Glucocorticoid Regulation of G_1 Cyclin-dependent Kinase Genes in Lymphoid Cells[1]

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Abstract

These experiments were undertaken to study cell cycle-dependent regulation of expression of genes encoding cyclin-dependent kinases (Cdks).[3] P1798 T-lymphoma cells were studied as a model system, since these cells undergo reversible G_0 arrest within 24 h after addition of 0.1 μM dexamethasone to mid log phase cultures. G_0 arrest is associated with inhibition of expression of several Cdks. The mRNAs encoding Cdk1 and Cdk4 decreased by 80–90% within 24 h. Fifty % inhibition of Cdk4 mRNA occurred within about 4 h, and 50% inhibition of Cdk1 mRNA was observed within 12–14 h. There was a slight decrease (<50%) in the abundance of the mRNAs encoding Cdk2 and Cdk5. Cdk6 mRNA did not decrease in glucocorticoid-treated cells. Cdk1 and Cdk2 protein levels were reduced by no more than 50–70% within 24 h after the addition of dexamethasone, and the amounts of Cdk5 and Cdk6 protein did not change. However, the amount of Cdk4 protein decreased by >90% under these circumstances. P1798 cells enter S phase in a synchronous fashion within 16–20 h after removal of dexamethasone. Cdk1, Cdk2, and Cdk5 mRNAs and proteins increased at or after the time that cells began to enter S phase. The mRNA encoding Cdk4 increased much more rapidly after removal of glucocorticoids, and a 5-fold increase in Cdk4 mRNA abundance was observed within 8 h after removal of the steroid. A corresponding increase in Cdk4 protein was observed, indicating that inhibition of *Cdk4* expression is more proximal to the glucocorticoid-induced blockade in G_1 progression. Glucocorticoids also inhibited *Cdk4* expression in cells that were arrested at the G_1 S boundary by thymidine block, but expression of *Cdk1* was not inhibited in such cells. This observation indicates that glucocorticoid regulation of *Cdk4* is not dependent on cell cycle progression, whereas inhibition of *Cdk1* is probably secondary to G_0 arrest. Nuclear run-on data indicate that dexamethasone inhibits transcription of *Cdk4* in P1798 cells. Furthermore, glucocorticoids inhibited expression of *Cdk4* in activated splenocytes, with no significant effect on *Cdk6* expression. Glucocorticoids regulate expression of several G_1 cyclin-dependent kinase genes. Some of these (such as *Cdk1*) are inhibited as cells withdraw into G_0. *Cdk4* appears to be directly regulated by glucocorticoids, and inhibition of this G_1 cyclin-dependent kinase may play a role in glucocorticoid-mediated G_0 arrest of lymphoid cells.

Introduction

The cell cycle may be viewed in its simplest context as consisting of alternating phases in which the genome is duplicated (S phase) and then distributed between daughter cells (M phase). Entry into each of these phases is governed by cell cycle-specific serine/threonine protein kinase complexes that catalyze rate-limiting steps in the initiation of either S or M phase (1–3). These kinases constitute the two major checkpoints that govern exit from G_1 and G_2 phases, and the mechanisms that entrain the activation and inactivation of these enzymes substantially define the molecular basis of cell cycle regulation. Consequently, the nature, function, and regulation of cell division control protein kinases has become a topic of great interest in cell biology, oncology, and endocrinology.

The most detailed understanding of regulation of cell cycle control kinases comes from analysis of yeast CDC[4] mutants (reviewed in Refs. 1, 2, and 4). The *CDC28* gene of *Saccharomyces cerevisiae* and *cdc2*$^+$ of *Schizosaccharomyces pombe* encode homologous M_r 34,000 serine/threonine protein kinases that comprise the catalytic subunit of the yeast CDC protein kinase complexes.

Yeast cells contain a single CDC kinase that catalyzes phosphorylation reactions that are critical to progression through cell cycle checkpoints at G_1 S and G_2 M. Cells of metazoan origin contain a dozen or more potential CDC serine/threonine protein kinases. Those kinases that are known to be regulated by cyclins are called Cdks. (The diversity of Cdks is reviewed in Ref. 5). Cdk1 is the M_r 34,000 vertebrate homologue of Cdc28/cdc2 (reviewed in Ref. 2). Related catalytic subunits, called Cdk2, Cdk3, Cdk4, Cdk5, Cdk6, and Cdk7, have been identified, and the corresponding cDNAs have been cloned (6–12).

The metazoan Cdks have specialized functions that apply to different phases of the cell cycle. Cdk1 is thought to be primarily involved in progression through the G_2 M checkpoint. Cdk1 is the catalytic subunit of the M-phase progression factor known as MPF (reviewed in Ref. 13). Cdk2 apparently functions in late G_1 or early S phase, whereas Cdk4 and Cdk6 are probably involved in progression through G_1 (14, 15). Because Cdk2, Cdk4, and Cdk6 func-

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[3] The following system of nomenclature will be used in reference to mammalian genes and gene products. The names of all genes will be given in italics with the first letter capitalized. All mammalian cyclin dependent kinases genes are abbreviated *Cdk* (*e.g.*, *Cdk4*). The names of gene products are the same as those of genes, except without italics, so that *Cdk4* is transcribed to yield Cdk4 mRNA, which is translated to yield Cdk4. Systematic nomenclature will be used in reference to yeast genes.

[4] The abbreviations used are: CDC, cell division cycle; Cdk, cyclin-dependent kinase; CAK, Cdk-activating kinase; Con A, concanavalin A; TGF, transforming growth factor; MEL, murine erythroleukemia.

tion subsequent to mitosis and prior to initiation of DNA replication, these three Cdks are called G_1 Cdks.

The activity of the G_1 CDC protein kinases is strictly regulated. At least four different regulatory motifs may be discerned: association with stimulatory subunits; association with inhibitory subunits; phosphorylation and dephosphorylation of critical tyrosine and threonine residues; and changes in the expression of the cognate genes. The best characterized regulatory mechanism involves changes in expression of the stimulatory subunits, the G_1 cyclins (reviewed in Refs. 14–16). There are at least two different classes of metazoan G_1 cyclins. Members of the D cyclin family, including cyclins D1, D2, and D3, associate with Cdk4 and Cdk6 (10, 17). The cyclin D-Cdk4/6 complex is capable of phosphorylating tumor suppressor gene products such as $p105^{Rb-1}$ *in vitro* and *in vivo* (18, 19). Cyclin E associates with Cdk2 (20). The cyclin E-Cdk2 complex can phosphorylate histone H1 and $p105^{Rb-1}$ (20, 21).

The activity of Cdks is also regulated by association with inhibitory subunits. The products of the *FAR1* and *SIC1* genes inhibit Cdc28 activity and cause G_1 arrest in budding yeast (22, 23); *rum1* encodes an inhibitor of G_1-specific, cdc2-catalyzed reactions in fission yeast (24). There are two families of mammalian proteins that function as inhibitors of CDC kinases (reviewed in Refs. 14, 15, and 25–28). One family of inhibitory proteins includes $p15^{INK4B}$ (29) and $p16^{INK4}$ (30). These inhibitors associate with Cdk4, Cdk6, or with cyclin D-Cdk4/6 complexes. Formation of the binary Ink4/4B-Cdk4/6 complex prevents binding of D cyclins and causes dissociation of preexisting cyclin D-Cdk4/6 complexes. A second class of inhibitors is exemplified by $p21^{Cip1}$ (31–34) and $p27^{Kip1}$ (35–38). These inhibitors bind to and inhibit cyclin-Cdk complexes containing cyclin D or cyclin E bound to Cdk2, Cdk4, or Cdk6.

Activation of Cdks also involves both phosphorylation and dephosphorylation reactions (reviewed in Ref 16 and 39–41). Yeast cdc2 is regulated by inhibitory phosphorylation of tyrosine 15 and by stimulatory phosphorylation of threonine 161. Mammalian G_1 Cdks are inactive until phosphorylated on critical threonine residues; phosphorylation of Thr-172 activates Cdk4 (42), and phosphorylation of Thr-160 activates Cdk2 (43). Phosphorylation of Cdk2 and Cdk4 is catalyzed by a Cdk-activating kinase (CAK), which is a complex between Cdk7 and cyclin H (43).

There is a direct relationship between phosphorylation of Cdks and association with either stimulatory or inhibitory subunits. For example, CAK phosphorylates Cdk2 or Cdk4 only when they are associated with appropriate cyclins. Cyclin kinase inhibitors of the "Ink" family ($p15^{Ink4B}$ and $p16^{Ink4}$) cause dissociation of cyclin D-Cdk4 complexes (29, 30) and prevent phosphorylation of Cdk4 by CAK. Binding of $p21^{Cip1}$ or $p27^{Kip1}$ also inhibits CAK-dependent phosphorylation of complexes containing Cdk2 (37) and probably Cdk4. The prevailing hypothesis is that cyclin kinase inhibitors have dual functions: to inhibit activation of newly synthesized Cdks and to inhibit kinase activity of cyclin/Cdk complexes that have already been activated.

The activity of Cdks is regulated by complex, interdependent, posttranslational mechanisms as described above. The activity of some of these kinases is also determined by changes in gene expression. Yeast Cdc28 and cdc2 are constitutively expressed (44, 45). Likewise, the abundance of mammalian Cdk1 and Cdk2 proteins is relatively constant throughout the cell cycle of cells in exponential growth (46–48). These observations have tended to discourage investigation into the mechanisms that control Cdk gene expression. Nevertheless, there are several observations that suggest that both the synthesis and the degradation of Cdks is regulated in interesting and important ways. *Cdk1* expression has been examined in the most detail.

Although Cdk1 protein content is relatively constant during the cell cycle, there are dramatic changes in both the rates of synthesis and degradation of the enzyme (47, 49, 50). Transcription of *Cdk1* is activated near the G_1 S boundary (46). This is associated with a corresponding increase in the rate of synthesis and the rate of degradation of bulk Cdk1. The net result is a near complete turnover of cellular Cdk1 with no net change in total Cdk1 abundance. The significance of this "housecleaning" process is not clear, but it has been observed that synthesis of Cdk1 is temporally associated with increased phosphorylation of Cdk1 (47). It may be that cell cycle progression is somehow tied to the ability to replace the entire cellular complement of the mitotic Cdk.

It has also been demonstrated that activation of *Cdk1* attends mitotic stimulation of cells (49, 50). Cdk2 mRNA abundance also increases under these circumstances (51). Mitotic activation of lymphoid cells is also associated with an increase in the mRNA encoding Cdk2 (51–53) and Cdk4 (53). Differentiation of murine erythroleukemia cells is dependent upon down-regulation of *Cdk4* (54). TGF-β inhibition of mink lung epithelial cell proliferation is also entrained by inhibition of *Cdk4* expression (55). These observations suggest that the regulation of Cdk genes in general, and *Cdk4* in particular, may be an essential component in the regulation of the transition from the proliferative state to the G_0 state.

We propose that regulation of Cdk gene expression may be central to withdrawal from the cell cycle and to reentry into the proliferative state. To test this hypothesis, we have initiated a series of experiments to analyze transcriptional regulation of mammalian Cdk genes, with particular emphasis on the relationship between such regulation and entry into the G_0 state. Our initial studies have focused upon glucocorticoid inhibition of murine T-lymphoma cell proliferation.

Glucocorticoids regulate proliferation of both normal and malignant lymphoid cells. Murine thymic lymphoma P1798 undergo G_0 arrest in the presence of dexamethasone (56). When exposed to dexamethasone in the presence of fetal bovine serum, P1798 cells do not die (57). Virtually all of the glucocorticoid-treated cells will reenter the cell cycle if the steroid is removed (58). Under these circumstances, one may regulate G_0 entry and exit by the addition or withdrawal of a single, chemically defined agent, dexamethasone. The reversibility of the process assures that one is observing aspects of physiological regulation of cell proliferation rather than manifestations of cell death. The data indicate that several Cdk genes are regulated by glucocorticoids. Of particular interest is *Cdk4*, which appears to be a direct target of glucocorticoids and may play a role in glucocorticoid inhibition of lymphoid cell proliferation.

Results

Glucocorticoids Regulate Cdk Expression. Asynchronous, mid log P1798 cultures were treated with dexamethasone for 24 h. This suffices to cause cell cycle arrest with a G_1 G_0 DNA content (as will be shown in Fig. 3). RNA was extracted from dexamethasone-treated and mid log cultures.

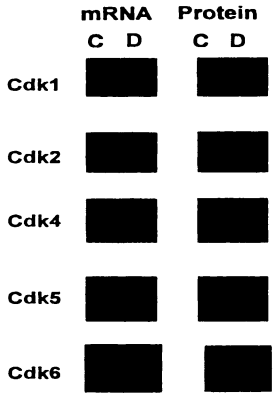

Fig. 1. Glucocorticoid regulation of Cdk expression. Mid log phase cultures were exposed to 0.1 μM dexamethasone for 24 h. Control (*C*) and dexamethasone-treated cultures (*D*) were divided into two aliquots, and protein and nucleic acid extracts were prepared from these. Extracts were assayed for the abundance of Cdk mRNAs or Cdk proteins. Northern and Western blotting were carried out as described in "Materials and Methods."

Samples were resolved by electrophoresis and assayed for the abundance of mRNAs encoding several members of the Cdk family. Representative data are shown in Fig. 1, in which those lanes designated *C* contain RNA from control cells, while lanes designated *D* contain RNA from dexamethasone-treated cells.

P1798 cells contain detectable levels of mRNA corresponding to most of the Cdks that were tested. The mRNAs encoding Cdk1 and Cdk4 were the most abundant in mid log phase P1798 cells, as evidenced by the fact that the Cdk1 blot was exposed to X-ray film overnight and that for Cdk4 was exposed for 2.5 days. The mRNA encoding Cdk2 was not very abundant in P1798 cells, and prolonged exposure (12 days) was required to obtain the autoradiographic signal shown in Fig. 1. Cdk3 mRNA was undetectable in P1798 cells as was the mRNA corresponding to PCTAIRE 3 (data not shown); PCTAIRE 1 mRNA was readily detected in P1798 cells (data not shown). Cdk5 and Cdk6 mRNAs were readily detected in P1798, although neither species was as abundant as Cdk1 or Cdk4. The Cdk5 blot shown in Fig. 1 was exposed for 7 days, whereas the Cdk6 blot was exposed for 3 days.

Except for Cdk6 mRNA, all of the Cdk mRNAs were significantly less abundant in glucocorticoid-treated cells. The abundance of Cdk1 mRNA decreased by 94% in this experiment. Cdk2 and Cdk5 mRNAs were reduced by about 40%. The mRNA encoding Cdk4 was much less abundant in cells that had been treated with dexamethasone. In this experiment, expression of Cdk4 mRNA was reduced to about 20% of control, and >90% inhibition was sometimes observed (as may be seen in Fig. 4).

Many of the Cdk proteins are quite stable, so that a decrease in the abundance of the cognate mRNA is not immediately accompanied by a corresponding decrease in protein content. The abundance of Cdk proteins was assayed in control and glucocorticoid-treated cells, as shown in Fig. 1. Dexamethasone had no significant effect on Cdk5 or Cdk6 protein (106 and 98% of control, respectively). The abundance of Cdk2 was reduced by about one-half after 24 h in the presence of dexamethasone (53% of control), whereas the abundance of Cdk1 was reduced by about 70%. Cdk4 protein was reduced to about 1% of control.

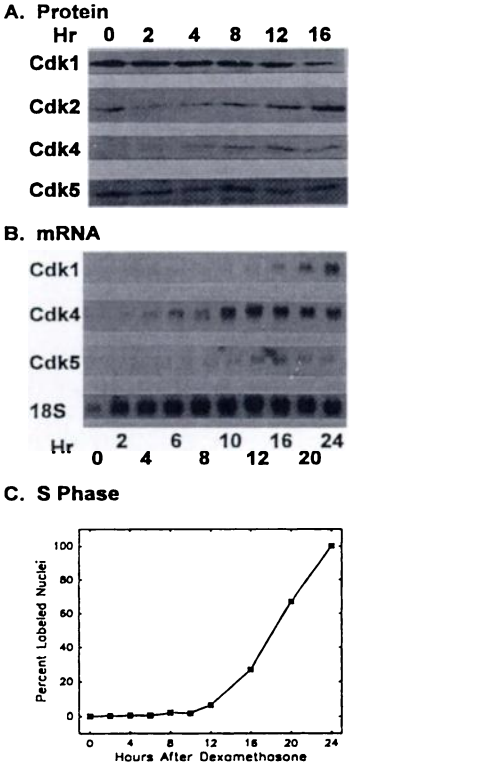

Fig. 2. Cdk expression during the transition from G_0 to S phase. P1798 cells were arrested in G_0 by 24 h exposure to 0.1 μM dexamethasone. Thereafter, the cells were washed in warm medium and suspended in fresh medium to a density of 10^6 cells/ml. Aliquots were withdrawn at intervals, and extracts were prepared for analysis of protein or mRNA abundance. In parallel, cells were labeled with 0.1 μCi/ml [^{3}H]thymidine. Nuclear labeling was assessed by autoradiography, as described in "Materials and Methods."

The ability of Cdk4-containing complexes to phosphorylate the retinoblastoma susceptibility gene product *in vitro* was also reduced by 80–90% (data not shown), which may be due in part to a reduction in the abundance of Cdk4 and in part to inhibition of cyclin D3 expression.[5]

Expression of Cdk4 Is Proximal to the Glucocorticoid Block in G_0. The effects of glucocorticoids are completely reversible if P1798 cells are exposed to dexamethasone in 5% fetal bovine serum (56–58). In the experiment depicted in Fig. 2, P1798 cells were exposed to dexamethasone for 24 h. Thereafter, the steroid was removed. One aliquot of the "rescued" culture was placed in [^{3}H]thymidine, and nuclear labeling was measured as an index of entry into S phase. Proteins were extracted from a different culture aliquot and RNA from a third. These extracts were assayed for the abundance of Cdk proteins (Fig. 2*A*) and mRNAs (Fig. 2*B*). Cultures that have been exposed to dexamethasone for 24 h contained virtually no detectable S phase cells, as shown in Fig. 2*C*. Initiation of S phase commenced 12–14 h after withdrawal of the steroid, and all of the cells entered S phase within 20–24 h. There was very little change in Cdk1 or Cdk5 protein within the first 16 h after removal of

[5] D. Reisman and E. A. Thompson. Glucocorticoid regulation of cyclin D3 gene transcription and mRNA stability in lymphoid cells, manuscript in preparation.

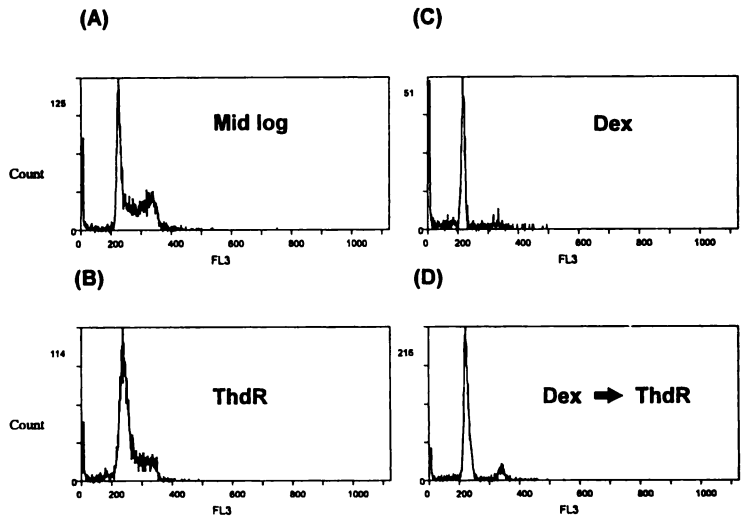

Fig. 3. Cell arrest at the G_1-S interface. A glucocorticoid/thymidine block protocol was used to synchronize P1798 cells at the G_1-S interface. *A*, a FACS histogram illustrating the DNA distribution in (about 2×10^4) mid log phase cells. Addition of 2 mM thymidine (*ThdR*) arrests (about 2×10^4) cells in G_1 and throughout S phase (*B*), whereas dexamethasone (*Dex*) arrests (about 1×10^4) cells entirely in G_0/G_1 (*C*). If dexamethasone is removed and 2 mM thymidine is added, P1798 cells (about 2.8×10^4) do not initiate DNA synthesis (*D*).

dexamethasone (Fig. 2*A*), although both Cdk1 and Cdk5 mRNAs began to accumulate 12–16 h after removal of the G_0 block (Fig. 2*B*), at the time when P1798 cells began to enter S phase (Fig. 2*C*). The abundance of Cdk2 mRNA was not measured in this experiment (due to technical difficulties in detecting this rare transcript in P1798 cells), but Cdk2 protein began to increase 12–16 h after removal of dexamethasone (Fig. 2*A*), which is consistent with the proposed role of Cdk2 in regulating entry into S phase.

The response of Cdk4 is much more rapid. Cdk4 protein increased within the first few hours after removal of dexamethasone, and maximum expression was observed 4–8 h after rescue (Fig. 2*A*). Similar results were observed with Cdk4 mRNA, which began to increase within 4 h and was maximally induced within 10 h after removal of dexamethasone (Fig. 2*B*). The data indicate that expression of Cdk4 achieves a maximum prior to entry into S phase, whereas the other Cdks reach maximum levels of expression at the time of entry into S phase or later. The data shown in Fig. 2 are consistent with the idea that inhibition of Cdk4 expression is closer to the dexamethasone-mediated block in cell cycle progression, whereas changes in the expression of the other Cdks are probably secondary to G_0 arrest. As will be shown presently, glucocorticoid inhibition of Cdk4 mRNA expression also occurs rapidly after the addition of dexamethasone to mid log phase cells.

Glucocorticoid Regulation of Cdk4 Expression Occurs Rapidly and Can Be Demonstrated in the Absence of Cell Cycle Progression. A modified thymidine block protocol was developed to measure the effects of glucocorticoids in the absence of cell cycle progression. Thymidine, when added at concentrations sufficient to block deoxyribonucleotide synthesis (*e.g.* 2 mM), prevents initiation of DNA replication and arrests cells in late G_1 as well as in S phase (59). P1798 cultures will not grow in 2 mM thymidine (data not shown). However, inhibition of ribonucleotide reductase arrests cells in late G_1 and throughout S phase, as shown in Fig. 3*B*, such that very little synchrony is achieved by addition of 2 mM thymidine. This lack of synchrony is usually overcome by a double thymidine block in which the cells are released from thymidine, allowed to complete S phase, and then arrested in late G_1 by a second addition of thymidine. However, the P1798 cell cycle has very short G_1 and G_2 phases. The duration of $G_2 + M + G_1$ phases is <4 h, whereas S phase is 7–8 h. As a result, some of the cells that are released from the initial thymidine blockade will reenter a second S phase before other cells can complete the initial S phase. Under these circumstances, double thymidine bock will never achieve a high degree of synchrony.

As an alternative, a dexamethasone/thymidine synchronization protocol was developed to achieve a very high degree of synchrony in late G_1. Initially, cells were arrested in G_0 by 24 h treatment with glucocorticoids. As reported previously (57), the majority of these glucocorticoid-treated cells arrested with a 2N DNA content (Fig. 3*C*). A minor subset of P1798 cells contains >80 chromosomes (60). These cells arrested in G_0 with an apparent 4N DNA content so that a small number of 4N cells was always observed in dexamethasone-treated cultures (Fig. 3, *C* and *D*).

After glucocorticoid-mediated G_0 arrest is complete, the cells were washed to remove dexamethasone and then transferred to steroid-free medium containing 2 mM thymidine. The cells exited G_0 upon removal of dexamethasone and proceeded to late G_1, where they arrested due to inhibition of deoxyribonucleotide biosynthesis. There was no increase in DNA content, since the cells cannot initiate DNA replication (Fig. 3*D*). However, there was an accumulation of late G_1-specific gene products such as thymidine kinase (Tk-1) mRNA.[6]

When P1798 cells were transferred from dexamethasone-containing medium to thymidine-containing medium, there was a rapid increase in the abundance of c-Myc mRNA as cells exited the steroid-induced G_0 state and proceeded to the thymidine block in late G_1. As shown in Fig. 4*A*, c-Myc mRNA abundance increased to a maximum within 2–4 h after removal of dexamethasone, and there was no significant change in c-Myc mRNA as long as the cells were maintained in the presence of thymidine. The abundance of Cdk4 mRNA also increased when cells were allowed to progress from G_0 to G_1. The abundance of Cdk4 mRNA began to increase within 1 h and reached a maximum

[6] K. Rhee and E. A. Thompson. Glucocorticoid regulation of a transcription factor that binds an initiator-like element in the thymidine kinase promoter, manuscript in preparation.

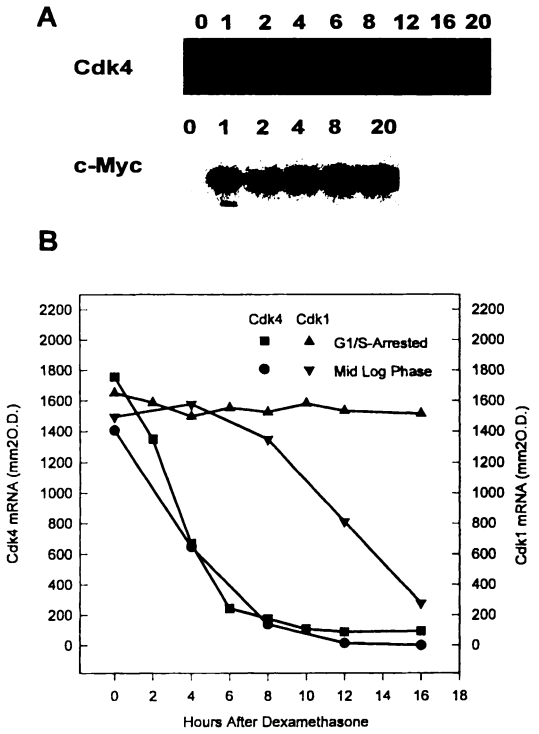

Fig. 4. Gene expression in G_1-S phase-arrested cells. The data in *A* illustrate progression from dexamethasone-induced G_0 state to the thymidine arrest point in late G_1. P1798 cells were treated with dexamethasone for 24 h, as in Fig. 3*C*. Dexamethasone was withdrawn at the time point designated *0* h, and RNA was extracted thereafter. The abundance of Cdk4 and c-Myc mRNAs was measured as an indicator of progression from G_0 to late G_1. In *B*, cells treated as in Fig. 3*D* were exposed to 0.1 μM dexamethasone. RNA was extracted at intervals and assayed for the abundance of Cdk4 (■) or Cdk1 (▲) mRNA. In parallel, mid log phase cultures were treated with dexamethasone and assayed for the abundance of the mRNAs encoding Cdk4 (●) or Cdk1 (▼). Northern blots were integrated and quantified as described in "Materials and Methods." All data are normalized to expression of 18S rRNA.

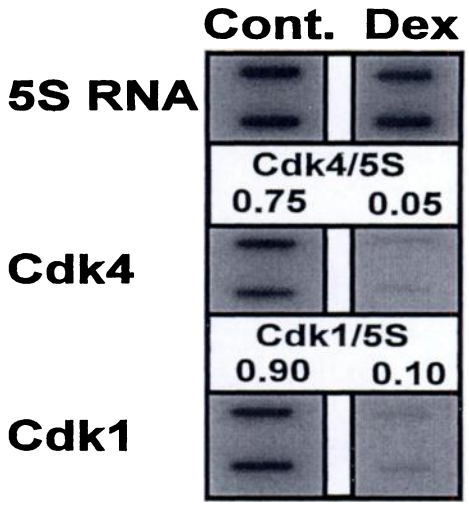

Fig. 5. Nuclear run-on transcription of *Cdk4*. Nuclei were prepared from mid log phase P1798 cells and from cells that had been exposed to 0.1 μM dexamethasone for 24 h. Nuclear run-on transcription was carried out, as described in "Materials and Methods," and the products were hybridized to filters containing full-length, double-stranded Cdk4 cDNA, Cdk1 cDNA, and 5S RNA genomic DNA. The autoradiographic signal was integrated and quantified as described in "Materials and Methods." The results were normalized to transcription of 5S RNA genes and are presented as the mean of two determinations.

within 6–8 h after removal of dexamethasone, as shown in Fig. 4*A*. There was no change in the amount of Cdk4 mRNA thereafter. The abundance of Cdk4 mRNA in thymidine-arrested cells was approximately equal to that observed in mid log populations.

Addition of dexamethasone to G_1 S-arrested cells resulted in a rapid decrease in the abundance of Cdk4 mRNA (squares in Figure 4B). The rate of disappearance of Cdk4 mRNA in G_1 S-arrested cells was similar to that observed in mid log populations (Fig. 4*B*, ●). The abundance of Cdk1 mRNA did not decrease in glucocorticoid-treated, G_1 S-arrested cells (Fig. 4*B*, ▲). However, the abundance of Cdk1 mRNA in glucocorticoid-treated, mid log populations decreased slowly, with 50% inhibition observed about 14 h after the addition of dexamethasone (inverted triangles, Fig. 4*B*, ▼). Eighty-five % inhibition of Cdk1 expression was observed within 16 h after the addition of dexamethasone to mid log phase cultures, which is consistent with the data shown in Fig. 1. These data indicate that glucocorticoid inhibition of Cdk1 expression correlates with withdrawal from the cell cycle. Glucocorticoid inhibition of Cdk4 expression is independent of cell cycle progression and may be due to a direct effect of dexamethasone upon transcription of *Cdk4* or the stability of Cdk4 mRNA.

Glucocorticoids Inhibit Transcription of Cdk4. Nuclear run-on transcription was carried out to ascertain the mechanism that accounts for glucocorticoid regulation of Cdk4 expression. Representative data are shown in Fig. 5. Transcription of 5S RNA genes was measured as an internal control, since transcription of 5S RNA genes is not regulated by dexamethasone (61). Transcription of *Cdk4* was profoundly inhibited, with >90% inhibition observed. Transcription of *Cdk1* was also inhibited. The extent of inhibition of *Cdk1* transcription is consistent with the observed decrease in Cdk1 mRNA abundance in dexamethasone-treated cells.

Glucocorticoids Inhibit Expression of Cdk4 in Normal Splenocytes. It was of interest to determine if glucocorticoid regulation of *Cdk4* expression was characteristic of lymphoid cells, in general, or was a unique property of P1798 cells. The effects of dexamethasone upon expression of *Cdk4* and *Cdk6* were analyzed in Con A-activated, normal murine splenocytes. Representative data are shown in Fig. 6.

Splenocytes were activated by exposure to Con A for 24 h. In parallel, cells were exposed to concanavalin and dexamethasone. The abundance of Cdk4 mRNA in the dexamethasone-treated cells (normalized to 18S rRNA to correct for loading efficiency) was about 12% of that observed in the absence of glucocorticoids. These data are similar to those observed in P1798 cells (Fig. 1). The abundance of Cdk6 mRNA was low, and detection of this transcript was difficult in activated splenocytes. However, within the limits of detection, dexamethasone did not inhibit expression of Cdk6 mRNA in Con A-stimulated splenocytes (Fig. 6). When corrected for loading of 18S rRNA, the abundance of Cdk6 mRNA in dexamethasone-treated splenocytes was 106% of control, which agrees with the data concerning glucocorticoid effects on Cdk6 mRNA abundance in P1798 cells (Fig. 1).

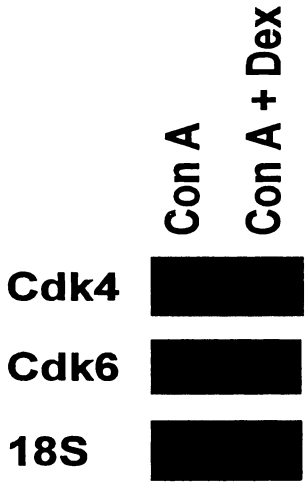

Fig. 6. Cdk4 and Cdk6 mRNA expression in activated splenocytes. Splenocytes were prepared from 3-month-old BALB/c female mice. The cells were suspended in medium containing 2 μg/ml Con A or 2 μg/ml Con A plus 0.1 μM dexamethasone (*Con A + Dex*). After 24 h, RNA was extracted and assayed for abundance of Cdk4 and Cdk6 mRNA. The autoradiogram depicting Cdk4 mRNA was exposed overnight, whereas that depicting Cdk6 mRNA was exposed for 5 days.

Discussion

Glucocorticoids inhibit expression of Cdks in both normal and transformed lymphoid cells. Regulation of Cdk4 mRNA can be demonstrated in the absence of cell cycle progression, whereas down-regulation of Cdk1 mRNA does not occur when dexamethasone is added to cultures that are arrested in late G_1. It is likely that glucocorticoid regulation of Cdk1 (and probably Cdk2) expression is secondary to redistribution of cells within the cell cycle. This conclusion is consistent with a number of observations concerning inhibition of Cdk1 and Cdk2 expression in cells that are arrested in G_0 by growth factor deprivation (46–51).

Transcription of *Cdk1* is known to be cell cycle dependent. Transcription of *Cdk1* decreases at the onset of M phase and is activated in late G_1 of the subsequent cycle (46). If glucocorticoids arrest lymphoid cell cycle progression in early G_1 or G_0, then one would expect transcription of *Cdk1* to be low in dexamethasone-treated cells. It has been determined that activation of *Cdk1* during mitotic activation of quiescent cells is not blocked by aphidicolin or by hydroxyurea (47), which arrest cell cycle progression in late G_1. The data present above indicate that thymidine blockade also permits activation of *Cdk1*.

Transcription of *Cdk2* has not been analyzed in as much detail as that of *Cdk1*, although there are many examples of induction of Cdk2 mRNA as a result of mitotic stimulation of quiescent cells (43, 44, 51–53). It is possible that *Cdk2* transcription is also regulated during glucocorticoid-mediated cell cycle arrest, although the abundance of Cdk2 mRNA is not greatly reduced in G_0 cells. In any event, the abundance of Cdk2 mRNA in P1798 cells is so low that more detailed analysis of *Cdk2* regulation has not been undertaken.

Regulation of *Cdk4* expression appears to reflect a direct effect of glucocorticoids upon transcription of *Cdk4*, rather than a cell cycle-dependent inhibition of expression. This is not to say that the glucocorticoid receptor binds to *Cdk4* so as to inhibit transcription, although this type of primary response is certainly one possibility. One can also imagine secondary mechanisms whereby glucocorticoids regulate the amount or activity of some factor that regulates *Cdk4* in *trans*. Expression of *Cdk4* is inhibited by TGF-β (56), which shares with glucocorticoids the ability to induce G_0 arrest in certain cell types. It has also been shown that irreversible differentiation of MEL cells involves inhibition of *Cdk4* expression (55). However, neither TGF-β nor agents that induce differentiation of MEL cells have any significant effect upon transcription of *Cdk4*. In both of these cases, expression of *Cdk4* is inhibited by some posttranslational process that appears to accelerate the rate of degradation of Cdk4 with no attendant change in the rate of Cdk4 synthesis. Regulation of *Cdk* genes is poorly understood at this time. Very little is known about transcription of *Cdk2*, and less is known about transcription of *Cdk4*. Elucidation of the mechanism(s) that account for the effects of dexamethasone should materially enhance our understanding of how these important genes are controlled.

The kinetics of regulation of Cdk4 mRNA and protein are consistent with the hypothesis that inhibition of *Cdk4* transcription may cause cell cycle arrest in glucocorticoid-treated P1798 cells. It has been shown that constitutive overexpression of *Cdk4* renders mink lung epithelial cells resistant to TGF-β (55). Likewise, differentiation of MEL cells is blocked by constitutive overexpression of *Cdk4* (54). We have isolated stable P1798 transformants that overexpress *Cdk4*; however, these transformants are not resistant to glucocorticoid-mediated inhibition of proliferation (data not shown). This observation may indicate that *Cdk4* is not the only target of dexamethasone. Glucocorticoids also rapidly inhibit transcription of c-*Myc* (56, 58, 62), and overexpression of *Cdk4* probably would not suffice to overcome the lack of c-Myc activity in glucocorticoid-treated P1798 cells.

P1798 cells express abundant Cdk6 protein, and this kinase is not regulated by glucocorticoids. Cdk4 and Cdk6 have similar properties *in vitro* (10, 17, 63, 64) and may be redundant *in vivo*. If this is so, then inhibition of *Cdk4* transcription might seem to be irrelevant in cells that express high levels of Cdk6. However, Cdk4 and Cdk6 have functions that are not dependent upon phosphorylation reactions. Both kinases bind Ink-type inhibitors ($p15^{Ink4B}$ and $p16^{Ink4}$), and cyclin D-Cdk4/6 complexes bind $p21^{Cip1}$ and/or $p27^{Kip1}$. A decrease in the abundance of Cdk4 protein would result in an increase in the "activity" of cyclin kinase inhibitors. There is still some confusion as to how Cdk inhibitors interact with their targets, but the prevailing model envisions a finely balanced, stoichiometric relationship between the concentrations of Cdk inhibitors and cyclin/Cdk complexes (65). Consequently, a rather small decrease in the abundance of any of single Cdk would perturb the balance and might create a situation in which the inhibitors were in excess. The activities of all Cdks would decrease as a result, and cell cycle arrest would ensue. We do not know if P1798 cells express any of the known cyclin kinase inhibitors, but it is interesting to speculate that inhibition of Cdk4 expression might alter the balance between Cdks and Cdk inhibitors, producing global inhibition of Cdk activity, and therefore contributing to G_0 arrest in P1798 cells.

Cell cycle progression is regulated by complex, interdependent mechanisms. *Cdk4* is one of several genes that appear to be involved in glucocorticoid-mediated G_0 arrest of lymphoid cells. Inhibition of Cdk4 may prevent critical phosphorylation reactions. Alternatively, a decrease in the abundance of Cdk4 may increase the activity of cyclin kinase inhibitors. It is also possible that inhibition of *Cdk4*

expression is irrelevant to lymphoid cell proliferation, although this would be surprising in light of our current understanding of how such proteins function. It may ultimately be of greater significance that glucocorticoids regulate transcription of *Cdk4*. Expression of this gene is linked to proliferation of several different cell types, and understanding how *Cdk4* expression is regulated may contribute to our knowledge of the biochemical mechanisms that underlie hormonal control of cell proliferation.

Materials and Methods

Cell Culture. P1798 cells were grown in suspension culture as described previously (56, 60). Unless stated otherwise, all of the experiments were performed with the P1798-S20 subpopulation (60), which has an average population doubling time of 11 h. Mid log phase cultures of 1–5 $\times 10^5$ cells/ml were used for all experiments. Cultures were treated with 0.1 μM dexamethasone for various periods of time. Flow cytometry was carried out as described previously (56). About 2 $\times 10^4$ nuclei were stained with propidium iodide and analyzed for DNA content using a Coulter Epics flow cytometer.

Analysis of mRNA Abundance. Total RNA was extracted with quanidinium isothiocyanate (66), and 20 μg was resolved by polyacrylamide gel electrophoresis in urea. RNA was blotted to nitrocellulose and probed with ^{32}P-labeled DNA made by random primer labeling of gel-purified cDNAs. The cDNA clone corresponding to mouse Cdk1 was obtained from E. M. Bradbury (University of California at Davis, CA; Ref. 67). Mouse Cdk2 cDNA was obtained from Hideyo Yasuda (Kanazawa University, Kanazawa, Japan) and mouse Cdk4 cDNA from C. J. Sherr (St. Jude Children's Research Hospital, Memphis, TN; Ref. 17). The cDNA for human Cdk5 was obtained from David Beach (Cold Spring Harbor, NY; Ref. 63), and the cDNA clone corresponding to mouse Cdk6 was provided by Ed Harlow (Massachusetts General Hospital, Boston, MA; Ref. 10). Mouse PC1 and PC3 cDNAs were obtained from J. R. Downing (St. Jude Children's Research Hospital, Memphis, TN; Ref. 68). Detection was achieved by autoradiography, and all films were quantified using the Applied Imaging Lynx 5000 digital imaging system. This system was also used to capture all of the bitmap images that were used to make the figures in this report.

Analysis of Protein Abundance. Cells were washed with PBS, and the cell pellet was lysed by addition of 25–50 μl of lysis buffer (PIPA) containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, 200 μM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml phenylmethylsulfonyl fluoride, and 10 μg/ml pepstatin A. The lysate was cleared by centrifugation at 15,000 rpm in a table top centrifuge for 10 min at room temperature. An aliquot containing 40 μg of protein was mixed with an equal volume of 2× sample buffer [made by mixing 2.5 ml 0.5 mM Tris-HCl (pH 6.8), 4.0 ml 10% SDS, 2.0 ml glycerol, 1.0 ml β-mercaptoethanol, 0.5 ml H_2O, and 0.05% bromophenol blue]. The mixture was heated at 95°C for 6 min. Proteins were resolved by electrophoresis on 6% polyacrylamide gels containing SDS for 1–3 h at 90–120 V. The gels were electroblotted onto nitrocellulose membranes. The membranes were blocked by soaking in 5% Carnation fat-free milk with 0.05% Tween 20 in Tris-buffered saline (pH 7.4) overnight at room temperature. The primary antibody was diluted with blocking buffer, and the filters were incubated for 3–5 h at room temperature. Thereafter, the membrane was washed 3× for 10 min with blocking buffer to remove excess primary antibody. The secondary antibody was diluted with blocking buffer, and the membrane was soaked in this solution for 60 min. The secondary antibody was decanted, and the membrane was washed 3× for 10 min with blocking buffer and twice for 5 min with 0.05% Tween 20 in Tris-buffered saline. The Amersham ECL Western blotting detection reagents were used according to the manufacturer's recommendations. Identity of Cdks was established by immunoreactivity with specific antibodies, molecular weight of the immunoreactant species, and (in a few cases) co-electrophoresis with recombinant standards. The antibody against Cdk4 was provided by Steve Hanks (Vanderbilt University Medical Center, Nashville, TN) and that against Cdk5 by Ed Harlow. Santa Cruz provided the antibodies against Cdk1, Cdk2, and Cdk6.

Nuclear Run-On Transcription and Labeling. Mid log phase P1798 cells were treated 24 h with 0.1 μM dexamethasone. Nuclei were isolated, and nuclear run-on transcription carried out as described previously (69). Autoradiographic data were quantified using the Lynx digital imaging workstation, as described above. Nuclear labeling studies to determine the percentage of cells in S phase were carried out as described previously (70).

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