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Cyclin D3 and c-MYC control glucocorticoid-induced cell cycle arrest but not apoptosis in lymphoblastic leukemia cells

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Abstract

Glucocorticoids (GC) induce cell cycle arrest and apoptosis in lymphoblastic leukemia cells. To investigate cell cycle effects of GC in the absence of obscuring apoptotic events, we used human CCRF-CEM leukemia cells protected from cell death by transgenic bcl-2. GC treatment arrested these cells in the G1 phase of the cell cycle due to repression of cyclin D3 and c-myc. Cyclin E and Cdk2 protein levels remained high, but the kinase complex was inactive due to increased levels of bound $p27^{Kip1}$. Conditional expression of cyclin D3 and/or c-myc was sufficient to prevent GC-induced G1 arrest and $p27^{Kip1}$ accumulation but, importantly, did not interfere with the induction of apoptosis. The combined data suggest that repression of both, c-myc and cyclin D3, is necessary to arrest human leukemia cells in the G1 phase of the cell division cycle, but that neither one is required for GC-induced apoptosis.

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Abbreviations: ActD, actinomycin D; Cdk, cyclin-dependent kinase; CDKI, cyclin-dependent kinase inhibitor; DHFR, dihydrofolate reductase; GC, glucocorticoid; GR, glucocorticoid receptor; PTMα, prothymosine α

Introduction

Glucocorticoids (GC) induce cell cycle arrest and apoptosis in immature lymphocytes and various malignancies of lymphoid origin.[1–4] Although these effects have led to the inclusion of GC analogs in essentially all therapeutic regimens for malignant lymphoproliferative disorders,[5,6] their underlying molecular mechanism has remained elusive. GC signaling initiates with the binding of GC to its receptor (GR), a ligand-activated transcription factor of the Zn-finger type.[7,8] After nuclear translocation, the activated GR influences the expression of a plethora of genes.[9] Which of these genes are responsible for apoptosis and/or cell cycle arrest, and whether these two events are independently regulated or as a consequence of each other is still unclear. Moreover, evidence is accumulating (see below) that these GC effects may not follow the same mechanistics in different systems (human lymphoblastic leukemia, mouse thymocytes or lymphoma, osteosarcoma, etc.).

Apoptosis is a process initiated by a large number of signals that activate specific membrane death receptors ('extrinsic pathway') and/or intracellular mechanisms controlled by members of the bcl-2 family and the mitochondria (called 'intrinsic pathway').[10–12] Both pathways converge at the level of specific proteases, called effector caspases, that are thought to be the executioners of most forms of apoptosis. GC-induced apoptosis in human lymphoblastic leukemia appears to employ the 'intrinsic pathway'[13,14] to activate the caspase cascade; however, the upstream components of this pathway have remained unclear.[2,3,15,16] Based on the observation that prevention of the GC-induced c-myc repression inhibits apoptosis in human CCRF-CEM cells,[17,18] one model proposes that apoptosis is the consequence of cell cycle arrest in highly proliferative leukemia cells ('conflicting signal'). However, tetracycline-regulated expression of c-myc did not prevent apoptosis but rather enhanced it.[19] Alternative models suggest that apoptosis, at least in these human leukemia cells, might be a cellular response to the widespread suppressive effects of GC on translation, RNA synthesis and/or metabolism which is further potentiated by GR autoinduction.[20,21]

The cell division cycle is controlled by serine/threonine protein kinases composed of a catalytic subunit called cyclin-dependent kinase (Cdk) and a regulatory unit named cyclin.[22] The activity of cyclin D-dependent kinases Cdk4 and Cdk6 is regulated by mitogenic hormones and by binding of cyclin-dependent kinase inhibitor (CDKI) of the INK4A family, like $p16^{INK4A}$, and of the Cip/Kip family members $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$.[23,24] Active Cdk4/6 complexes phosphorylate and inactivate pRB, p107 and p130, thus promoting the activity of E2F transcription factors and the expression of genes essential for the onset of S phase and mitosis.[25] A second important cell cycle and growth regulator is the proto-oncogene c-myc. It is regulated by mitogenic hormones and seems to play an important,[26–29] although not necessarily essential,[30] role in the G1/S transition. c-Myc promotes progression through the cell cycle by induction of D-type cyclins,[31] cyclin E[32] and cyclin A.[33] It stimulates cyclin E/Cdk2 activity by relieving the kinase complex from $p27^{Kip1}$ inhibition via reassortment of this inhibitor to cyclin D/$Cdk4$[34,35] or by induction of the regulatory subunit Cul1 of the $p27^{Kip1}$ degrading ubiquitin–ligase complex SCF^{SKP2}.[36]

It has been known for a long time that GC treatment causes cell cyle arrest in human lymphoblastic leukemia cells; however, the mechanism has remained largely unknown. In P1798 mouse T-lymphoma cells, GC treatment in combination with serum withdrawal caused cell cycle arrest and apoptosis associated with repression of c-myc and cyclin D3.[37] Cell cycle arrest and apoptosis were prevented by ectopic expression of c-myc together with cyclin D3 (but not either one alone) in this system. GC-induced cell cycle arrest and apoptosis have also been investigated in two human osteosarcoma cell lines expressing transgenic rat GR.[38,39] Cell cycle arrest was associated with either repression of Cdk4, Cdk6, cyclin D3 and c-myc (in U2OS that undergo apoptosis as well) or accumulation of $p21^{Cip1}$ and $p27^{Kip1}$ (in SaOS that do not apoptose), and this was correlated with the presence or absence of functional pRB, respectively.

Regarding a possible interplay between cell cycle arrest and apoptosis in human CCRF-CEM T-ALL cells, we showed that transgenic bcl-2 delayed apoptotic events such as caspase activation, but did not affect G1 arrest.[14] This implied that G1 arrest is not a consequence of apoptotic cleavage of critical cell cycle regulatory proteins but left open whether the regulatory events leading to cell cycle arrest constitute the death signal upstream of bcl-2. In addition, $p16^{INK4A}$-induced G1 arrest dramatically sensitized CCRF-CEM cells to apoptosis[40] supporting the hypothesis that G1 phase might facilitate apoptosis.

In the work reported herein, we investigated how GC interfere with cell cycle progression and how this relates to apoptosis induction in human CCRF-CEM cells. We observed corresponding alterations in a number of cell cycle regulators, most of which might be related to GC repression of cyclin D3 and c-myc. Using several transfected and transduced CCRF-CEM cell lines, we analyzed the functional significance of cyclin D3 and c-myc repression for GC-induced cell cycle arrest and whether this is a prerequisite for GC-induced apoptosis in human leukemic cells.

Results

GC-mediated G1 arrest is associated with accumulation of $p27^{Kip1}$ and repression of c-myc, cyclin D3 and their downstream targets

To address the mechanism of GC-induced proliferation arrest, we investigated the effect of GC on cell cycle regulatory genes in human CCRF-CEM T-ALL cells with conditional expression of bcl-2. In these cells, apoptosis is delayed by 24 h,[14] allowing the analysis of gene expression in the absence of apoptosis-induced secondary changes. Treatment with 100 nM dexamethasone for 36 h induced accumulation of cells in G1 (Figure 1a). Cyclin D3, the only D-type cyclin expressed in CCRF-CEM cells,[41] was repressed to undetectable levels (Figure 1b). Its partner molecule Cdk4 was reduced while Cdk6 expression appeared unaltered (Figure 1b) but, as expected in the absence of cyclin D3, their kinase activity was markedly reduced (shown for Cdk4 in Figure 1d). pRB, the major Cdk4/6 target, was mainly present in its faster migrating, hypophosphorylated form (Figure 1b). Most likely as a consequence of pRb hypophosphorylation, the expression of its downstream target E2F-1 was also markedly reduced. In addition, expression of the proto-oncogene c-myc, another important regulator of G1-phase progression and cell growth, was almost completely repressed. The suppression of the cyclin D3/pRb and c-myc downstream targets E2F-1, dihydrofolate reductase (DHFR) and prothymosin-α (PTMα) underscored that the cyclin D3/pRB and c-myc pathway were inactivated by GC (Figure 1c).

Cyclin A protein was absent (Figure 1b), but unexpectedly, cyclin E and its partner Cdk2 were still expressed in GC-treated, G1-arrested cells. The CDKI $p27^{Kip1}$, however, was increased after GC treatment, explaining why these cyclin E/Cdk2-expressing cells did not enter S phase. To address this phenomenon more directly, we immunopurified Cdk2 complexes from GC-treated cells and found that $p27^{Kip1}$ coimmunoprecipitated with Cdk2 which was markedly inactivated in GC-treated cells (Figure 1e). This finding might explain why GC-treated cells did not enter S phase in spite of considerable cyclin E/Cdk2 levels. Other CDKIs, namely $p21^{Cip1}$ and $p57^{Kip2}$ that are regulated by GC in other experimental systems,[39,42,43] were either not expressed ($p21^{Cip1}$) or not elevated ($p57^{Kip2}$) in these GC-treated cells (not shown).

The data so far suggested that most of the observed alterations in cell cycle regulator expression and/or function might be secondary to repression of cyclin D3 and c-myc on the one hand and accumulation of $p27^{Kip1}$ on the other hand. Thus, regulation of these three proteins might be critical for GC-mediated cell cycle arrest and was therefore investigated in more detail.

Cyclin D3 and c-myc are independently regulated by GC, but $p27^{Kip1}$ is not

We first determined whether cyclin D3, c-myc and $p27^{Kip1}$ were regulated at the mRNA level. As shown in the time course experiments in Figure 2, c-myc was repressed at the mRNA level already after 3 h, cyclin D3 mRNA declined much more slowly and $p27^{Kip1}$ steady-state mRNA levels were not regulated by GC at all (Figure 2a). Cyclin D3 and $p27^{Kip1}$, in spite of their late (cyclin D3) or absent ($p27^{Kip1}$) regulation at the mRNA level, showed pronounced changes in protein expression (Figure 2b). Cyclin D3 started to decline after 6 h, and $p27^{Kip1}$ levels increased at 12 h after initiation of GC treatment. To study the molecular mechanism of c-myc and cyclin D3 mRNA repression, CCRF-CEM cells were treated with dexamethasone for 3 or 12 h and transcription was then blocked by addition of actinomycin D (ActD). The decline in mRNA levels, as measured by Northern blot analysis, suggests that the stability of c-myc mRNA was not altered (Figure 2c) whereas cyclin D3 mRNA stability was reduced by GC (Figure 2d).

Since c-myc repression clearly preceded the regulation of cyclin D3 and $p27^{Kip1}$ and since c-myc has been shown to influence expression of D-type cyclins and $p27^{Kip1}$,[34,35,37] we investigated whether cyclin D3 and $p27^{Kip1}$ regulation might be secondary to c-myc repression in CCRF-CEM cells. To address this possibility, we treated CCRF-CEM cells carrying a conditional *MYC* allele[19] with GC and/or doxycycline and probed the cells for expression of cyclin D3 and $p27^{Kip1}$. As

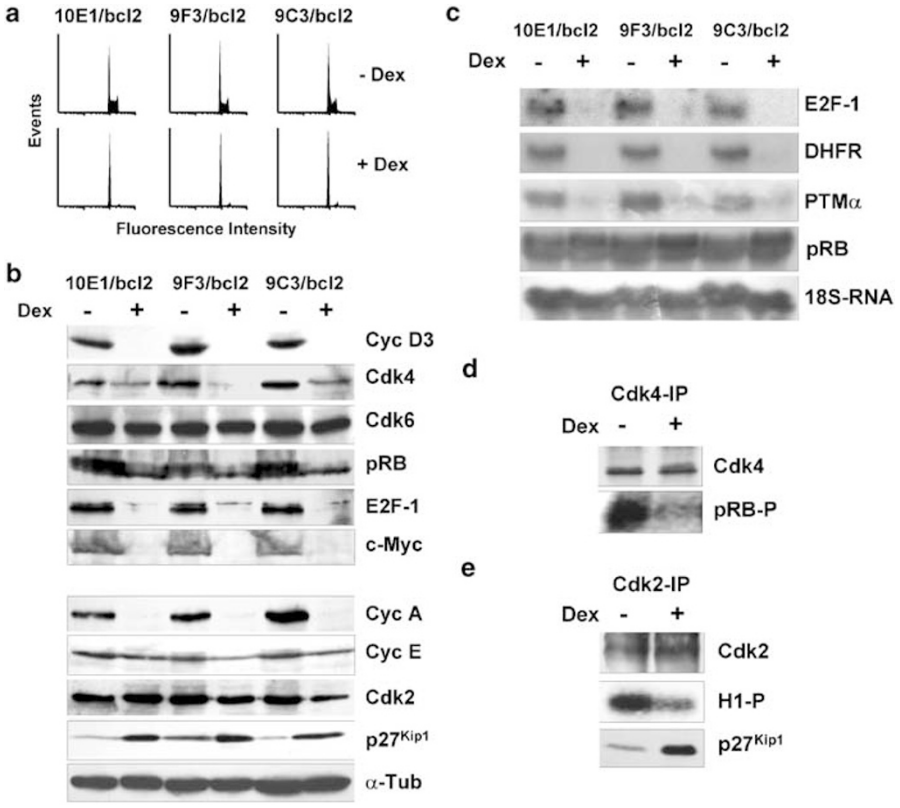


Figure 1 GC-mediated cell cycle arrest is associated with regulation of multiple G1/S transition regulatory proteins. Bcl-2-expressing CCRF-CEM cells 9C3/bcl2, 9F3/bcl2 and 10E1/bcl2, treated with 100 nM dexamethasone for 36 h were analyzed by FACS analysis (**a**), immunoblotting (**b**), Northern blotting (**c**), immunoprecipitation and kinase assays (**d** and **e**). Apoptosis and cell cycle distribution were measured by FACS analysis of PI-stained nuclei (**a**). Protein levels were analyzed by antibodies directed against cyclin D3, Cdk4, Cdk6, pRB, E2F-1, c-myc, cyclin E, Cdk2, $p27^{Kip1}$, cyclin A and α-tubulin (**b**). mRNA steady-state levels were examined by Northern blotting using ^{32}P-labeled cDNA probes to E2F-1, pRB, DHFR, PTMα and 18S-RNA as a loading control (**c**). 9F3/bcl2 cells were cultured for 36 h in the presence or absence of 100 nM dexamethasone. Cdk4 and Cdk2 kinase complexes were immunoprecipitated by polyclonal antibodies for kinase activity assays and for immunoblot analysis of precipitated proteins. GST-pRB and histone H1 were used as substrates for Cdk4 and Cdk2, respectively. Labeled substrate was detected by autoradiography. Precipitated kinases and Cdk2-bound $p27^{Kip1}$ were detected by immunoblot analysis (**d** and **e**)

shown in Figure 3a, addition of doxycycline induced ectopic c-myc expression and prevented GC-induced reduction of c-myc, but did not elevate cyclin D3 levels, suggesting that repression of cyclin D3 by GC is not a consequence of c-myc downregulation. In contrast, $p27^{Kip1}$ induction was abrogated by transgenic c-myc. The induced c-myc protein was functional since it restored the expression of PTMα, a known c-myc target gene,[44] in GC-treated cells (Figure 3b). We concluded from these experiments that downregulation of cyclin D3 is not a consequence of suppressed c-myc but that induction of $p27^{Kip1}$ is downstream of c-myc repression in these GC-treated human leukemia cells.

To determine whether c-myc and $p27^{Kip1}$ regulation might be secondary to cyclin D3 repression, we generated stably transfected CCRF-CEM sublines with tetracycline-inducible cyclin D3 expression. Two of these cell clones, referred to as 4E9/d3 and 7F4/d3, showed doxycycline-dependent induction and tight regulation of ectopic cyclin D3 mRNA (Figure 4a) and protein (Figure 4b). As proof of functionality of the transgene, induction of cyclin D3 in these clones correlated with increased ^{3}H-thymidine incorporation (Figure 4c). This indicates that a higher proportion of cells were in S phase and suggests that cyclin D3 is a rate-limiting factor for S-phase entry in these cells. Regarding a possible upstream role of cyclin D3 repression in GC regulation of c-myc and $p27^{Kip1}$, Figure 4d shows that ectopic cyclin D3 did not prevent GC-induced reduction of c-myc, but abrogated GC treatment-associated $p27^{Kip1}$ accumulation.

Since we had provided evidence that Cdk2 kinase activity (but not protein) was reduced in GC-treated cells because of increased association with $p27^{Kip1}$ (Figure 1e), we wondered whether ectopic cyclin D3 (which prevents $p27^{Kip1}$ accumulation) might restore Cdk2 activity in GC-treated cells. As shown in Figure 4e, Cdk2 activity was completely restored by ectopic cyclin D3 in GC-treated cells and this was associated with lower levels of $p27^{Kip1}$ bound to Cdk2. The reduction of Cdk4 activity in GC-treated cells was also prevented by cyclin D3.

We concluded from these experiments that cyclin D3 and c-myc are regulated independently of each other, whereas $p27^{Kip1}$ induction was a secondary, but potentially important phenomenon. We next investigated the functional role of the repression of cyclin D3 and c-myc in GC-induced cell cycle arrest and/or apoptosis.

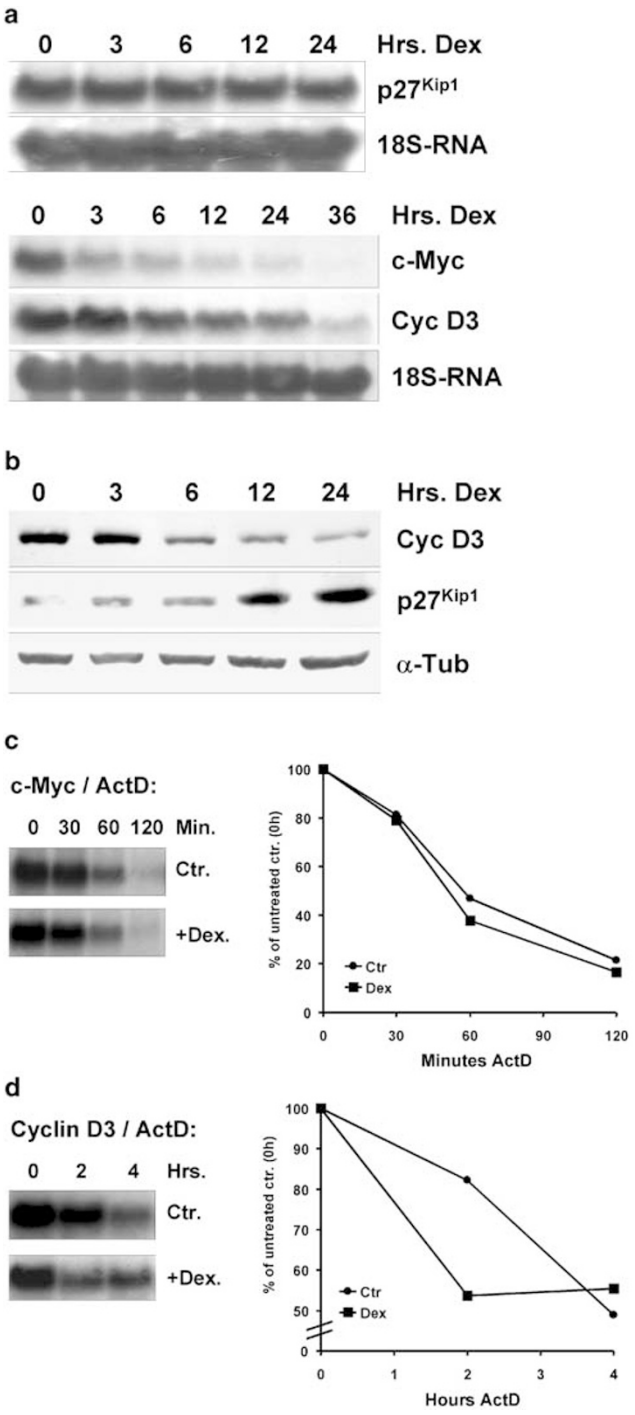


Figure 2 c-Myc, $p27^{Kip1}$ and cyclin D3 are regulated on a transcriptional, on a post-transcriptional level and by mRNA stability, respectively. Bcl-2-expressing cells were cultured for the times indicated in the presence of 100 nM dexamethasone. RNA samples were subjected to Northern blot analysis with probes for c-myc, cyclin D3, $p27^{Kip1}$ and 18S-RNA (**a**). Protein expression was assessed by immunoblotting with antibodies directed against cyclin D3, $p27^{Kip1}$ and α-tubulin as loading control (**b**). CCRF-CEM cells were cultured with 100 nM dexamethasone for 3 h (**c**) and 12 h (**d**) and then treated with actinomycin D for the times indicated. Decline of c-myc and cyclin D3 mRNA was measured by Northern blot analysis and quantified in a Packard Instant Imager

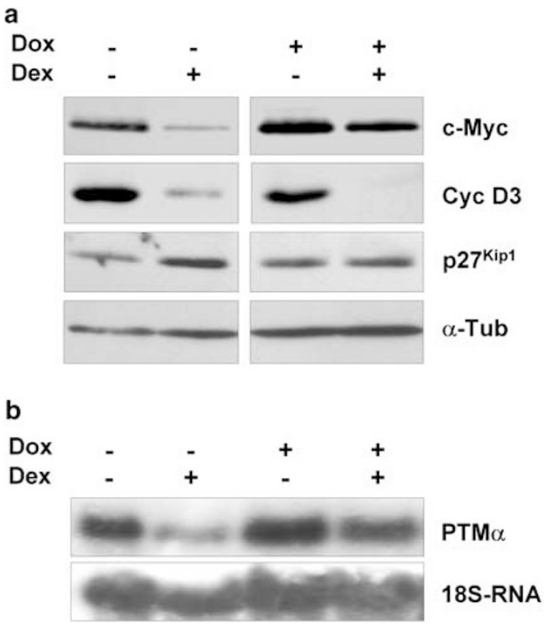


Figure 3 GC regulation of cyclin D3 is independent of c-myc. Tetracycline-regulated conditional c-myc-expressing D64/myc cells were maintained in the presence or absence of 200 ng/ml doxycycline and/or 100 nM dexamethasone for 36 h. c-Myc, cyclin D3, $p27^{Kip1}$ and α-tubulin protein levels were determined by immunoblot analysis (**a**). To proove functionality of ectopic c-myc, D64/myc cells were cultured for 24 h in the presence or absence of 100 nM dexamethasone and/or 200 ng/ml doxycycline. Total RNA from these cells was subjected to Northern blot analysis with ^{32}P-labeled cDNA probes to PTMα and 18S RNA (**b**)

Conditional expression of cyclin D3 does not prevent apoptosis but restores cell cycle progression in GC-treated CCRF-CEM cells

To determine the role of cyclin D3 in apoptosis induction, cells with conditional expression of cyclin D3 were treated with dexamethasone for 48 h in the presence or absence of doxycycline. Although addition of doxycycline maintained cyclin D3 levels in GC-treated cells comparable to those in untreated controls (Figure 5b), the induction of apoptosis, as measured by forward/sideward light scatter FACS analyses, propidium iodide (PI) and annexin-V staining, was not affected (Figure 5a). Moreover, transgenic cyclin D3 had no detectable effect on sensitivity to different dexamethasone concentrations in dose–response curves (data not shown).

Next, we wanted to assess the influence of cyclin D3 overexpression on cell cycle progression in GC-treated cells, but the onset of cell death made measurements of such effects difficult. To circumvent this problem, we delayed cell death by transducing the cyclin D3-expressing cell lines 4E9/d3 and 7F4/d3 and the parental rtTA-expressing cell line 2C8 (as a control) with a retrovirus coexpressing bcl-2 and enhanced green fluorescent protein (EGFP). A FACS-sorted homogenous green fluorescent population of infected cells (Figure 6a) with elevated bcl-2 expression (Figure 6b) and delayed cell death (data not shown) was then cultured for 36 h in the presence or absence of dexamethasone and/or doxycycline (to induce the transgenic cyclin D3). In the absence of doxycycline, DNA synthesis, as measured by incorporation of ^{3}H-labeled thymidine, was repressed by GC

to about 50% of untreated controls. Ectopic expression of cyclin D3 almost completely restored DNA synthesis in the presence of GC (Figure 6c). FACS cell cycle distribution analysis of PI-stained nuclei showed that ectopic expression of cyclin D3 prevented GC-mediated accumulation of cells in G1 (Figure 6d).

The combined data suggested that GC-induced repression of cyclin D3 in CCRF-CEM leukemia cells is necessary for GC-mediated cell cycle arrest but not for apoptosis. Since c-myc levels remained suppressed in cyclin D3-expressing GC-treated cells (see Figure 4d), we investigated whether c-myc is involved in the regulation of S-phase entry.

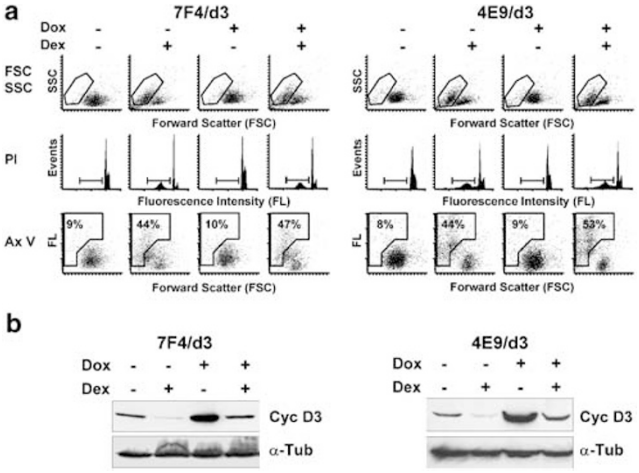


Figure 5 Cyclin D3 does not prevent GC-induced apoptosis. CCRF-CEM derivatives with doxycycline-inducible expression of cyclin D3 (7F4/d3 and 4E9/d3) were cultured in the presence or absence of 200 ng/ml doxycycline, with and without 100 nM dexamethasone for 48 h and subjected to determination of apoptosis by forward/sideward light scatter analysis and PI and annexin-V staining (**a**). Similarly treated cells (100 nM dexamethasone, 200 ng/ml doxycycline for 36 h) were subjected to immunoblot analysis with antibodies against cyclin D3 and α-tubulin as a loading control (**b**). The data presented are representative for three independent experiments

Ectopic expression of c-myc restores cell cycle progression in GC-treated CCRF-CEM cells

We have previously reported that restoring GC-induced repression of c-myc by tetracycline-induced expression of transgenic c-myc does not reduce, but rather increases, GC-induced apoptosis in CCRF-CEM leukemia cells.[19] Although c-myc was markedly induced by doxycycline, these cells expressed some ectopic c-myc in the absence of doxycycline due to leakyness of the system.[19] In the present study, we used this observation to our advantage because it allowed us to study the effects of a weak constitutive overexpression of c-myc in GC-treated cells. As shown previously, this weak

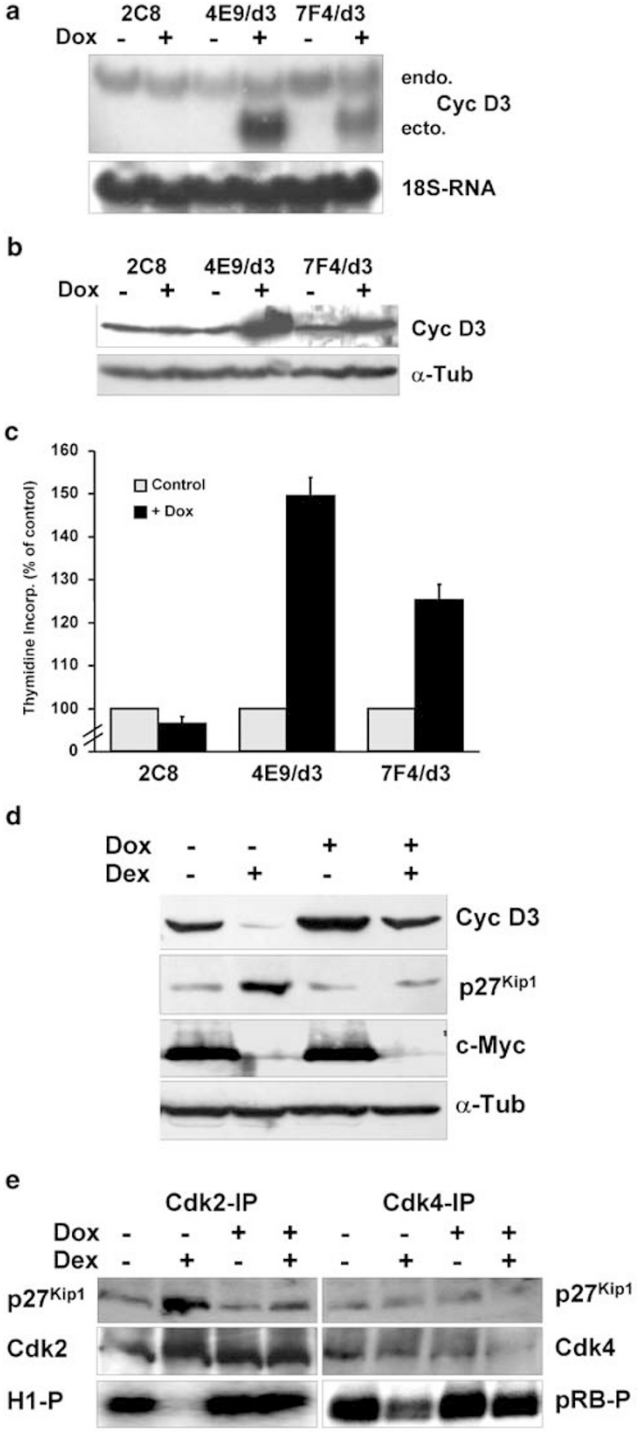
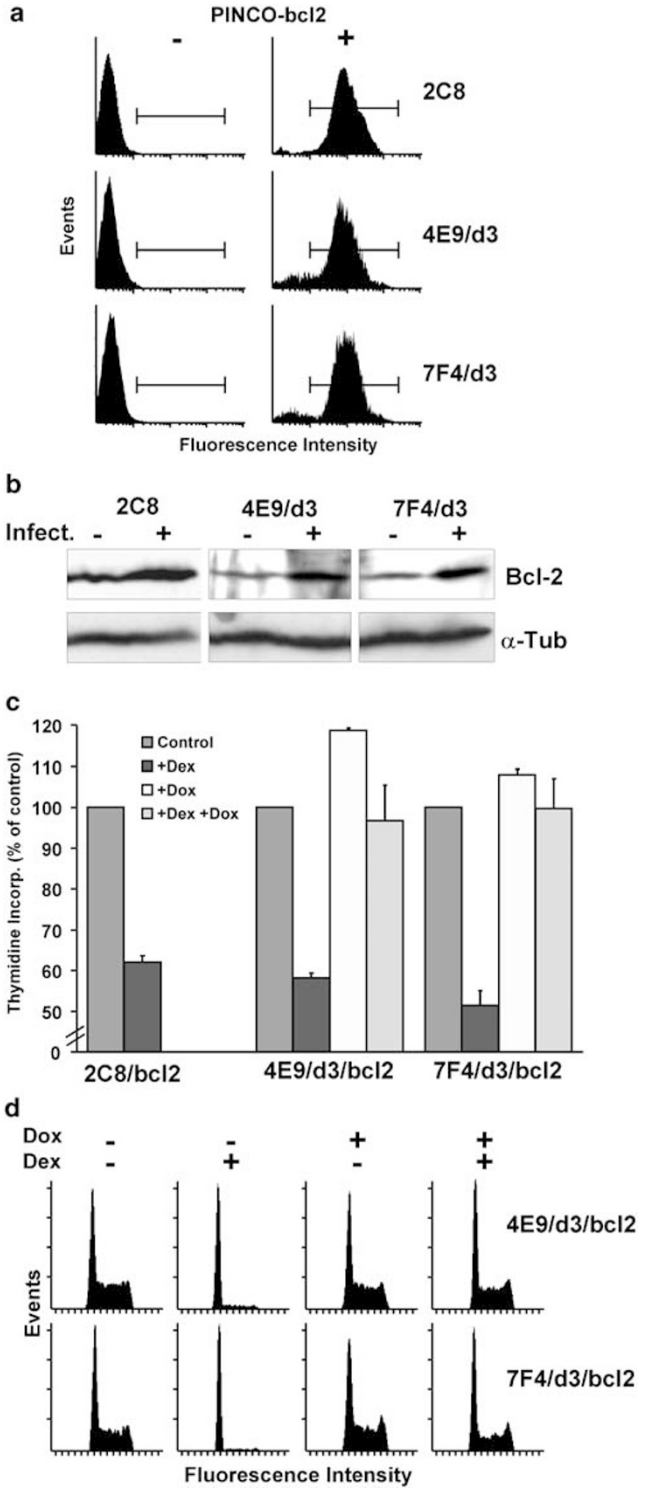


Figure 4 Characterization of CCRF-CEM derivatives with doxycycline-regulated expression of cyclin D3. The rtTA-expressing parental C7H2-2C8 cell line and its cyclin D3-transfected derivatives 4E9/d3 and 7F4/d3 were cultured in the presence or absence of 200 ng/ml doxycycline for 48 h. Samples were subjected to Northern blot analysis with ^{32}P-labeled cDNA probes to cyclin D3 and 18S-RNA (**a**) and immunoblot analysis with cyclin D3- and α-tubulin-specific antibodies (**b**). DNA synthesis was measured by incorporation of ^{3}H-labeled thymidine. A representative experiment performed in triplicate is shown (**c**). Cyclin D3-expressing 7F4/d3 cells were cultured in the presence or absence of 200 ng/ml doxycycline and/or 100 nM dexamethasone for 36 h. Cyclin D3, c-myc, p27^{Kip1} and α-tubulin protein levels were determined by immunoblot analysis (**d**). 4E9/d3 cells were cultured for 24 h in the presence or absence of 100 nM dexamethasone with or without 200 ng/ml doxycycline. Cdk4 and Cdk2 kinase complexes were immunoprecipitated by polyclonal antibodies. GST-pRB and histone H1 were used as substrates for Cdk activity assays. Precipitated kinases and Cdk2-bound p27^{Kip1} were detected by immunoblot analysis (**e**)

constitutive expression did not interfere with GC-induced apoptosis, but when cell death was prevented by ectopic bcl-2 (Figure 7a and b) we found that thymidine incorporation was restored to levels similar to untreated cells. As shown in Figure 7c, GC only slightly reduced thymidine uptake in CCRF-CEM cells with constitutive, low-level c-myc expression within 40 h, while it significantly repressed thymidine uptake in the control cell line 2C8/bcl2. This suggested that interfering with c-myc downregulation by transgenic c-myc prevented GC-induced cell cycle arrest, despite continuous repression of cyclin D3.



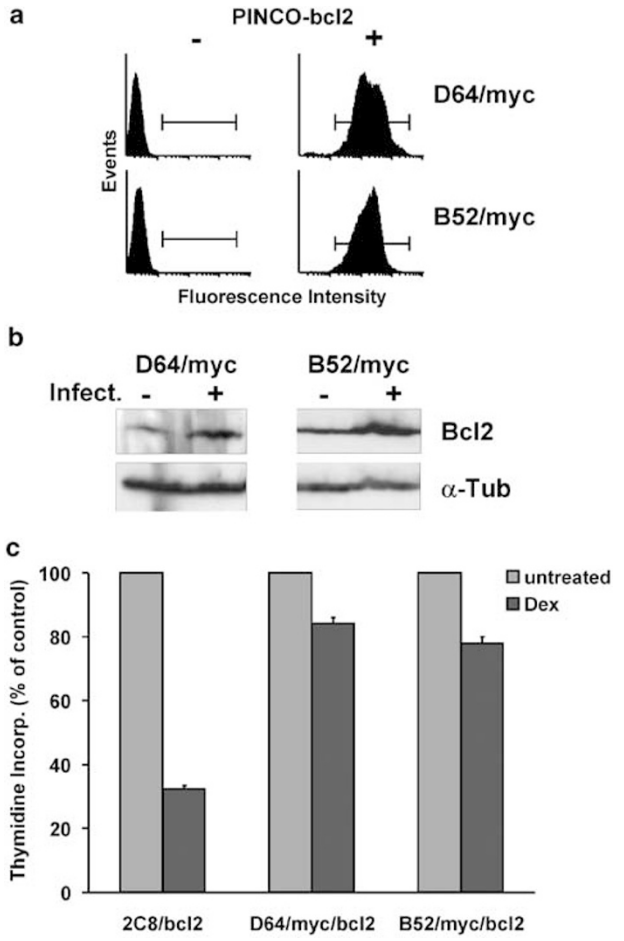


Figure 7 Transgenic c-myc restores cell cycle progression in GC-treated CCRF-CEM cells. PINCO-bcl-2-infected green fluorescent B52/myc and D64/myc cells were enriched via FACS sorting (**a**) and subjected to immunoblot analysis with monoclonal anti-bcl-2 and anti-α-tubulin antibodies (**b**). FACS-sorted D64/myc/bcl2, B52/myc/bcl2 and 2C8/bcl2 cells were cultured for 40 h in the presence or absence of 100 nM dexamethasone. DNA replication was measured by incorporation of radioactive ^{3}H-thymidine. A representative experiment performed in triplicate is shown (**c**)

Figure 6 Conditional expression of cyclin D3 restores cell cycle progression in GC-treated CCRF-CEM cells. C7H2 subclones 2C8, 4E9/d3 and 7F4/d3 were left untreated (**a**, left panel) or infected with the amphotropic retrovirus PINCO-bcl-2 (**a**, right panel) and subjected to FACS analyses. Uninfected and infected FACS-sorted cells were subjected to immunoblot analysis with a monoclonal anti-bcl-2 antibody and an antibody directed against α-tubulin as loading control (**b**). The sorted, retroviral bcl-2-expressing cells were further cultured for 36 h in the presence or absence of 200 ng/ml doxycycline and/or 100 nM dexamethasone and analyzed for incorporation of radioactive ^{3}H-thymidine. A representative experiment performed in triplicate is shown (**c**). For cell cycle determination, the same cells were maintained for 44 h in the presence or absence of 200 ng/ml doxycycline and/or 100 nM dexamethasone and subjected to FACS analysis of PI-stained nuclei. The data are representative for three independent experiments (**d**)

Coexpression of c-myc and cyclin D3 does not prevent GC-induced apoptosis

To test whether ectopic expression of cyclin D3 together with c-myc would prevent GC-induced cell death, we transduced conditional c-myc-expressing CCRF-CEM cells with a retrovirus expressing cyclin D3 and EGFP (Figure 8a and b). When treated with GC for 36 h, FACS-sorted cyclin D3-expressing cells underwent about equal amounts of apoptosis compared to uninfected cells regardless of whether transgenic c-myc was induced by doxycycline or not (Figure 8c). Thus, GC-induced apoptosis did not depend on repression of these two genes. Moreover, since cell cycle arrest is prevented by transgenic expression of either cyclin D3 or c-myc, GC-induced apoptosis occurs independently of GC-induced cell cycle effects.

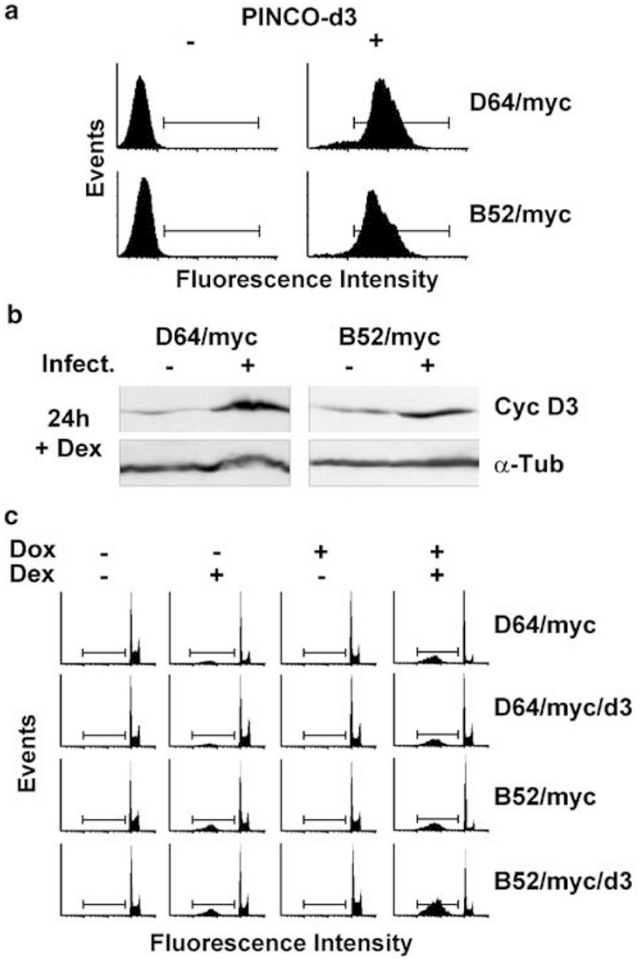


Figure 8 Coexpression of cyclin D3 and c-myc does not prevent GC-induced apoptosis. B52/myc and D64/myc cells were infected with a PINCO-d3 retrovirus for constitutive expression of cyclin D3 and enriched via FACS sorting (**a**). Sorted cells were treated with 100 nM dexamethasone for 24 h (to repress endogenous cyclin D3) and subjected to immunoblot analysis for cyclin D3 and α-tubulin (**b**). D64/myc/d3 and B52/myc/d3 cells were cultured for 36 h in the presence or absence of 100 nM dexamethasone and/or 200 ng/ml doxycycline. Apoptosis was detected by FACS analysis of PI-stained nuclei (**c**)

Discussion

In this study, we addressed the functional role of two GC-suppressed genes, cyclin D3 and c-myc, on cell cycle progression and apoptosis in human leukemia cells. We found that GC-induced G1 arrest depended on repression of both cyclin D3 and c-myc, and that cell cycle arrest and apoptosis were not causally related. Both cell cycle regulators prevented accumulation of the cell cycle inhibitor $p27^{Kip1}$, which was critical for inhibition of cyclin E/Cdk2 kinase activity in GC-treated cells.

Cyclin D3 is the only D-type cyclin expressed in CEM cells,[41] and is thus required to inactivate pRB and to allow subsequent progression through G1 and entry into S phase. CEM cells carry a homozygous deletion of the *INK4A* locus that renders them deficient for the G1-CDK inhibitor $p16^{INK4A}$.[45] As in other cell types with similar deficiencies, pRB then often remains wild type, but is functionally inactivated by high cyclin D/CDK activities.[46] In such cells, expression of $p16^{INK4A}$ results in a G1 arrest that depends on the presence of wild-type pRB.[47] We have previously shown that CEM cells can be arrested by $p16^{INK4A}$ expression,[40] which implies that pRB is wild-type and D-type cyclins are essential for proliferation in these cells. Thus, marked repression of cyclin D3 may well explain G1 arrest in CCRF-CEM cells. Cyclin D3, however, was downregulated with fairly slow kinetics reaching its nadir only after 36 h of GC treatment and making it an unlikely direct transcriptional GC target. This conclusion is further supported by the observation that cyclin D3 repression in human CCRF-CEM cells is due to reduced mRNA stability, a GC effect also described for P1798 mouse T-lymphoma cells.[48]

c-Myc, on the other hand, was rapidly downregulated by GC in CCRF-CEM cells. This regulation has been ascribed to increased c-myc-specific mRNA degradation[49] and/or, more recently, to a direct transcriptional mechanism.[50] However, in human CCRF-CEM leukemia cells, mRNA stability was not altered by GC treatment suggesting that a direct transcriptional GC effect was responsible. As outlined in the Introduction, several mechanisms, such as reduction of $p27^{Kip1}$ stability, induction of cyclin D, cyclin E and cyclin A, have been proposed to explain how c-myc might contribute to cell cycle progression; hence, its repression is consistent with cell cycle arrest.

Surprisingly, we found that both transgenic cyclin D3 and c-myc on their own restored cell cycle progression. Since c-myc did not revert cyclin D3 repression and *vice versa,* the cell cycle proceeded in the apparent absence of cyclin D3 and c-myc, respectively. Identification of $p27^{Kip1}$ as a potential downstream target of both c-myc and cyclin D3 might explain this phenomenon. The association of $p27^{Kip1}$ with cyclin E/Cdk2 suggests that this CDKI acts as a critical repressor of cyclin E/Cdk2 activity in GC-treated leukemia cells. The fact that both c-myc and cyclin D3 prevented the GC-induced accumulation of $p27^{Kip1}$ and that transgenic cyclin D3 restored Cdk2 activity in GC-treated leukemia cells further supports this assumption.

The mechanism of how cyclin D3 and c-myc mediate the above effect on $p27^{Kip1}$ expression is unclear. As equal amounts of this CDKI copurified with Cdk4, irrespective of

whether the cells expressed ectopic cyclin D3 or were treated with GC, sequestration of $p27^{Kip1}$ by cyclin D3/Cdk4 complexes does not seem to be a likely mechanism. Alternatively, $p27^{Kip1}$ accumulation could have been prevented by induction of cyclin E via the pRB/E2F pathway as suggested previously.[51] c-Myc, on the other hand, might counteract $p27^{Kip1}$ accumulation by induction of cyclin E[32] or by accelerated ubiquitin-mediated proteolysis.[36] Further studies are required to shed light on the connection between c-myc and $p27^{Kip1}$ and to clarify whether $p27^{Kip1}$ accumulation is indeed indispensable for GC-induced cell cycle arrest in human leukemia cells. We are currently using RNA interference to study $p27^{Kip1}$ function in GC-treated CCRF-CEM cells.

In contrast to human leukemia cells, GC repress $p27^{Kip1}$ in mouse thymocytes and increase entry into the cell cycle. When cell cycle progression is blocked by inhibition of Cdks, thymocyte apoptosis is prevented suggesting that cell cycle progression might be required for thymocyte apoptosis (reviewed in Winoto and Littman[52]). However, Montani *et al*.[53] observed that human peripheral T lymphocytes, arrested in the G0/G1 phase of the cell cycle, were susceptible, while proliferating T cells were resistant to dexamethasone-mediated apoptosis.

While both cyclin D3 and c-myc repression was essential for GC-induced G1 arrest in human CCRF-CEM leukemia cells, neither one, nor their combination, was required for apoptosis induction. Thus, we show for the first time that GC-induced apoptosis is not a mere consequence of GC-induced cell cycle arrest, but an independent effect of this hormone on human ALL cells.

Materials and Methods

Cell lines, culture conditions and reagents

CCRF-CEM cells and CCRF-CEM-derived clones were cultured in RPMI 1640 (BioWhittaker, Verviers, Belgium) containing 10% fetal calf serum (FCS; Gibco BRL, Paisley, UK), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine (Gibco BRL) at 5% CO_2 and 37°C in saturated humidity. CCRF-CEM-C7H2,[54] rtTA expressing C7H2-2C8,[19] conditional bcl-2[14] and c-myc[19] cell lines have been described previously. Phoenix packaging cells for helper-free production of amphotropic retroviruses, kindly provided by G. Nolan, Stanford,[55] were cultured in DMEM (Sigma, Vienna, Austria), containing 5% bovine calf serum. Dexamethasone was stored as 10 mM stock solution in 100% ethanol, and doxycycline was kept as 1 mg/ml solution dissolved in phosphate buffered saline (PBS). All reagents were from Sigma (Vienna, Austria) unless indicated otherwise. For each experiment, mid log phase cultures (5×10^5 cells/ml) were centrifuged and resuspended in fresh medium at a concentration of approximately 2.5×10^5 cells/ml.

RT-PCR, plasmids and cDNAs

The tetracycline-inducible plasmid pUHD10-3-cycD3 was constructed by amplifying the complete coding region of cyclin D3 by PCR using cDNA from CCRF-CEM T ALL cells and primers 5′-TTT GGA TCC CCC TGC CTG TTC GCT G-3′ and 5′-TTT GTC GAC GCG GGG ATG GGT AGG A-3′ and by subcloning into the *Bam*HI and *Sal*I sites of the tetracycline-responsive plasmid pUHD10-3.[56] pKS-tkHyg is a pBluescriptII-KS (Stratagene, La Jolla, CA, USA) vector containing the hygromycin B resistance gene under the control of a thymidine kinase promoter and a Simian virus (SV40) polyadenylation site. PINCO-bcl-2 was constructed by cloning full-length bcl-2 from pUHD-bcl-2[14] into the *Eco*RI site of the EGFP-expressing retroviral vector PINCO.[54]

Stable transfections

For stable introduction of cyclin D3, C7H2-2C8 cells were transfected by electroporation, as detailed previously.[13] Briefly, approximately 1×10^7 mid-log phase cells were electroporated with 30 μg of the linearized pUHD10-3-cycD3 plasmid and 25 μg of pKS-tkHyg plasmid at 300 V and 500 μF using a Bio Rad Gene Pulsar (BioRad, Hercules, CA, USA). 2 days after transfection, the cells were exposed to hygromycin B selection and resistant clones were analyzed for doxycycline inducibility of cyclin D3 by Northern blot and immunoblot analysis.

Production of retroviruses and retroviral infection

About 1×10^6 Phoenix packaging cells were transfected with 5 μg PINCO-bcl-2 using the calcium phosphate coprecipitation method.[57] After transfection, cells were cultured for 24 h in DMEM and for another 24 h in RPMI 1640. The retrovirus-containing supernatants were filtered through 0.45 μm syringe filters (Sartorius, Göttingen, Germany) and used to infect CCRF-CEM leukemia cells. Transient transfection and infection was repeated three times and analyzed by fluorescence microscopy. Green fluorescent CCRF-CEM cells were sorted with a Becton Dickinson FACS-Vantage.

Northern blot analysis

Northern blot analysis was performed as described.[58] Briefly, 10 μg of total RNA was separated on a denaturing, formaldehyde-containing 1% agarose gel and blotted overnight onto Zetabind™ nylon membranes (Cuno, Meridien, CO, USA). After UV crosslinking, filters were prehybridized to block nonspecific binding at 65°C and hybridized for at least 12 h to ^{32}P-labeled, heat-denatured cyclin D3, c-myc, pRB, E2F-1, PTMα, DHFR and 18S-RNA cDNA probes. The washed blots were exposed to Agfa Curix X-ray films with an amplifying screen for several hours to days and quantified in a Packard Instant Imager. Between hybridizations, the blots were stripped by boiling in 0.1% SDS.

Determination of apoptosis and proliferation

Apoptosis was determined by PI staining of nuclei[59] and forward/sideward scatter analysis using a Becton Dickinson FACScan. Briefly, cells were centrifuged and resuspended in hypotonic PI solution containing 0.1% NP-40. Cellular debris and small particles were excluded from FACS analysis, and stained nuclei in the sub-G1 marker window were considered to represent apoptotic cells. Annexin-V binding was determined using the TACS annexin-V–FITC kit (TREVIGEN, Gaithersburg, MD, USA), as described by the manufacturer. DNA synthesis was determined by incubating 5×10^4 cells with 4 μCi/ml ^{3}H-thymidine (NEN, Boston, MA, USA) for 6 h after which incorporated tritium was counted by liquid scintillation.

Immunoprecipitation and Cdk assays

For detection of Cdk-bound proteins and Cdk kinase activity, 2×10^7 cells were lysed in IP buffer (50 mM HEPES pH 7.5, 1% Triton X-100, 150 mM

NaCl, 2 mM EDTA, 25 mM β-glycerophosphate, 10% glycerol, 1 mM PMSF, 1 mM DDT, 50 mM NaF, 5 mM Na_3VO_4, 50 μg/ml aprotinin, 10 μg/ml leupeptin) for 30 min on ice. Lysates were clarified by centrifugation and precleared with Pansorbin™ (Calbiochem, La Jolla, CA, USA). Lysates were incubated at 4°C overnight in the presence of 1 μg of polyclonal rabbit antibodies (Pharmingen, Hamburg, Germany) directed against Cdk2 and Cdk4 and precipitated by 2.5 μg Pansorbin™. The pellets were washed three times in IP buffer and either resuspended in sodium dodecyl sulfate (SDS) sample buffer (SSB) for immunoblotting or washed twice in kinase buffer (25 mM HEPES pH 7.5, 2 mM $MnCl_2$, 20 mM $MgCl_2$, 1 mM NaF, 10 mM β-glycerophosphate, 1 mM Na_3VO_4). Pellets were resuspended in kinase buffer containing 10 μCi [γ^{32}-P]ATP and 2 μg histone H1 (Sigma) or 2 μg recombinant GST-pRB and incubated at 30°C for 30 min. Reactions were stopped by addition of 4 × SSB buffer and boiling (5 min at 95°C). Labeled proteins were resolved on denaturating polyacrylamide gels, which were dried and subjected to autoradiography in a Packard Instant Imager.

Immunoblotting

Identical numbers of cells were lysed on ice in lysis buffer (1% NP-40, 10 mM sodium fluoride in PBS) containing a cocktail of protease inhibitors and centrifuged at a maximum speed. The supernatant was mixed with 2 × SSB containing 10% β-mercaptoethanol and boiled. Samples were separated by SDS-PAGE on 7.5–15% polyacrylamide gels, and transferred to nitrocellulose membranes by a Hoeffer semi-dry transfer apparatus. The membranes were blocked with TRIS-buffered saline (TBS) blocking buffer containing 1% Tween 20 and 5% nonfat dry milk, incubated with primary antibodies specific for human cyclin D3, cyclin E, cyclin A, $p27^{Kip1}$, Cdk2, Cdk4, Cdk6, E2F-1, c-myc, bcl-2 (Pharmingen), pRB and α-Tubulin (Oncogene Research, Cambridge, MA, USA), washed and incubated with anti-mouse or anti-rabbit horseradish-peroxidase-conjugated secondary antibodies (Amersham, Buckinghamshire, UK). The blots were developed by enhanced chemiluminescence (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions and exposed to Agfa Curix X-ray films. Stripping and reprobing was performed as described by the manufacturer.

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