclin D2* mRNA was constitutively expressed at high levels throughout the cell cycle, a pattern confirmed by immunoblotting of protein lysates. Bone marrow cells from *cyclin D2*-deficient strains of mice failed to proliferate in response to infection with a retrovirus carrying BCR/ABL and failed to generate transformed lymphoid cell lines *in vitro*. These results establish that BCR/ABL promotes cell cycle progression by altering expression of *cyclin D2* and that *cyclin D2* induction plays a critical role in proliferation of hematopoietic cells by BCR/ABL.

INTRODUCTION

Virtually all of the patients with CML,³ a malignancy of the pluripotent hematopoietic stem cell, carry the Philadelphia chromosome, a result of translocation between chromosomes 9 and 22 (1–4). The t(9;22) fuses the *BCR* and *ABL* genes, and the chimeric gene encodes a constitutively active 210 kDa protein tyrosine kinase consisting of an amino terminal BCR domain fused to the ABL protein (5, 6). The most significant effect of the gene fusion is to activate the intrinsic tyrosine protein kinase activity of c-ABL and to relocate the nuclear ABL protein to a predominantly cytoplasmic distribution (7). Definitive demonstration that BCR/ABL is responsible for CML comes from experiments in mouse models. When transplanted with bone marrow infected with a BCR/ABL-expressing retrovirus, mice develop a granulocytic leukemia that closely resembles human CML (8, 9). Transplantation of bone marrow from diseased mice into secondary recipients leads to the development of CML in some mice and acute myeloid or other lymphoid malignancies in others (10, 11).

Through multiple protein-protein associations, BCR/ABL inappropriately activates a variety of signal transduction cascades. BCR/ABL expression overrides the normally tight regulation of hematopoiesis by the cytokine receptor and integrin pathways, and provides a survival signal that in cultured hematopoietic cell lines potentially blocks apoptotic programs (12–14). BCR/ABL alters cell proliferation and programmed cell death, two critical aspects of tumor development, by interfering with both G₁ and G₂-M checkpoints of the cell cycle. In cytokine-dependent hematopoietic cells, BCR/ABL can replace growth factors and drive the G₁ to S phase transition, implying that BCR/ABL activates mitogenic signaling pathways (15). Expression of BCR/ABL can protect cells from apoptosis induced by a variety of

agents including cytokine deprivation, DNA damage, and chemotherapeutic agents (13, 16–20). BCR/ABL is reported to potentiate the γ irradiation-induced cell cycle arrest at the G₂-M transition, raising speculation that apoptosis is blocked because the prolonged G₂-M checkpoint enables the cell to repair DNA damage (20).

Much of the work of the past decade has focused on the specific phosphorylated targets of the BCR/ABL kinase and on signal transduction pathways activated by the oncoprotein that lead to cell proliferation. Signal transduction pathways impinge on transcription factors and generate alterations in patterns of gene expression, but a systematic analysis of the transcriptional consequences of BCR/ABL-transformation has not been reported. We set out to identify the transcriptional targets of BCR/ABL signaling by expression profiling using oligonucleotide arrays and discovered that *cyclin D2* expression is deregulated by BCR/ABL at both the mRNA and protein level. Furthermore, we have demonstrated that *cyclin D2* gene expression is essential for BCR/ABL-induced proliferation of mouse bone marrow cells *in vitro*.

MATERIALS AND METHODS

Mouse Strains. *Cyclin D1* and *cyclin D2*-deficient strains of mice have been described previously (21, 22). The mice were treated in accordance with a protocol approved by the Committee on Animal Care of the Massachusetts Institute of Technology Division of Comparative Medicine.

Hematopoietic Cell Lines. IL-3-dependent BaF3, TonB210.1, and 32D cells were cultured in RPMI 1640 with 10% FBS and 7.5% conditioned medium from WEHI-3B cells (as a source of IL-3). BCR/ABL-transformed BaF3 cells (B210) and 32D Cells (32D/P210) were grown in RPMI 1640 with 10% FBS. The TonB210.1 cell line expresses BCR/ABL under the regulation of the tetracycline-inducible promoter, as described previously (23). For conditional expression of BCR/ABL, TonB210.1 cells were cultured in RPMI 1640 with 10% FBS, 7.5% conditioned medium from WEHI-3B cells, and 2 μ g/ml doxycycline for 72 h. After induction of BCR/ABL, TonB210 cells were maintained in RPMI 1640 with 10% FBS and 2 μ g/ml doxycycline.

Antibodies. Anticyclin D1, anticyclin D2, and anticyclin D3 antibodies were obtained from Santa Cruz Biotechnology. Antiactin antibody, antirabbit-horseradish peroxidase, and antimouse-horseradish peroxidase were purchased from Amersham. All of the PE- and biotin-conjugated primary antibodies, and PE-linked streptavidin were obtained from PharMingen.

Expression Profiling. BaF3 and BCR/ABL-transformed BaF3 cells (viability > 95%) were γ irradiated (500 cGy) and cultured in RPMI 1640 with 10% FBS at 37°C with 5% CO₂ for 4.5 h. The vital dye Hoechst 33342 (Sigma Chemical Co.) was added to a final concentration of 2.5 μ g/ml, and the cells were cultured for another 1.5 h before isolating cells at different cell cycle stages using a FACS. Cells with a DNA content consistent with the G₁ and G₂-M phases of the cell cycle were collected separately. Total RNA was extracted from the sorted cells as well as normal cycling cells using RNA STAT-60 according to the manufacturer's specifications (Tel-Test "B," Inc.). Total RNA (20 μ g) of each sample was used to prepare cRNA probes by *in vitro* transcription. Mouse oligonucleotide arrays (representing 6502 Genes; Affymetrix) were hybridized as per the manufacturer's protocols. The arrays were analyzed using a Hewlett-Packard confocal laser scanner. Scanned outputs representing the level of expression of different genes in different samples were analyzed by both the GeneExpress software (Affymetrix) and a median

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³ The abbreviations used are: CML, chronic myelogenous leukemia; IL, interleukin; FBS, fetal bovine serum; PE, phycoerythrin; FACS, fluorescence-activated cell sorter.

Table 1 Relative transcript level of genes in BCR/ABL expressing BaF3 cells compared to the parental BaF3 cells, according to cell cycle status

Status	Genes >3-fold increased		Genes >3-fold decreased	
	Genes	Fold change	Genes	Fold change
Cycling	<i>Annexin V</i>	5.39	Sim. ^a to EF2	0.082
	<i>Heat shock protein</i>	3.61	VALRS	0.154
	<i>Scl</i>	3.43	Sim. to EIF5A	0.158
	<i>Spi2</i>	3.08	Major basic protein (mbp-1)	0.166
G1			Gelsolin	0.233
	<i>Spi2</i>	8.73	Sim. to EF2	0.249
	<i>T-cell receptor γ chain</i>	5.1	Sim. to XP-E	0.279
	α -tubulin	3.8	Transglutaminase	0.281
	<i>Pim 1</i>	3.76	Gelsolin	0.219
	<i>PEM</i>	3.3	Major basic protein (mbp-1)	0.232
G2/M	<i>ID1A</i>	7.5	IL-3 receptor	0.243
	<i>Spi2</i>	7	Transglutaminase	0.275
	<i>Sim. to putative S/T kinase</i>	4		
	<i>Sim. to peptidyl prolyl cis-trans isomerase</i>	3.7	Sim. to IFN inducible protein	0.2
	<i>Cyclin D2</i>	3.5		
	α -tubulin	3.5		
	<i>Prostaglandin endoperoxidase</i>	3.3		
	<i>Rsp-1</i>	3.3		
	<i>Coproporphyrinogen oxidase</i>	3.1		
	<i>Cytochrome c (MC1)</i>	3		
	<i>Sim. to XBP-1</i>	3		

^a Sim., similar.

ratio prime algorithm.⁴ Three prediction groups for gene expression data were determined: reliable, less reliable, and least reliable. For the expression pattern of a given gene to be deemed reliable, the intensity of at least five probe pairs for different regions of the same gene had to be greater than threshold in both experiments. The final results of the comparison between two samples by median ratio prime method were additionally analyzed by visual inspection. The difference in the expression level of a given gene was called positive if the minimum difference in the mean intensities of the probe between two samples was 200 and the fold change was ≥ 3 . The groups of genes that were predicted to have reliable differential expression by this analysis are presented (Table 1).

Protein Extraction and Immunoblotting. Cell populations were washed twice with PBS and resuspended in 5 volumes of lysis buffer [100 mM NaCl, 20 mM Tris (pH 7.4), 10 mM NaF, 1 mM EDTA, 1 mM ZnCl₂, 1 mM sodium vanadate, 1 mM MgCl₂, 1% NP40, 10% glycerol, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride], and incubated at 4°C for 30 min with constant agitation. The lysate was centrifuged at 12,000 $\times g$ for 5 min, and the supernatant was stored at -70°C until later use. Total protein concentration of each sample was assayed by BCA protein assay kit (Pierce). Equal amounts of protein lysate for each sample (50 μ g) was separated by gel electrophoresis on a 15% SDS-polyacrylamide gel, and the protein was transferred to nitrocellulose membrane. The blots were probed with the indicated antibodies using TBST buffer [20 mM Tris (pH 7.6), 137 mM NaCl, and 0.1% Tween 20] and developed using the enhanced chemiluminescence reagent kit (Amersham).

Analysis of Cell Surface Markers by FACS. Freshly harvested bone marrow cells were mixed with 3 volumes of RBC lysis buffer (Sigma Chemical Co.), vortexed gently, and kept at room temperature for 15 min before centrifugation at 200 $\times g$ for 5 min. The pellets were resuspended and washed once in cold wash buffer (PBS/1.0% FBS). The final pellet was resuspended in cold wash buffer containing 5 μ g/ml antimouse CD32/CD16 (Fc Block; PharMingen) to a final density of 2 $\times 10^7$ cells/ml. Cultured cells were washed once with the cold wash buffer and resuspended in the cold wash buffer containing 5 μ g/ml antimouse CD32/CD16 to a final density of 2 $\times 10^7$ cells/ml. Cells were aliquoted (10⁶ cells/well in 50 μ l) into a V-bottomed 96-well plate. Primary antibodies were diluted in the wash buffer to a concentration of 1–10 μ g of antibody/ml. A volume of 50 μ l was added to each well and mixed by pipetting. The plates were rocked at 4°C for 30 min in the dark. The cells were washed twice (for fluorochrome-linked primary antibody) or three times (for biotin-linked primary antibody) with 200 μ l of wash buffer/well for each washing. In case of biotin-linked primary antibody, the cells in

each well were incubated with 100 μ l of streptavidin-linked PE diluted to 2.5 μ g/ml in wash buffer for 30 min in the dark at 4°C and thereafter washed three times with the wash buffer. The final cell pellet was resuspended with 100 μ l of wash buffer/well. The cells were transferred to tubes containing 400 μ l of wash buffer and analyzed by FACS.

Bone Marrow Proliferation Assay. Femurs from mice were harvested aseptically. Bone marrow cells were flushed from the femur with bone marrow medium (DMEM, 15% FBS, 5% conditioned medium from WEHI-3B cells, 50 units/ml penicillin, 50 μ g/ml streptomycin, 2 mM glutamine, 6 ng/ml mouse IL-3, 10 ng/ml human IL-6, and 50 ng/ml human stem cell factor). The cells were passed through a 21-gauge needle to obtain a single cell suspension. The bone marrow cells were plated in 10 ml of bone marrow medium and incubated overnight at 37°C and 5% CO₂. Nucleated cells were counted after RBCs were lysed by diluting an aliquot of cells with 10 volumes of PBS containing 2% acetic acid. Equal numbers of bone marrow cells from each genotype (~10⁷ cell) were plated in six-well plates and infected with 5 ml of a BCR/ABL retroviral supernatant by centrifugation at 1000 $\times g$ for 1.5 h. The spin infection was repeated within 24 h (24). After the final infection, the cells were cultured in the bone marrow medium and incubated at 37°C and 5% CO₂ for 72 h. The cells were washed once with PBS and resuspended in selection medium (RPMI 1640, 10% FBS, 50 μ M 2-mercaptoethanol, 50 units/ml penicillin, 50 μ g/ml streptomycin, and 2 mM glutamine), and plated in 48-well culture dishes in a dilution series starting at 2 $\times 10^5$ cells/ml. The selection medium was changed every 3 days. The growth of nonadherent hematopoietic cells was determined by observing cells under the microscope and by counting of cells at day 7. Wells with $>2 \times 10^5$ cells/ml also demonstrated acidification of the medium and were scored as positive. The percentage of positive wells for each genotype was determined, and percentage positive wells relative to wild type was calculated by dividing the percentage of positive wells for a given genotype with the wild type.

RESULTS

Expression Profile of BaF3 Cells versus BaF3/BCR/ABL. BCR/ABL is known to enhance cell proliferation and inhibit apoptosis by driving cell cycle progression through both the G₁-S and G₂-M phase transitions. Therefore, BCR/ABL likely activates or suppresses genes that regulate the cell cycle. We set out to investigate genes of which the transcription is dysregulated by BCR/ABL in BaF3 cells, an IL-3-dependent bone marrow-derived cell line that has been widely used to investigate the transforming properties of the BCR/ABL

⁴ Unpublished.

oncogene. Expression of BCR/ABL in BaF3 cells allows these cells to proliferate in the absence of IL-3 and form tumors in immunodeficient nude mice and leukemias in syngeneic BALB/c recipients (25). Total RNA from BaF3 and BCR/ABL-transformed BaF3 cells (B210) were isolated from cells arrested at distinct stages of the cell cycle. To isolate cells at different cell cycle stages based on DNA content, cells were subjected to γ irradiation (500 cGy) in the absence of IL-3 to induce cell cycle arrest, labeled with Hoechst 33342, and sorted by FACS to separate cells in the G₁ and G₂-M phases of the cell cycle. Total RNA was extracted from cycling cells as well as those with a G₁ and a G₂-M DNA content, and analyzed for patterns of mRNA expression using oligonucleotide arrays. Of 6502 genes analyzed, the transcription of a small set of 28 genes was found to reliably differ by >3-fold between the two cell types (Table 1). Of these 28 genes, 18 were more highly expressed and 10 were expressed at reduced levels in BCR/ABL-transformed BaF3 cells compared with the parental cell line. Genes of which the expression was reduced in BCR/ABL-transformed cells included the actin-severing protein gelsolin, the IL-3 receptor, and a novel membrane protein homologous to an IFN-inducible protein (Table 1).

Among the genes of which the expression was elevated in BCR/ABL-transformed cells was the hematopoietic-specific transcription factor SCL, the antiapoptotic protein kinase Pim-1, and the negative regulator of basic helix-loop-helix transcription factors ID1A. Up-regulation of certain genes, which have been reported to be induced by BCR/ABL, like *c-myc*, fell below threshold reliability in our analysis and, thus, are not listed (26). Most interestingly, we noted that the transcript level of *cyclin D2* was 3.5-fold higher in cells having the DNA content predicted for G₂-M phase cells. This was an unusual result given that the expression of D-type cyclins is typically low during G₂-M phase and peaks during the G₁ phase of the cell cycle. The discovery that *cyclin D2* expression was dysregulated in the BCR/ABL-expressing cell line suggested to us that cyclin D2 might be a critical effector of transformation.

Induction of Cyclin D2 Protein in BCR/ABL-transformed Hematopoietic Cells. To confirm that our observation of altered mRNA levels resulted in altered levels of protein expression, we examined the levels of D-type cyclin proteins in BCR/ABL-transformed hematopoietic cell lines and their parental counterparts. We analyzed protein lysates from the original BaF3-derived cell lines from which mRNA was isolated for the array analysis, as well as protein lysates from a BCR/ABL-transformed cytokine-dependent myeloid cell line, 32D.

In agreement with the results from the array hybridizations, immunoblot analysis demonstrated that the expression of cyclin D2 protein was greater during the G₂-M phase of the cell cycle in BCR/ABL-transformed cells than the corresponding parental lines (Fig. 1A, compare Lanes 2 and 4 in each panel). In each of the parental cell lines, the expression level of cyclin D2 protein was highest in the G₁ phase and lower in G₂-M phase (Fig. 1A, compare Lanes 1 and 2 in each panel). In contrast, in BCR/ABL-expressing cells (B210 and 32D/p210), the expression level of cyclin D2 protein was maintained at the same elevated level in both the G₁ and G₂-M phases (Fig. 1A, compare Lanes 3 and 4 in each panel). This implies that BCR/ABL transformation alters the normal cell cycle modulation of cyclin D2 expression and induces its constant expression throughout the cell cycle.

BCR/ABL transformation led to increased expression of cyclin D3 in all of the cell types tested but did not appear to disrupt the phase-specific modulation of D3 expression observed in normal cells, because levels peaked in G₁ and were lower in G₂-M (Fig. 1A, compare Lanes 3 and 4 in each panel). The expression profile generated by array hybridization showed that *cyclin D3* mRNA was over-expressed 2.3-fold during the G₂-M phase in BCR/ABL-transformed

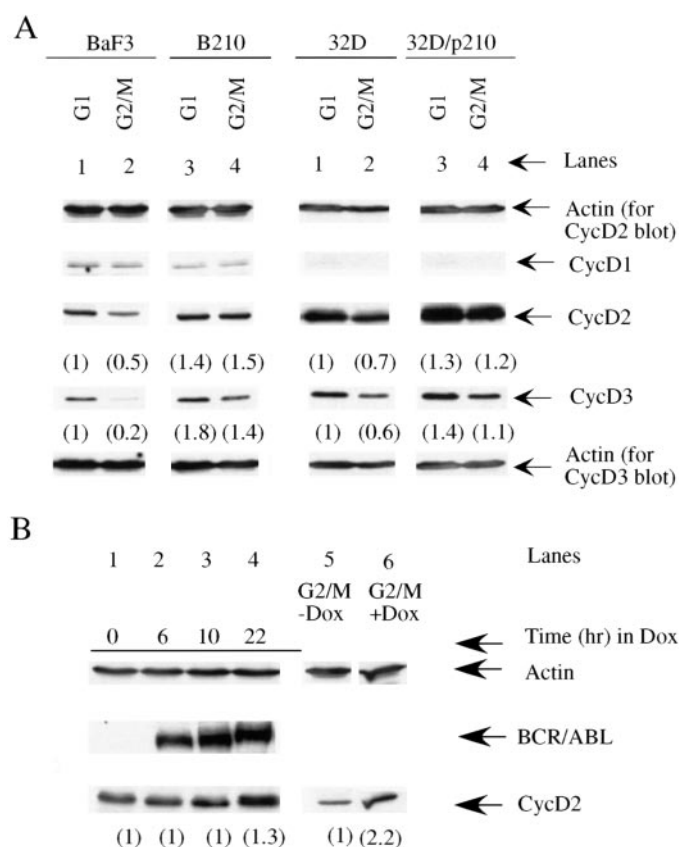


Fig. 1. A, immunoblot of D-type cyclins in hematopoietic cell lines transformed by BCR/ABL. Total protein lysates from parental and BCR/ABL-transformed hematopoietic cell lines were probed with antibodies against cyclins D1, D2, and D3, as indicated. Lanes 1 and 3, and 2 and 4 represent total protein lysates extracted from cells carrying the DNA content predicted for cells in the G₁ and G₂-M phases of the cell cycle, respectively. An actin immunoblot was performed to enable normalization of protein loading. The numbers below the bands represent their normalized fold change in comparison with the level of expression in G₁ phase of the parental cell types (BaF3 or 32D). The actin bands for normalization of cyclin D2 and cyclin D3 are different; they are labeled accordingly. B, time course of induction of cyclin D2 protein by BCR/ABL in TonB210.1 cells. Uninduced TonB210.1 cells growing in IL-3 were washed and treated with 1 μ g/ml doxycycline. Total cellular lysate was made at different time points, and the blot was probed with antibodies against cyclin D2, BCR/ABL, and actin (Lanes 1–4). The numbers in parentheses for Lanes 1–4 indicate the fold change in cyclin D2 protein relative to its steady state level in IL-3 supplemented medium (Lane 1). Cellular lysate from G₂-M phase TonB210.1 cells growing without doxycycline (Lane 5) or with doxycycline (Lane 6) was probed with antibody against cyclin D2 and actin. The numbers in parentheses for Lanes 5 and 6 indicate the fold change in cyclin D2 in G₂-M cells in the uninduced (Lane 5) and induced states (Lane 6).

cells, and, thus, this gene fell below the threshold we set for reliability of our array data and is not listed in Table 1. *Cyclin D3* might also be a downstream target of BCR/ABL signaling, but, unlike *cyclin D2*, its normal cell cycle variation did not appear to be disrupted by BCR/ABL to the same extent as D2.

The expression of cyclin D1 protein, which is normally absent in hematopoietic cell types, was low in general in all of the cells tested, irrespective of the presence of BCR/ABL (Fig. 1A). Our observations suggested that, among the D-type cyclins, cyclin D2 might be a critical factor in BCR/ABL-induced cell proliferation in hematopoietic cell types.

Tetracycline-dependent BCR/ABL Expression Demonstrates Coupling to Cyclin D2 Induction. We tested the link between BCR/ABL expression and cyclin D2 induction using the TonB210.1 cell line, a BaF3 derivative carrying a BCR/ABL gene under the regulation of a tetracycline-inducible promoter. In the uninduced state, expression of BCR/ABL is barely detectable in TonB210.1 cells, and they remain dependent on IL-3. After induction with doxycycline, the cells

express high levels of BCR/ABL oncoprotein and are transformed into a cytokine-independent and tumorigenic cell type. Therefore, beginning in the uninduced state, we deprived the TonB210.1 cells of IL-3, added doxycycline to induce BCR/ABL expression, and examined protein lysates for cyclin D2 expression. Doxycycline induction of BCR/ABL expression corresponded with modest overall induction of cyclin D2 in cycling cells, which was more marked when cells with G₂-M-phase DNA content were analyzed (Fig. 1B). These data confirm the association of BCR/ABL expression and cyclin D2 induction.

BCR/ABL Fails to Induce Proliferation in Bone Marrow Cells from Cyclin D2^{+/-} and Cyclin D2^{-/-} Mice *In Vitro*. Because BCR/ABL altered the cell cycle regulation of cyclin D2 in all of the cell lines tested, we asked whether transformation by BCR/ABL was dependent on its ability to induce cyclin D2. To do so, we determined whether BCR/ABL could induce proliferation in bone marrow cells lacking cyclin D2. Bone marrow cells from *cyclin D2*^{+/+}, *cyclin D2*^{+/-}, and *cyclin D2*^{-/-} mice were infected with a retrovirus carrying either the BCR/ABL oncogene or a control vector expressing the green fluorescent protein. Infected cells were plated in 48-well plates and serially diluted to achieve different seeding densities to enable a quantitative assessment of the capacity for BCR/ABL to induce proliferation of primary bone marrow cells in culture. Morphological and flow cytometric analysis of infected cell populations after 7 days in culture demonstrates that this short term assay reflects the proliferation of mostly B220⁺ lymphoid cells in response to BCR/ABL, as reported previously (27, 28). Nonadherent hematopoietic cells proliferated in response to infection with the BCR/ABL retrovirus but not the control green fluorescent protein virus (not shown) in samples from wild-type animals. Wells achieving high cell densities (> 2 × 10⁵/well) and acidification of the culture medium within 1 week were considered as positive responders to the proliferative influences of BCR/ABL. Bone marrow cells from the *cyclin D2*^{+/+} mice proliferated robustly in response to BCR/ABL. However, bone marrow cells from the *cyclin D2*^{+/-} and the *cyclin D2*^{-/-} mice failed to proliferate in response to BCR/ABL and were >10-fold less likely to show proliferation by this assay (Fig. 2A).

To test whether the inability of BCR/ABL to induce cell proliferation was specific to *cyclin D2* deficiency, we performed a comparable retroviral infection of bone marrow from mice differing in genotype at the *cyclin D1* locus. Bone marrow cells from *cyclin D1*^{+/+}, *cyclin D1*^{+/-}, and *cyclin D1*^{-/-} mice proliferated equivalently in response to BCR/ABL (Fig. 2B). These data suggest that the defect in BCR/ABL-induced cell proliferation from primary bone marrow was a consequence of deficiency of *cyclin D2*, because deficiency of *cyclin D1* had no effect on hematopoietic cell proliferation. As measured by this assay, there was no redundancy in D-type cyclin function in enabling cell cycle progression in response to BCR/ABL, and there appears to be specific coupling of BCR/ABL to cyclin D2 expression in bone marrow cells.

BCR/ABL Fails to Generate B220⁺ Pre-B-Cell Lines from Cyclin D2-deficient Bone Marrow. The proliferation assay used above measures short-term effects of BCR/ABL on bone marrow populations over a 1-week period. When cell cultures infected with ABL oncoproteins are kept for several weeks *in vitro*, the initial phase of proliferation is followed by a period of marked cell death. After 2–3 weeks, continuously growing cell lines emerge that express the B220 surface antigen and represent transformed pre-B lymphocytes (27, 29). The capacity for BCR/ABL to generate stable transformed pre-B lymphoid cell lines *in vitro* has been widely exploited to characterize the transforming mechanisms of BCR/ABL and its variants (27, 28, 30). To determine whether *cyclin D2* was required for BCR/ABL to generate transformed pre-B lymphoid cell lines *in vitro*, we maintained all of the infected cell populations in culture and subsequently

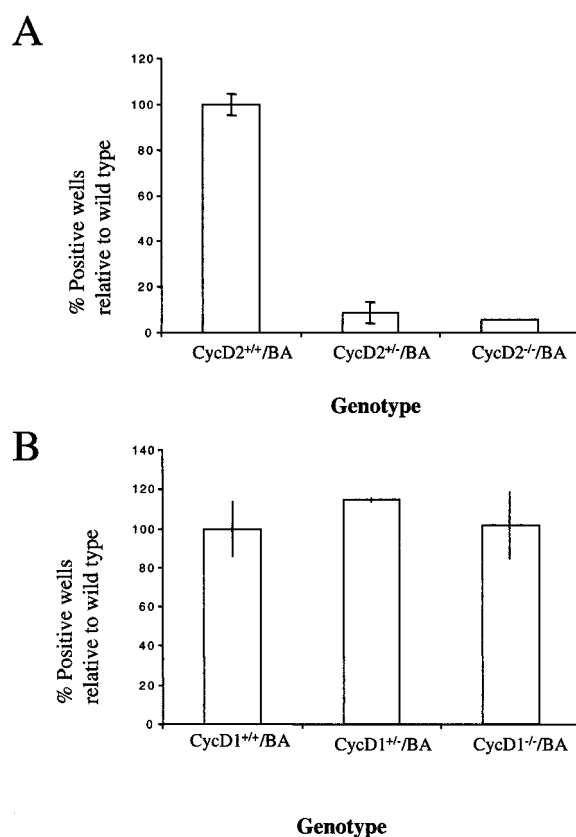


Fig. 2. *In vitro* bone marrow proliferation assay after infection by the BCR/ABL retrovirus. A, normalized percentage of positive wells after infection with BCR/ABL of bone marrow cells from *cyclin D2* wild-type (CycD2^{+/+}), heterozygous (CycD2^{+/-}), and homozygous null (CycD2^{-/-}) mice. B, normalized percentage of positive wells after infection with BCR/ABL of bone marrow from *cyclin D1* wild-type (CycD1^{+/+}), heterozygous (CycD1^{+/-}), and homozygous null (CycD1^{-/-}) mice. The percentage of positive wells was calculated relative to results for wild-type bone marrow; bars, \pm SD.

characterized the growth properties and surface immunophenotype of the cell populations that emerged. Infected bone marrow cells from *cyclin D2*^{+/+} and *cyclin D1*^{+/+}, *cyclin D1*^{+/-}, and *cyclin D1*^{-/-} mice generated continuously growing cell lines that proliferated robustly in culture. By flow cytometry, all were found to express the B220 antigen, consistent with the expected nature of bone-marrow-derived cell lines resulting from BCR/ABL expression (Table 2). In contrast, the scant cells from *cyclin D2*^{+/-} and *cyclin D2*^{-/-} mice that proliferated weakly in response to BCR/ABL were largely negative for the B220 antigen. Of these, only the cells from the *cyclin D2*^{+/-} mice generated adequate numbers of cells to allow a more thorough investigation of surface marker expression. Infected cells from *cyclin D2*^{+/-} mice expressed markers of primitive hematopoietic progenitors (CD34, c-kit, and *Sca1*) and lacked expression of markers of more committed lineages, except for a small percentage of cells expressing the erythroid marker Ter119 (9.08%; Table 2). These data argue that *cyclin D2* was required for BCR/ABL to generate stable transformed pre-B lymphoid cell lines and that *cyclin D2*-deficient marrow was highly resistant to the transforming effects of BCR/ABL under these standard assay conditions.

Mice with *cyclin D2* deficiency have no obvious abnormality in hematopoiesis at baseline (21). However, an alternative explanation for the deficiency of *cyclin D2*-deficient marrow is the absence of adequate numbers of progenitor cells susceptible to BCR/ABL transformation. To evaluate cellular composition of bone marrow from *cyclin D2*-deficient mice, we analyzed the expression of multiple surface markers by FACS. Using a broad panel of antibodies directed

Table 2 Analysis of hematopoietic lineage markers on bone marrow cells after transduction with BCR/ABL

Cell surface markers	Percentage of cells expressing specific cell surface markers after transduction with BCR/ABL ^a					
	CycD2 ^{+/+} /BCR/ABL	CycD2 ^{+/-} /BCR/ABL	CycD2 ^{-/-} /BCR/ABL	CycD1 ^{+/+} /BCR/ABL	CycD1 ^{+/-} /BCR/ABL	CycD1 ^{-/-} /BCR/ABL
B220	98.1	0.03	4.29	97.2	78.6	97.5
CD19	99.3	0.03	nd ^b	nd	nd	nd
Mac1	3.76	1.42	nd	nd	nd	nd
Ter119	0.27	9.08	nd	.61	1.75	1.3
CD34	0.17	57.2	nd	1.58	2.3	0.67
c-kit	0.21	96.65	nd	6.05	8.7	3.8
Sca1	2.57	91.92	nd	39.2	31.5	31

^a The percentage of cells expressing specific surface markers were represented as mean value.

^b nd, not done.

against surface antigens expressed on most of the major lymphoid, myeloid, and progenitor lineages, no significant differences were detected among mice with different genotypes at the *cyclin D2* locus (Table 3). In particular, there were equivalent populations of B220+ cells, suggesting that *cyclin D2* deficiency did not lead to a profound defect in this population of lymphocytes in the bone marrow. This result implies that the major populations of bone marrow cells from *cyclin D2*^{+/-} and *cyclin D2*^{-/-} mice were present in normal proportions and participated normally in hematopoiesis but were intrinsically resistant to transformation by BCR/ABL.

BCR/ABL-infected Bone Marrow Cells from Cyclin D2^{+/-} and Cyclin D2^{-/-} Mice Proliferate Slowly and Have Deficient Cell Cycle Progression. To compare the proliferation rates of different populations of bone marrow cells infected by BCR/ABL, we inoculated a fixed number of cultured cells (10⁵ cells/ml) from each genotype into fresh medium and counted the number of cells every 24 h. BCR/ABL-infected cells from the *cyclin D2*^{+/+} and *cyclin D1*^{-/-} mice proliferated equivalently and had significantly faster growth rates than *cyclin D2*^{+/-} and *cyclin D2*^{-/-} cells (Fig. 3A). We also determined the cell cycle profile of these cell populations by staining with propidium iodide to reveal DNA content. BCR/ABL-transformed bone marrow cells from *cyclin D2*^{+/+} or *cyclin D1*^{-/-} mice yielded normal profiles for actively proliferating cell populations (Fig. 3B, upper and lower panels), whereas cells from the *cyclin D2*^{+/-} mice were largely arrested in the G₁ phase of the cell cycle, demonstrating a marked deficiency in G₁ progression. Therefore, BCR/ABL appeared to couple to the cyclin D2 protein to drive cell cycle progression in hematopoietic cells, and deficiency of cyclin D2 resulted in a mark deficiency in cell proliferation in response to BCR/ABL.

DISCUSSION

Through the use of oligonucleotide arrays for transcription profiling of BCR/ABL-transformed cells, we determined a small subset of genes that were differentially expressed compared with parental counterparts and, therefore, were likely candidates to explain the sustained biological consequences of BCR/ABL signal transduction. Enhanced expression of *cyclin D2* was prominent among the small set of gene candidates identified. By analysis of protein lysates, we confirmed that the altered pattern of *cyclin D2* mRNA expression was also reflected in altered levels of protein. By showing that bone marrow cells from a strain of mice deficient in *cyclin D2* failed to proliferate in response to BCR/ABL and failed to yield transformed cell lines, we have demonstrated the critical importance of cyclin D2 in mediating the effects of this leukemia oncoprotein on hematopoietic cells *in vitro*. These data validate the utility of expression array analysis for identifying important transcriptional targets of oncogene action.

D-type cyclins are differentially expressed in response to growth factors or cytokines and, therefore, translate extracellular signals into

cell cycle progression (31). In parental BaF3 cells, the level of cyclin D2 responds to stimulation with the mitogenic cytokine IL-3. Levels peak during G₁ and are later reduced in the G₂-M phase, reflecting normal cell cycle variation (32). In cells expressing BCR/ABL, the level of cyclin D2 remained high throughout the cell cycle, implying that the constitutive growth signal from the oncoprotein BCR/ABL induced *cyclin D2* transcription irrespective of the phase of the cell cycle and relieved the cell cycle phase-specific control. Classical studies of the cell cycle dynamics of Philadelphia chromosome-positive hematopoietic progenitors point to enhanced cycling and reduced sensitivity to agents that normally maintain stem cells in a quiescent state (33). Cytokine-independent proliferation has been demonstrated recently for the earliest CD34+ progenitors in CML patients, arguing that BCR/ABL alone can drive cell cycle progression in hematopoietic cells (34). By constitutive activation of cyclin D2 expression, BCR/ABL would provide for enhanced G₁ progression in the absence of cytokines and increased sensitivity to cytokines that drive G₁ progression. Additionally, inappropriate levels of cyclin D2 expression during G₂-M might sequester cyclin-dependent kinase inhibitors and promote mitotic progression in BCR/ABL-transformed cells.

Several members of the D class of G₁ phase cyclins (D1, D2, and D3) appear to have significant functional redundancy. Loss of function of any one member in homozygous null mice results in only modest phenotypic defects largely restricted to specific classes of cells, particularly the retina and breast for D1-deficient mice, and the testes and ovaries for D2-deficient mice (21, 22). Furthermore, expression of cyclin E in place of cyclin D1 can rescue these specific defects (35). Recently, it has been shown that *cyclin D2*-deficient mice have normal numbers of bone marrow lymphocytes that proliferate normally in response to lipopolysaccharide treatment (36). How-

Table 3 Analysis of hematopoietic lineage markers on normal bone marrow cells from *cyclin D2*-deficient mice

Markers	Percentage of bone marrow cells expressing specific cell surface markers ^a			P
	CycD2 ^{+/+}	CycD2 ^{+/-}	CycD2 ^{-/-}	
B220	20.7 ± 3.87	23.5 ± 6.24	23.4 ± 1.49	0.856
CD4	3.55 ± 1.15	4.225 ± 1.925	4.493 ± 1.508	0.932
CD8	2.08	2.36	2.3 ± 0.17	0.747
CD18	85.9 ± 2.6	78.65 ± 6.25	74.45 ± 2.95	0.3
CD19	21.03	27.96	19.15 ± 0.635	0.122
GR1	53.606 ± 3.546	50.41 ± 5.348	49.235 ± 2.689	0.713
NK	6.83	6.89	7.875 ± 0.255	0.334
Thy1	15.51	19.81	16.375 ± 1.185	0.452
Mac1	57.86	66.71	57.97 ± 0.72	0.132
Ter119	12.783 ± 2.616	11.04 ± 2.494	13.922 ± 3.255	0.791
CD34	5.816 ± 1.294	5.226 ± 0.476	4.817 ± 0.586	0.693
c-kit	19.1 ± 3.874	16.46 ± 1	16.167 ± 0.79	0.592
Sca1	11.16 ± 1.607	10.736 ± 0.87	13.182 ± 2.143	0.589

^a The percentage of cells expressing specific surface markers were represented as mean ± SE.

^b The P was calculated by single factor ANOVA (Microsoft Excel program).

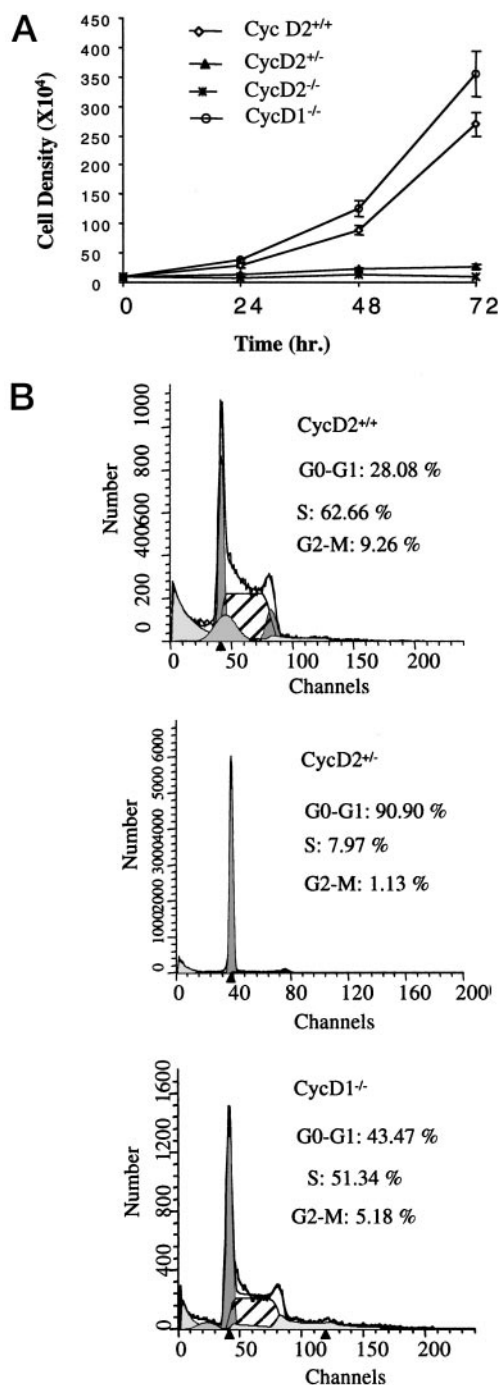


Fig. 3. Growth pattern and cell cycle profile of *BCR/ABL*-infected bone marrow cells. A, equal numbers of *BCR/ABL*-infected bone marrow cells (10^5 /ml) from each genotype were plated, and viable cell counts were determined every 24 h; bars, \pm SD. B, equal numbers of cells (10^6) from each genotype were stained with propidium iodide, and their cell cycle distribution was determined by analyzing total DNA content of the stained cells by FACS. Percentage of cells in various stages of the cell cycle is indicated.

ever, the B lymphocytes in these mice have an intrinsic defect in cell proliferation in response to activation of the B-cell receptor and the CD40 antigen, which can partially be compensated by up-regulation of cyclin D3 (36). These data suggest that specific proliferative signals activate particular D-type cyclins and that loss of a single D-type cyclin can under some conditions be compensated for by up-regulation of another cyclin of the same class, thereby maintaining homeostasis of bone marrow populations in the steady state. In our assays, deficiency of *cyclin D2* led to a profound cell intrinsic defect in

proliferation by *BCR/ABL*. Although cyclin D3 is expressed in hematopoietic cells, this alternative D-type cyclin could not compensate for the lack of cyclin D2 expression for *BCR/ABL*-mediated proliferation of pre-B lymphoid cells in our studies. Whether the transformation profile of bone marrow cells from mice deficient in *cyclin D3* will be altered in response to *BCR/ABL* awaits the availability of these mice for similar experiments. Ultimately, analysis of how cyclin D2 or D3 deficiency influences leukemia in reconstituted animals will be needed to link D-type cyclin induction with the myeloproliferative phenotype *in vivo*.

A particularly perplexing result of our experiments was the resistance of the *hemizygous cyclin D2^{+/-}* bone marrow cells to proliferation by *BCR/ABL*. This suggests that a specific threshold of cyclin D2 protein is required to mediate *BCR/ABL*-induced cell cycle progression. Therefore, cyclin D2 may be an appealing target for treatment of *BCR/ABL*-related leukemias, because effecting only a modest 50% reduction in cyclin D2 levels or protein function may be sufficient to inhibit transformation of hematopoietic cells by *BCR/ABL* while leaving normal hematopoiesis relatively unperturbed.

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NOTE ADDED IN PROOF

Similar conclusions linking *BCR-ABL* expression and activation of cyclin D2 in lymphoblasts have been reported independently in a recent issue of *Cancer Research*: Deininger, M. W., Vieira, S. A., Parada, Y., Banerji, L., Lam, E. W., Peters, G., Mahon, F. X., Kohler, T., Goldman, J. M., and Melo, J. V. Direct Relation between *BCR-ABL* Tyrosine Kinase Activity and Cyclin D2 Expression in Lymphoblasts. *Cancer Res.* 61: 8005–8013, 2001.

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Critical Role for *Cyclin D2* in BCR/ABL-induced Proliferation of Hematopoietic Cells

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