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Cyclin C/Cdk3 Promotes Rb-Dependent G0 Exit

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Summary

G0 is a physiological state occupied by resting or terminally differentiated cells that have exited the cell cycle. In contrast to the well-characterized cyclin/cdk-mediated inactivation of pRb that controls the G1/S transition, little is known about regulation of the G0/G1 transition. However, pRb is likely to participate in this process because its acute somatic inactivation is sufficient for G0-arrested cells to reenter the cell cycle. One physiological regulator of this event may be cyclin C because its highest mRNA levels occur during G0 exit. Here we show that a non-cdk8-associated cellular pool of cyclin C combines with cdk3 to stimulate pRb phosphorylation at S807/811 during the G0/G1 transition, and that this phosphorylation is required for cells to exit G0 efficiently. Thus, G1 entry is regulated in an analogous fashion to S phase entry, but involves a distinct cyclin/cdk combination.

Introduction

Experimentation during the past twenty years has resulted in a detailed mechanistic understanding of the controls that govern cell cycle progression (reviewed in Sherr, 2000). Transition between cell cycle phases is regulated by a series of temporally coordinated phosphorylation events that target members of the pocket protein family, including pRb, p107, and p130. The kinases responsible for pocket protein phosphorylation are cyclin dependent kinases (cdks), which are activated in part by their association with cyclins. In early G1, pRb is phosphorylated to a limited extent by cdk4 and cdk6 in association with D-type cyclins. This "hypophosphorylated" pRb binds to E2F family members thereby preventing transcription of E2F-responsive genes both by physically blocking E2F's transactivating domain and by recruiting chromatin-modifying enzymes that actively repress transcription (Trimarchi and Lees, 2002). In mid-G1, additional cdk4/6 phosphorylation releases some of the associated proteins, and in late G1 cyclin E/cdk2 phosphorylates pRb at additional sites to produce hyperphosphorylated pRb, an isoform that no longer has affinity for E2F. Dissociation from pRb removes both the blockade of E2F's transactivation domain and the nidus for enzymes that produce transcriptionally silent chromatin. This combination of events activates transcription of E2F responsive genes and stimulates S phase entry.

While the mechanisms governing early and late G1 progression are well understood, much less is known about the G0 to G1 transition. G0 is generally considered to be a distinct state that cells do not enter while actively cycling (Lajtha, 1963), implying the existence of molecular signals that regulate the transition between G0 and G1. By analogy to S phase progression, pocket protein phosphorylation may also govern this phase change. For example, p130 changes its phosphorylation status as cells leave G0 and enter G1 (Canhoto et al., 2000; Mayol et al., 1995, 1996) in concert with shifts in the association between p130 and the E2F family members E2F-4 and E2F-5 (Moberg et al., 1996; Sardet et al., 1995). A specific role for pRb in G0 arrest is indicated by the observation that acute inactivation of pRb alone is sufficient for G0-arrested cells to reenter the cell cycle (Sage et al., 2003). This suggests that the hypo- or, perhaps, unphosphorylated form of pRb that appears in G0 (Ezhevsky et al., 1997) may undergo phosphorylation-mediated inactivation in order for cells to enter G1. In fact, some of the kinases responsible for phosphorylating un- and hypophosphorylated pRb and p130 have been identified. Cdk4 phosphorylates both pocket proteins early in G1 (Ezhevsky et al., 1997; Farkas et al., 2002), however the contribution of these events to the G0 to G1 transition has not been rigorously demonstrated. Furthermore, mutation of all known cdk4 phosphorylation sites in p130 produces a protein that can still be inactivated in a regulated manner in early G1 (Farkas et al., 2002). This suggests that other cyclin/cdk combinations may also be active in the G0 to G1 transition.

One possible regulator of G0 events is cyclin C because its highest mRNA expression levels occur during G0 exit (Liu et al., 1998b; Rickert et al., 1996). Cyclin C was identified in genetic screens for mammalian or *Drosophila* genes that could rescue a triple *CLN*-deficient strain of *S. cerevisiae* (Leopold and O'Farrell, 1991; Lew et al., 1991). These same screens discovered cyclins D1 and E, and although they have found places as regulators of cdks that have a direct effect on cell cycle progression, cyclin C has not. Instead, it has been shown to regulate the activity of cdk8 (Tassan et al., 1995), which phosphorylates the C-terminal domain (CTD) of RNA polymerase II (Leclerc et al., 1996; Rickert et al., 1996). This function is consistent with the close structural similarities between cyclin C/cdk8 and the SRB11/SRB10 complex which is a component of the *S. cerevisiae* RNA polymerase II holoenzyme (Liao et al., 1995) and is required for optimal CTD phosphorylation (Dahmus, 1996; Serizawa et al., 1995). Cyclin C/cdk8 also phosphorylates cyclin H, which negatively regulates TFIIH (Akoulitchev et al., 2000).

Although cyclin C's contribution to transcriptional regulation is well established, this activity does not readily explain its capacity to rescue G1 cyclin deficiency in yeast. On one hand, cyclin C's ability to stimulate CDC28 in *S. cerevisiae* may simply be a consequence of the

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same structurally conserved cyclin domains that activate cdk8 in its native mammalian or insect contexts. On the other hand, cyclin C may also have an unappreciated G0/G1 cyclin activity. In this study, we tested cyclin C's ability to regulate events in G0 and early G1. We found that during exit from G0, cyclin C directs pRb phosphorylation in a temporal pattern that precedes pRb phosphorylation by cyclin D/cdk4, cyclin D/cdk6, and cyclin E/cdk2. Furthermore, this activity does not involve cdk8 but rather is mediated by cdk3, and targets specific pRb substrate sites that must be phosphorylated in order for cells to exit G0. Together, these results indicate that cyclin C/cdk3 plays an important role in regulating the G0 to G1 transition and does so, in part, through specific phosphorylation of pRb.

Results

Cyclin C-Associated pRb Kinase Activity

To test whether cyclin C has an associated pRb kinase activity that varies with cell cycle phase, we examined human T98G cells that express wild-type pRb and can be arrested in G0. After three days of serum deprivation, T98G cells were stimulated with serum and lysates were collected at various times thereafter. Cyclin C immune precipitates from these lysates had pRb kinase activity that was present at low levels in G0 cells, increased in early G1, decreased in late G1 and S phases, and then reappeared as cells reentered G1 (Figure 1A). This pattern of activity was precisely the inverse of pRb kinase activities associated with cyclins D1 and E, which increased at the G1/S boundary (Figure 1A).

As the pRb-kinase activity would predict, endogenous cyclin C forms complexes with endogenous pRb. In serum-stimulated T98G cells, cyclin C preferentially bound to hypophosphorylated pRb in G0/early G1 and in late G2/M, coincident with periods of maximal pRb kinase activity (Figure 1B). In confirmation of the cyclin C/pRb interaction, cyclin C immune precipitates from 3T3 cells contained pRb (Figure 1C). The former interaction was specifically prevented by a competing cyclin C peptide. Also, the nominal pRb band of ≈110 kD only appeared in lysates from wild-type 3T3 cells and not 3T3 cells from $Rb^{-/-}$ mice (Figure 1C). Similarly specific interactions occurred in human cells i.e., the presence of pRb in cyclin C immune precipitates from Rb-positive T98G cells, and its absence in cyclin C immune precipitates from Rb-negative SAOS-2 cells (Figure 1D). (Evidence for the specificity of the anticyclin-C antibody is shown in Supplemental Data available at http://www.cell.com/cgi/content/full/117/2/239/DC1.)

Cyclin C Promotes G0 Exit

The pRb kinase activities associated with G1 cyclins stimulate S phase entry by inactivating pRb. However, despite its associated pRb kinase activity, cotransfection of cyclin C with pRb in SAOS-2 cells did not promote S phase entry (Figures 2A and 2B). Because of the timing of cyclin C-associated pRb kinase activity, we considered the possibility that its effects might be occur prior to those of G1 cyclins i.e., at the G0/G1 transition. One technique for assessing G0 status is simultaneous quantitation of DNA and RNA content by fluorescence activated cell sorting (Darzynkiewicz and Traganos, 1990; Ladd et al., 1997). Cells with 2n DNA content and low RNA content are resting and in G0 while cells with 2n DNA and higher RNA content are in G1. By this measure, expression of pRb alone in SAOS-2 cells placed 33–36% of cells in G0 (Figure 2C). However, overexpression of cyclin C along with pRb reduced the proportion of G0 cells to 12%. Conversely, suppression of endogenous cyclin C using RNAi (Figure 2A) increased the proportion of cells in G0 to 49% (Figure 2C). Overexpression of cyclin C without pRb did not alter RNA content in SAOS-2 cells, nor did overexpression of cyclin C in exponentially growing T98G cells, indicating that cyclin C does not stimulate nonspecific enhancement of RNA synthesis (data not shown).

Cell cycle phase was also determined by monitoring patterns of G1-specific gene expression. Cells transfected with Rb alone expressed no detectable c-*fos*, thymidine kinase, or DHFR, and low levels of E2F-1 mRNAs (Figure 2D). Cotransfection of Rb with cyclin C led to substantial increases in levels of each of these mRNAs. That cyclin C did not induce a nonspecific, generalized increase in mRNA transcription was again indicated by the unchanged levels of cyclin E and GAPDH expression in cells transfected with Rb or Rb plus cyclin C (Figure 2D).

G0 Exit Requires pRb Phosphorylation at S807/811

Coexpression of cyclin C and pRb in SAOS-2 cells stimulated pRb phosphate incorporation (data not shown). Using mass spectrometric and phosphospecific antibody analyses, we identified S807/811 as being among the sites whose phosphorylation was enhanced by cyclin C expression. Suppression of endogenous cyclin C by RNAi reversed constitutive S807/811 phosphorylation in transfected SAOS-2 cells and overexpression of cyclin C enhanced it (Figure 3A). Cyclin C immune precipitates from SAOS-2 cells transfected with cyclin C and pRb phosphorylated GST-Rb at S807/811 in vitro indicating that the cyclin C-associated kinase activity can directly phosphorylate pRb at those sites (Figure 3B).

To test the functional significance of S807/811 phosphorylation, we constructed pRb variants in which S807 and S811 were changed individually or in tandem to nonphosphorylatable amino acids. Coexpression of either variant with cyclin C reduced the phosphospecific antibody signal and expression of the double mutant resulted in no signal (Figure 3C). Expression of the double mutant in SAOS-2 cells promoted G0/G1 arrest to the same extent as wild-type pRb, as reported by others (Knudsen and Wang, 1996), as did expression of either single mutant (Figure 3D). However, expression of either single mutant along with cyclin C increased the proportion of cells in G0 to 30% (Figure 3E). Coexpression of the double mutant with cyclin C increased the G0 proportion to 38%, the same level observed when pRb was expressed without cyclin C (Figure 2C), indicating that the double mutant had a completely suppressive phenotype.

Cdk8 Is Not the Cyclin C-Associated pRb Kinase

Until now, cdk8 was the only kinase associated with cyclin C. Surprisingly, then, cdk8 immune precipitates

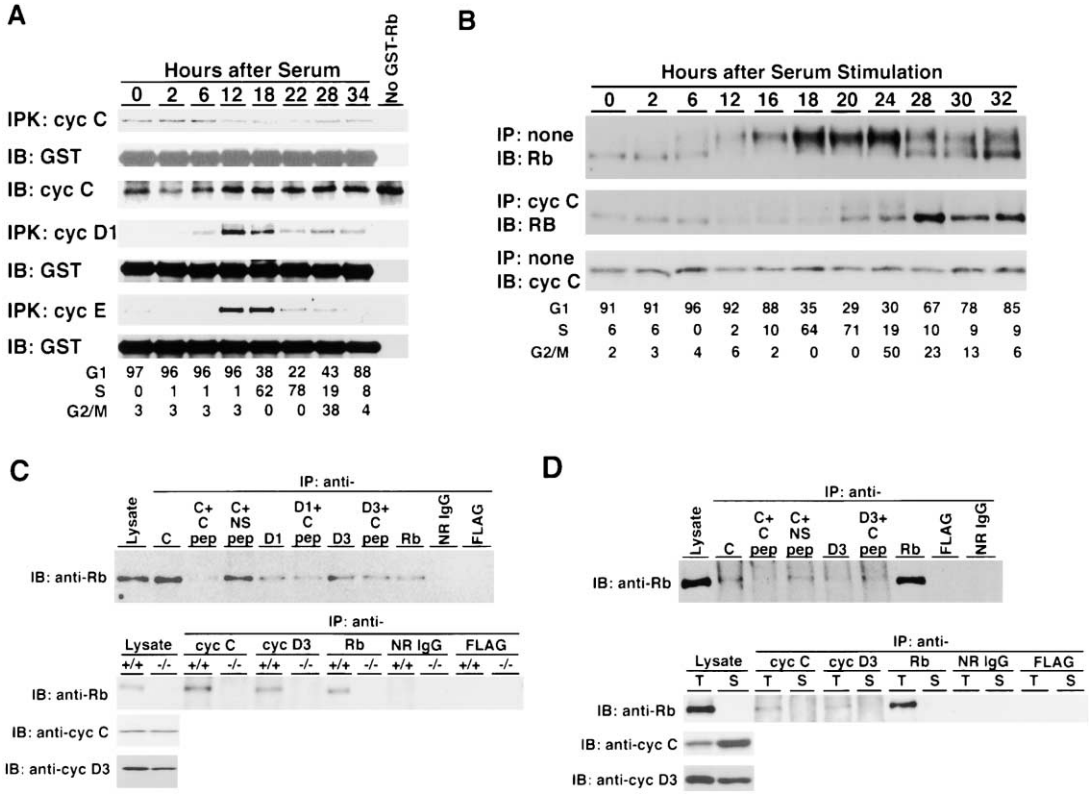

Figure 1. Cyclin C-Associated pRb Kinase Activity

(A) T98G cells were placed in serum-free medium for three days then stimulated with 15% FBS. Lysates were prepared at the indicated times after serum stimulation and subjected to immune precipitation using anticyclin C, anticyclin D1, and anticyclin E. Precipitates were tested for in vitro kinase activity using GST-Rb(379–928) as substrate (IPK). Kinase reactions were analyzed by SDS-PAGE followed by transfer to PVDF membranes. After exposing the membranes to X-ray film, they were probed using anti-GST or anticyclin C. Cells at each time point were also analyzed for DNA content and the proportion of cells with a G1, S, or G2/M DNA content is indicated. Exposure time for the cyclin D1 and cyclin E blots was overnight while the exposure time for the cyclin C blot was 30 min.

(B) T98G cells were placed in serum-free medium for 72 hr and then restimulated with 15% FBS. Lysates were prepared at the indicated times after restimulation and analyzed by immunoblot using anti-pRb (upper image), by immune precipitation using anticyclin C followed by immunoblot using anti-pRb (middle image), or by immunoblot using anticyclin C. An aliquot of cells from each time point was analyzed for DNA content by FACS and the proportion of cells with a G1, S, or G2/M DNA content is indicated.

(C) Upper image, Murine 3T3 fibroblasts were placed in 0.1% FBS for three days, then stimulated with 15% FBS for 6 hr at which time lysates were prepared. Lysates were subjected to immune precipitation using anticyclin C (C), anticyclin C with a competing cyclin C peptide (C + C pep), anticyclin C with a nonspecific peptide (C + NS pep), anticyclin D1 (D1), anticyclin D1 with the cyclin C peptide (D1 + C pep), anticyclin D3 (D3), anticyclin D3 with the cyclin C peptide (D3 + C pep), and anti-Rb G3-245 (Rb). Immune precipitates were analyzed by immunoblot using anti-Rb. Normal rabbit IgG (NR IgG) served as an isospecific control for anticyclin C, anticyclin-D1, and anticyclin-D3; anti-FLAG IgG_1 (FLAG) served as an isotype control for anti-Rb. Lower image, lysates from wild-type 3T3 cells (+/+) and 3T3 cells from *Rb*$^{-/-}$ embryos (−/−) were prepared and subjected to immune precipitation using anticyclin C, anticyclin D3, and anti-Rb. Precipitates and whole lysates were analyzed by immunoblot using anti-Rb, anticyclin C, and anticyclin D3 as indicated. Normal rabbit IgG (NR IgG) and anti-FLAG (FLAG) served as controls.

(D) Upper image, T98G cells were placed in serum-free medium for three days, then stimulated with 15% FBS for 6 hr at which time lysates were prepared. Lysates were subjected to immune precipitation using antibodies and peptides described in (C). Precipitates were analyzed by immunoblot using anti-Rb. Lower image, Lysates from Rb^+ T98G cells (T) and Rb- SAOS-2 cells (S) were prepared and subjected to immune precipitation using the antibodies described in (C). Precipitates and lysates were analyzed by immunoblot using anti-Rb, anticyclin C, and anticyclin D3.

from T98G cells were unable to phosphorylate GST-pRb (Figure 4A) despite the presence of cdk8 in the anticyclin C precipitate (Figure 4B). Consistent with cdk8's lack of pRb kinase activity, we found no evidence for pRb/cdk8 complexes: no pRb was detected in anti-cdk8 precipitates and no cdk8 was detected in anti-pRb precipitates (Figure 4B). Furthermore, although cdk8 depletion prevented anticyclin C precipitates from phosphorylating a peptide derived from the RNA polymerase II C-terminal domain, it did not alter pRb kinase activity (Figure 4C). Thus, cdk8 is not the cyclin C-associated Rb kinase.

Cyclin C Forms Complexes with cdk3 to Phosphorylate pRb

In preliminary work, we found that cyclin C bound to a protein from cell extracts that was recognized by anti-PSTAIR antibodies. Because this domain is conserved in several cdks, including cdk3, and because cdk3 has effects on cell cycle progression (Meyerson et al., 1992; van den Heuvel and Harlow, 1993), we tested the possibility that it might be a cyclin C-associated kinase. Since endogenous cdk3 levels are extremely low in most cell types, we ectopically expressed FLAG-tagged cyclin C

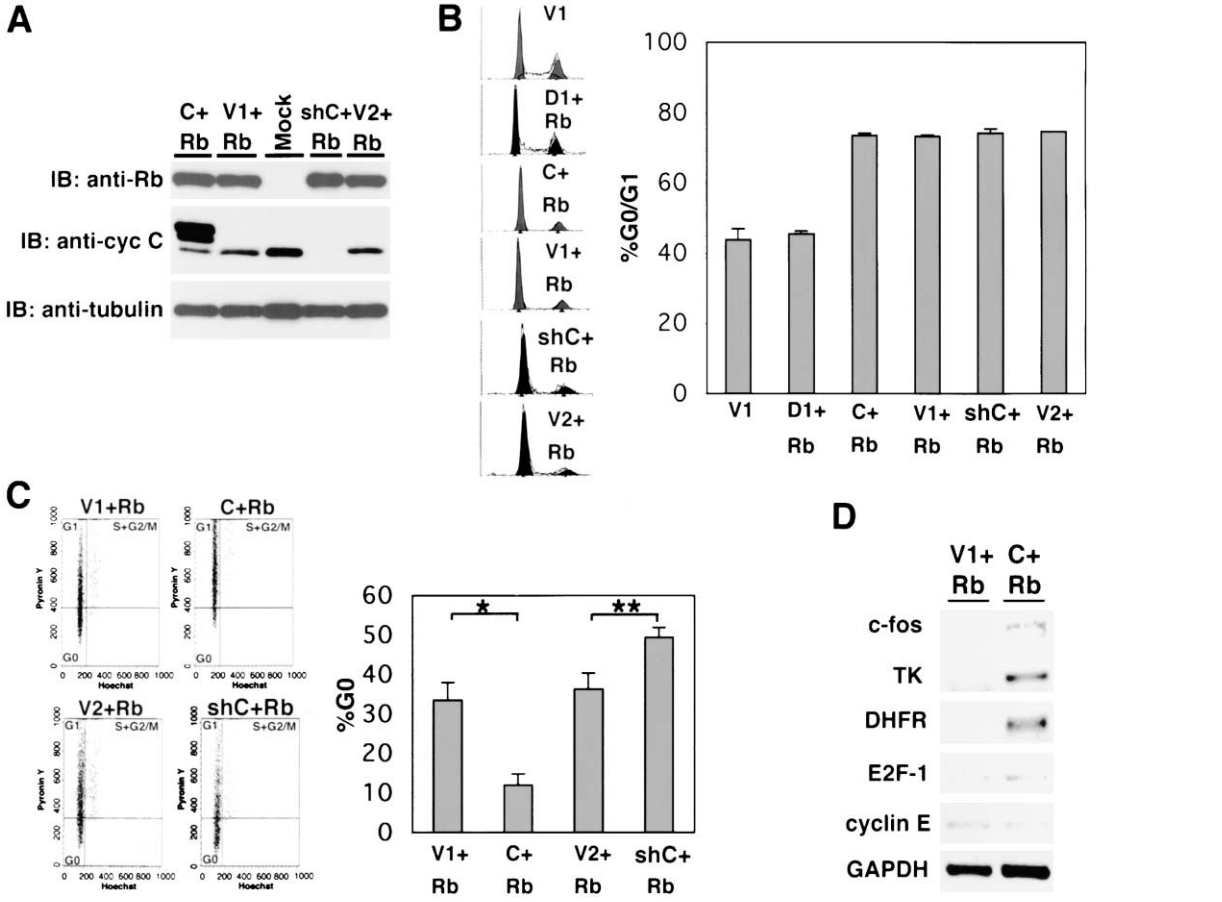

Figure 2. Cyclin C Promotes G0 Exit

(A) SAOS-2 cells were cotransfected with CD19 and: Rb and cyclin C; Rb and a vector control for the cyclin C expression vector (V1); nothing (mock); Rb and shC (an expression vector encoding an shRNA directed against cyclin C); and Rb and the vector control for the shC expression vector (V2). Immune blotting of extracts from $CD19^+$ cells was performed using the indicated antibodies. (Evidence that all three bands recognized by the anticyclin C antibody are cyclin C-derived is presented in Supplemental Data available on *Cell* website.)

(B) SAOS-2 cells were cotransfected with eGFP and the indicated plasmids described in (A) as well as vector alone (V1) and cyclin D1 + Rb (D1 + Rb). DNA content of $eGFP^+$ cells was determined by PI staining and FACS analysis. Left, representative FACS profiles; right, combined results of 4 independent experiments.

(C) SAOS-2 cells were cotransfected with a plasmid expressing CD19 and the indicated plasmids. Forty-eight hr later, cells were stained with Hoechst dye, Pyronin Y, and anti-CD19. Left, dot plots of Hoechst/Pyronin Y staining in $CD19^+$ SAOS-2 cells transfected with the indicated plasmids. The G0 quadrant was defined by cells with a 2n DNA content that had an RNA content lower than cells with >2n DNA (i.e., S + G2/M). Right, combined results from four independent experiments. (*$p < 0.0001$, **$p < 0.02$ by Student's two-tailed t test.)

(D) SAOS-2 cells were cotransfected with CD19 and the indicated plasmids. Forty-eight hr later, RNA was extracted from $CD19^+$ cells and analyzed by RT-PCR for the indicated mRNAs.

and HA-tagged cdk3 in SAOS-2 cells and performed coimmune precipitation experiments. Anticyclin C precipitates contained HA-tagged cdk3, and anti-cdk3 precipitates (using three different antibodies) contained cyclin C (both endogenous and ectopically expressed) (Figure 5A). The specificity of this interaction was demonstrated by the absence of interactions between cotransfected FLAG-tagged cyclin C and HA-tagged cdk2 (Figure 5B).

Overexpression of cdk3 with cyclin C in SAOS-2 cells led to enhanced phosphorylation of pRb at S807/811, and overexpression of dominant-negative cdk3, with or without coexpressed cyclin C, completely prevented it (Figure 5C). Although S807/811 is also a cdk4 substrate, expression of dominant-negative cdk4 did not prevent S807/811 phosphorylation, nor did expression of dominant-negative cdk2 (Figure 5C). Suppression of cdk3 expression by RNAi in T98G cells reduced the levels of endogenous pRb S807/811 phosphorylation during the G0/G1 transition, and abrogated the S807/811 kinase activity in cyclin C immune precipitates (Figure 5D). Thus, cdk3 is the endogenous cyclin C partner that phosphorylates pRb at S807/811.

Cdk3 Promotes G0 Exit

As expected, neither dominant-negative cdk3, cdk2, nor cdk4 had any effect on S phase entry by SAOS-2 cells coexpressing Rb and cyclin C (Figure 6A). However, expression of dominant-negative cdk3 along with pRb and cyclin C increased the proportion of cells with 2n DNA content that are in G0 to 83% (Figure 6B). Dominant-negative cdk2 or cdk4 had no effect on G0 status.

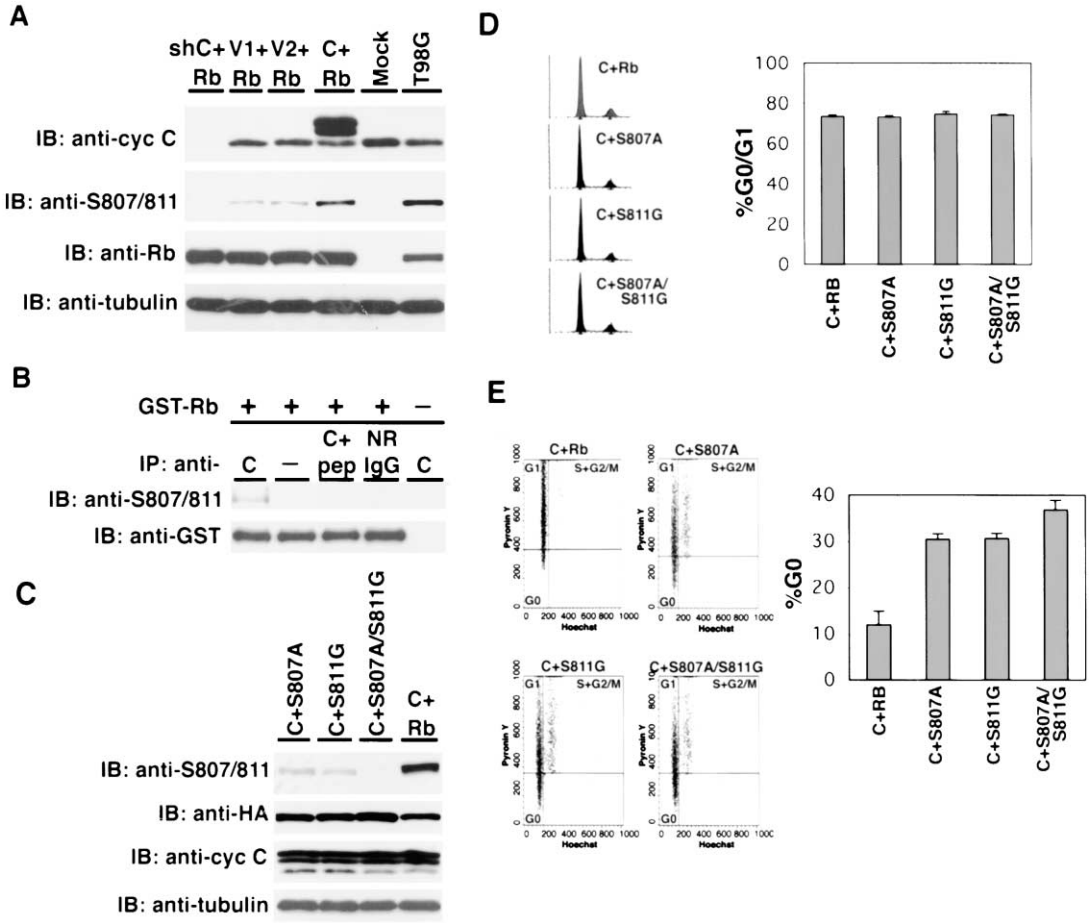

Figure 3. Cyclin C-Induced G0 Exit Requires pRb Phosphorylation at S807/811

(A) SAOS-2 cells were transfected with CD19, Rb and a plasmid encoding shRNA directed against cyclin C (shC), or Rb and a cyclin C expression vector. (V1 is a vector control for cyclin C; V2 is a vector control for shRNA.) Lysates from $CD19^+$ cells were analyzed by immunoblot for cyclin C and tubulin. (Note expression of endogenous cyclin C in the vector control lanes and its loss in the shRNA lane). Phosphorylation status of Rb at S807/811 was determined by immunoblot using a phosphospecific antibody. Mock, mock-transfected SAOS-2 cells; T98G, T98G lysates as a positive control for the phosphospecific Rb Ab.

(B) Lysates of SAOS-2 cells transfected with cyclin C and Rb were subjected to immune precipitation using anticyclin C (with or without a competing peptide) or control normal rabbit IgG (NR IgG). Precipitates were mixed with GST-Rb and the products of an in vitro kinase reaction were analyzed by immunoblot (IB) using S807/811 phosphospecific Rb antibody or anti-GST.

(C) SAOS-2 cells were transfected with cyclin C and Rb or the indicated nonphosphorylatable Rb variants. After 48 hr, lysates were analyzed by immunoblot for phosphorylation at S807/811, for total expression of pRb, for total expression of cyclin C, and for tubulin.

(D) PI staining and FACS analysis of SAOS-2 cells transfected with eGFP and the indicated nonphosphorylatable pRb variants. Left, representative FACS profiles; right, combined results of four independent experiments.

(E) DNA/RNA analysis of SAOS-2 cells expressing either the singly or doubly mutated Rb variants along with cyclin C. Left, representative FACS profiles; right, combined results of four independent experiments. ($p < 0.00005$ comparing C + Rb to C + 807A or C + 811G, $p < 0.002$ comparing C + 807A/811G to C + 807A or C + 811G by Student's two-tailed t-test).

To examine the effects of endogenous cyclin C/cdk3 on the G0/G1 transition, expression of cyclin C or cdk3 was suppressed by RNAi in G0-arrested T98G cells. Suppression of cyclin C delayed serum-induced S phase entry by 8 hr, and this delay was entirely the result of an 8 hr delay in G0 exit (Figure 7A). Suppression of cdk3 produced a nearly identical result (Figure 7B). Although the data of Figures 3–6 suggest that pRb is the relevant cdk3 target, we obtained additional confirmation by comparing the effects of cdk3 expression on G0 exit in wild-type versus $Rb^{-/-}$ 3T3 cells. Restoring cdk3 expression to wild-type 3T3 cells (which do not express endogenous cdk3) accelerated G0 exit (Figure 7C). At 2 hr after serum stimulation, 50% of cdk3-expressing wild-type cells were in G0 compared to 90% of control transfected wild-type cells ($p = 0.0005$ by Student's t-test); at 4 hr only 10% of cdk3-expressing cells were in G0 compared to 70% of control cells ($p = 0.006$). By comparison, although cdk3 expression appeared to slightly reduce the proportion of $Rb^{-/-}$ 3T3 cells in G0, none of the differences with control-transfected cells were significant. Thus, cdk3's ability to promote G0 exit depends on the presence of pRb. Furthermore, even prior to serum stimulation, a lower proportion of $Rb^{-/-}$ 3T3 cells were in G0 compared to wild-type 3T3 cells, and this difference persisted at 2 hr and 4 hr after serum

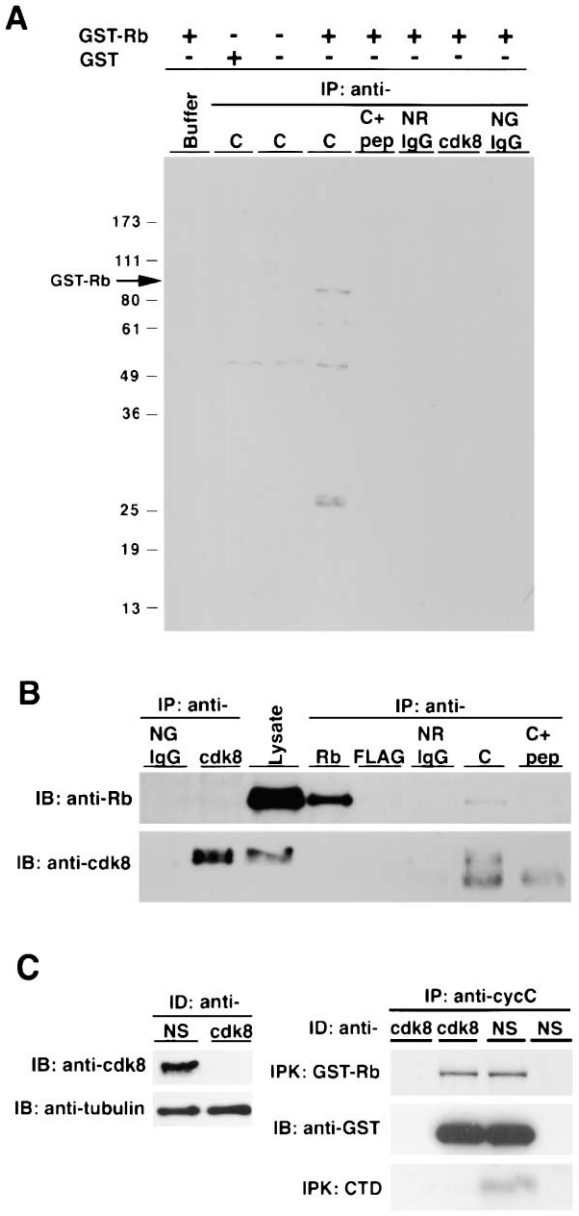

Figure 4. The Cyclin C-Associated pRb Kinase Is Not cdk8

(A) Lysates were prepared from T98G cells that had been placed in serum-free medium for three days then stimulated with 15% FBS for 6 hr. Immune precipitations were performed using anticyclin C (C), anticyclin C with a competing cyclin C peptide (C + pep), or anti-cdk8 (cdk8). Precipitates were tested for their ability to phosphorylate GST or GST-Rb(379–928). Cyclin C precipitates phosphorylated GST-Rb(379–928), but not GST alone. Normal rabbit IgG (NR IgG) was a control for anticyclin C; normal goat IgG (NG IgG) was a control for anti-cdk8.

(B) Lysates were prepared from T98G cells as described in (A), and subjected to immune precipitation using anti-cdk8 (cdk8), anti-Rb (Rb), anticyclin C (C), and anticyclin C with a cyclin C competing peptide (C + pep). Precipitates were analyzed by immunoblot using anti-Rb or anti-cdk8. The lower band in the anti-cdk8 immunoblot of the cyclin C immune precipitation is nonspecific (note its persistence in the C+ pep lane and its absence from the cdk8 immune precipitate). Anti-FLAG was used as an isotype control for anti-Rb; other controls were as described in (A).

(C) T98G lysates were depleted of cdk8 by two rounds of incubation with anti-cdk8 (ID, immune depleted using the anti-cdk8 or nonspecific normal goat IgG (NS) as a control). Cyclin C immune precipitates from lysates depleted using anti-cdk8 or nonspecific normal goat IgG (NS) were tested for in vitro kinase activity (IPK) using GST-Rb or a tandemly repeated CTD peptide as substrate. Immune blot (IB) using anti-GST indicated that equal amounts of GST-Rb were present in the kinase assays in lanes 2 and 3. As a control for nonspecific phosphorylation, GST-Rb was omitted from the reactions in lanes 1 and 4.

stimulation. Similar differences were observed in the cdk3-transfected cells. Thus, the absence of pRb impairs G0 arrest.

Discussion

Motivated by the possibility that cyclin C might regulate mammalian cell cycle progression, we have demonstrated that, similar to G1 cyclins, cyclin C forms complexes with pRb and stimulates its phosphorylation. However, cyclin C-directed pRb phosphorylation occurs earlier in the cell cycle than authentic G1 cyclin-directed activities and does not promote S phase entry. Rather, cyclin C stimulates cdk3-mediated phosphorylation of pRb at S807/811 (at a minimum), and this activity is required for efficient transition from G0 to G1.

Although cyclin C was cloned on the basis of its ability to rescue G1 cyclin-deficient yeast (Leopold and O'Farrell, 1991; Lew et al., 1991), subsequent analyses have provided little support for its mammalian G1 cyclin activity. One report suggested that in vitro translated cyclin C might bind to pRb with low affinity (Dowdy et al., 1993), but another indicated that cyclin C did not stimulate pRb hyperphosphorylation in vivo (although the authors conceded their inability to detect cyclin C protein in transfectants) (Hinds et al., 1992). Instead, cyclin C has been extensively characterized as a component of the RNA polymerase II holoenzyme (Leclerc et al., 1996; Rickert et al., 1996; Tassan et al., 1995). Homologies between cyclin C/cdk 8 and the SRB11/SRB10 complex in *S. cerevisiae*, which is required for proper CTD phosphorylation and RNA polymerase II function (Liao et al., 1995), have further steered attention away from cyclin C's potential contributions to cell cycle regulation. However, IL-3 treatment of resting BAF-B03 murine pro-B cells induced cyclin C expression as cells reentered the cycle, and while ectopic expression of cyclin C alone had no effect on BAF-B03 cell proliferation, coexpression with c-Myc stimulated IL-3-independent growth (Liu et al., 1998b). Furthermore, these cell cycle effects (as well as others; Liu et al., 1998a) were cdk8-independent, suggesting that they did not occur by means of CTD phosphorylation.

Our present work provides direct support for cyclin C's role as a physiological cell cycle regulator, in particular during the G0 to G1 transition. Our inference is based on cyclin C's effects on G1-specific gene expression and on an assay that demonstrates cyclin C-induced increase in total RNA abundance in cells with a 2n DNA content. We chose the latter assay because it had been validated in a system with a well-characterized G0 phase, namely resting hematopoietic stem cells (Ladd

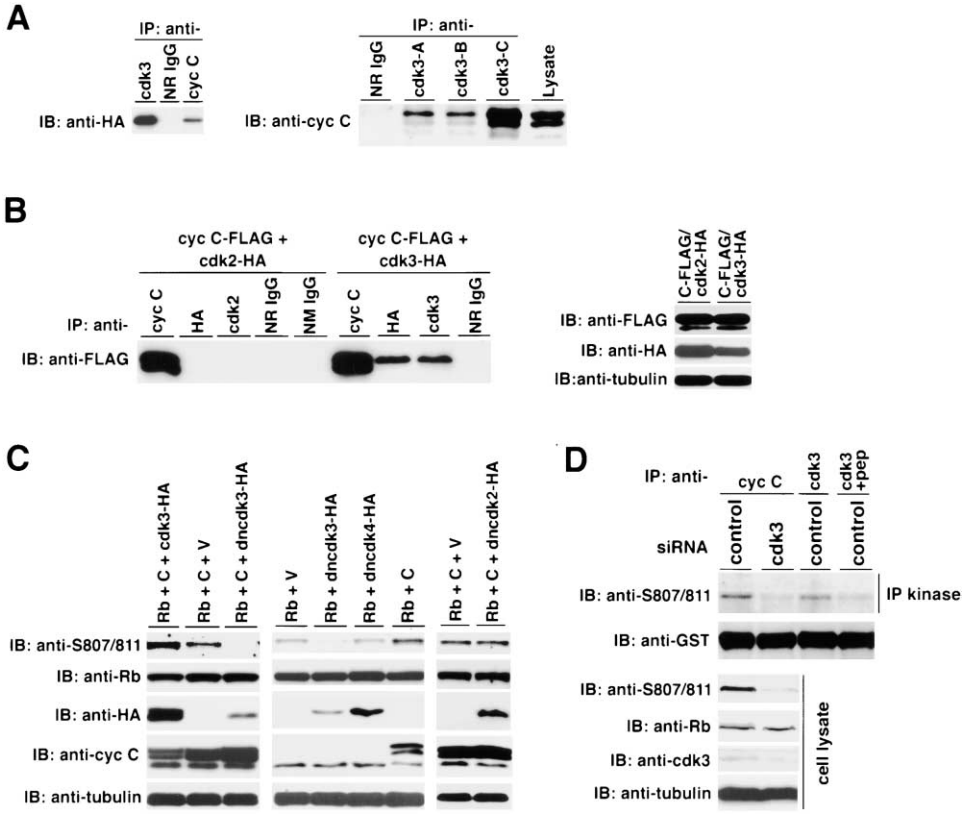

Figure 5. The Cyclin C-Associated pRb Kinase Is cdk3

(A) SAOS-2 cells were cotransfected with FLAG-tagged cyclin C and HA-tagged cdk3, and lysates were subjected to immune precipitation followed by immune blot analysis. Left, anticyclin C precipitates analyzed by anti-HA blot (NR IgG, normal rabbit IgG as a control for both anti-cdk3 and anticyclin C antibodies). Right, precipitates using three different anti-cdk3 antibodies (denoted A, B, and C) analyzed by anticyclin C blot (NR IgG, was used as a control for the anti-cdk3 antibodies).

(B) SAOS-2 cells were cotransfected with FLAG-tagged cyclin C and HA-tagged cdk2 or HA-tagged cdk3. Left, lysates were subjected to immune precipitation using the indicated antibodies and precipitates were analyzed by anti-FLAG immune blot to detect the presence of ectopic cyclin C. (NR IgG was used as a control for the anti-HA and anti-cdk3 antibodies; NM IgG, normal mouse IgG, was used as a control for the anti-cdk2 antibody). Right, total lysates were analyzed by immune blot to document levels of ectopic protein expression.

(C) SAOS-2 cells were cotransfected with Rb, cyclin C, HA-tagged wild-type cdk3 (cdk3-HA), HA-tagged dominant- negative cdk3 (dncdk3-HA), HA-tagged dominant-negative cdk4 (dncdk4-HA), HA-tagged dominant-negative cdk2 (dncdk2-HA) or control vectors (V). Lysates were analyzed by immunoblot.

(D) G0-arrested T98G cells were transfected with siRNA oligonucleotides directed against cdk3 or control oligonucleotides. Forty-eight hr later, cells were stimulated for 6 hr with 15% FBS and lysates were subjected to immune precipitation using anticyclin C or anti-cdk3 in the presence and absence of a competing cdk3 peptide. In vitro kinase reactions were performed using GST-Rb as substrate. The ability of the precipitates to phosphorylate GST-Rb at S807/811 was determined by immunoblot. Whole-cell lysates were also examined for pRb phosphorylation at S807/811, total pRb, and total cdk3.

et al., 1997). Additional validation comes from our own observation that suppressing cyclin C or cdk3 in T98G cells resulted in an 8 hr delay in G0 exit after serum stimulation. The existence of G0 was originally inferred because arrested cells stimulated to reenter the cell cycle took the same time to reach S phase that actively cycling cells took to traverse from M to S. Therefore, it was thought that resting cells must have been in a different state i.e., distinct from G1, when they were resting (Lajtha, 1963). In the experiments presented here (Figures 7A and 7B), S phase entry occurred 20 hr after control cells were treated with serum. When cyclin C or cdk3 were suppressed by RNAi, cells entered S phase 28 hr after serum stimulation. In fact, though, according to the RNA/DNA assay, these cells did not enter G1 until 8 hr after serum stimulation (as opposed to almost immediate entry by control cells) so that the G1-to-S transition time remained unchanged at 20 hr. Thus, as expected, G1-to-S transition time was the same in siRNA-treated cells as it was in control cells, and their overall delay in S phase entry is evidence for time spent in G0. Furthermore, the inferred duration of G0 in siRNA-treated cells, 8 hr, is reflected precisely by the time during which the RNA/DNA ratio remains low, indicating that this assay accurately identifies the G0 state.

Given the fact that cyclin C/cdk 8 exerts important effects on RNA polymerase II, what is the likelihood that cyclin C's influence on RNA abundance during cell cycle progression might still be mediated through cdk8's CTD kinase activity? This is unlikely for at least two reasons. First, these shifts in bulk RNA content reflect changes in ribosomal RNA abundance that depend on the activi-

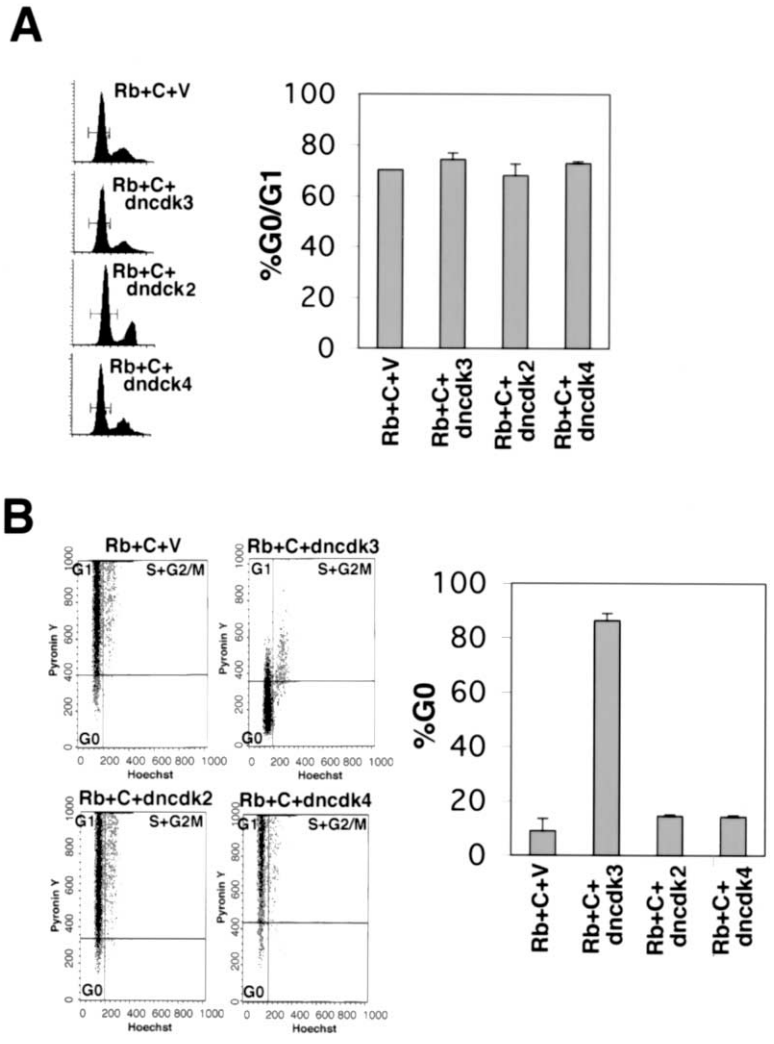

Figure 6. Dominant-Negative cdk3 Blocks G0 Exit

(A) SAOS-2 cells were cotransfected with CD19, pRb, cyclin C, dominant-negative cdk3 (dncdk3), dominant-negative cdk2 (dncdk2), dominant-negative cdk4 (dncdk4) or control vectors (V). DNA content was monitored in CD19 positive cells by Hoechst staining followed by FACS analysis. Left, representative FACS profiles; right, combined results of four independent experiments.

(B) SAOS-2 cells transfected as described in (A) were stained with Hoechst dye and Pyronin Y, and CD19 positive cells were analyzed by FACS. Left, representative FACS profiles; right, combined results of four independent experiments.

ties of RNA polymerases I and III, not II. In that regard, it is worth noting that pRb directly represses the activities of RNA polymerases I and III (Cavanaugh et al., 1995; White et al., 1996). This suggests that the regulation of cell cycle entry from G0 is tightly coordinated with the transcriptional machinery and that phosphorylation of pRb by cyclin C/cdk3 inactivates both its blockade of G0 exit and its suppression of rRNA synthesis. These observations are consistent with the notion that G0 is a resting, quiescent state comparable to stationary phase in yeast (Herman, 2002), and that resumption of active cell cycling is predicated on rearming the cell for translation (Pardee, 1989). While the tandem control of these events might not be unexpected, it is surprising that pRb itself contributes to regulating both. The fact that part of the tumor suppressor activity of $p19^{ARF}$ may involve suppression of rRNA synthesis indicates that the coordination of cell cycle progression and ribosome synthesis through a single molecular switch point may be a general phenomenon (Sugimoto et al., 2003). The cyclin C/cdk3-mediated increase in RNA abundance is not a nonspecific effect because expression of cyclin C alone i.e., without Rb, in SAOS-2 cells and in exponentially growing T98G cells did not result in increased RNA content.

The involvement of cdk3 in this process rather than cdk8 is surprising, although consistent with previously reported cdk8-independent effects of cyclin C in BAF-B03 cells (Liu et al., 1998b). Like cyclin C, cdk3 is structurally related to other cell cycle regulators (Meyerson et al., 1992), and a variety of observations suggest that it can influence cell cycle progression. Cdk3 complements CDC28 in *S. cerevisiae* (Meyerson et al., 1992), and dominant-negative cdk3 causes growth arrest in SAOS-2 cells prior to S phase entry (van den Heuvel and Harlow, 1993). However, phosphorylation of pRb by cdk3 is insufficient to inactivate its suppression of S phase entry (Connell-Crowley et al., 1997). Rather, our data indicate that cdk3 is stimulated by cyclin C in G0 to phosphorylate pRb at S807/811 to promote G1 entry. Notably, dominant-negative cdk3 placed a higher proportion of SAOS-2 cells in G0 than shRNA directed against cyclin C (compare Figure 6B to Figure 2C). Thus, cdk3 may interact with additional regulatory subunits or may be activated to some extent even without associating with cyclin C in SAOS-2 cells. However, in T98G cells, suppression of cyclin C or cdk3 placed the same proportion of cells in G0. In this more physiological cell type, cyclin C and cdk3 may be specifically matched.

Despite our evidence implicating cdk3 in the G0 to

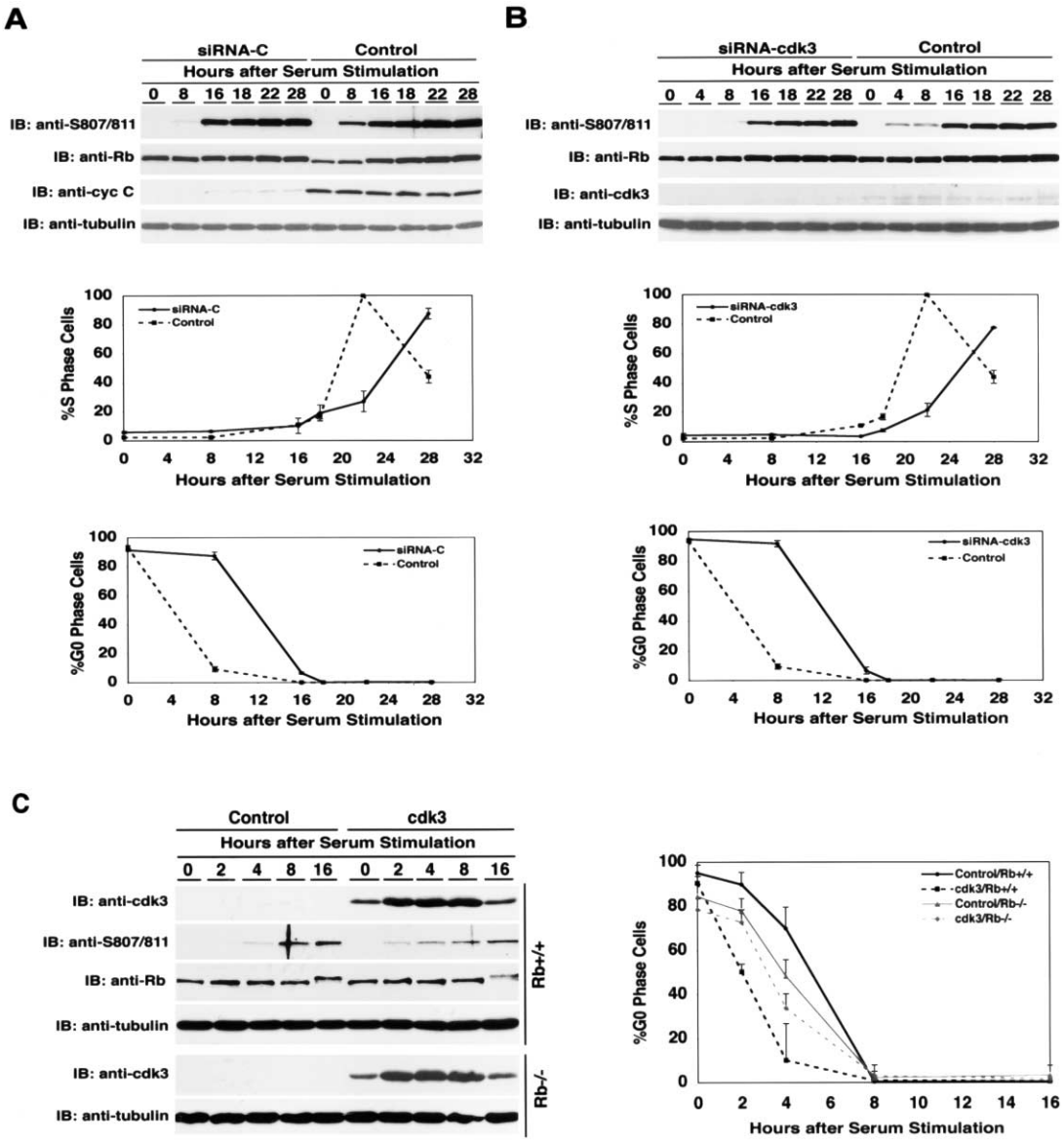

Figure 7. Endogenous Cyclin C and cdk3 Target pRb to Promote Efficient G0 Exit

(A) T98G cells were serum-starved for 72 hr then transfected with siRNA oligonucleotides directed against cyclin C (siRNA-C) or control oligonucleotides (Control). After an additional 48 hr in serum-free medium, transfected cells were treated with 15% FCS. At the indicated times, some cells were collected and extracts were analyzed by immunoblot for Rb phosphorylation at S807/811, total Rb, cyclin C, and tubulin (upper image). Additional cells were analyzed for DNA content by propidium iodide staining and the proportion of cells in S phase was determined (middle image). A final group of cells was analyzed for RNA and DNA content and the proportion of cells in G0 was determined (lower image). Results in the lower images were derived from four independent experiments; error bars, standard deviation.

(B) Serum starved T98G cells were treated exactly as described in (A), except that RNA oligonucleotides directed against cdk3 were used and cdk3 levels were determined in cell lysates.

(C) 3T3 cells from wild-type or *Rb*$^{-/-}$ mice of the same strain were transfected with a control vector or a cdk3 expression vector along with eGFP. Thirty-six hr later, cells were placed in 0.1% serum for two days and then stimulated with 15% FBS. Left, lysates were collected at the indicated times and analyzed by immunoblot using the indicated antibodies. (The phosphospecific S807/811 pRb antibody raised against the human protein recognizes murine pRb phosphorylated at the analogous positions, S800/804.) Right, parallel cultures were harvested at the same time points and eGFP-positive cells were analyzed for RNA and DNA content to determine the proportion of cells in G0. Combined results are shown for three independent experiments; error bars, standard deviation; outcomes of tests for statistical significance are described in the text.

G1 transition, its essential contribution to this process is questioned by the fact that most inbred laboratory mouse strains lack functional cdk3 (Ye et al., 2001). While this may cast doubt on an essential function for cdk3, it is likely that the "rescue" of these mice reflects functional redundancy among cdks. Nonetheless, compensation for loss of cdk3 may not be complete, because overexpressing cdk3 accelerated G0 exit in 3T3

cells from cdk3-deficient mice. Thus, the effects of cdk3 loss are still detectable in cdk3-deficient mouse cells, and functional redundancy among the cdks is not fully compensatory for stimulating G0 exit.

This point is also relevant in considering our observation that suppression of cyclin C or cdk3 in T98G cells resulted in only a transient inability to exit G0. Again, there may be redundant specificities in the cyclin/cdk system, which could achieve some of the same regulatory effects as cyclin C/cdk3. However, the 8 hr delay in G0 exit in the absence of cyclin C or cdk3 indicates that cyclin C/cdk3 promotes G0 exit much more efficiently than putatively redundant kinases. Similar delays rather than permanent blocks to S phase entry have been observed when G1 cyclins are targeted by intracellular antibody administration (Pagano et al., 1992) or when cdk4 is inactivated by targeted disruption (Tsutsui et al., 1999). In fact, a potential source of redundancy in our system might be cdk4 because it also phosphorylates pRb at S807/811 (Brantley and Harbour, 2000; Pan et al., 1998). Phosphorylation at these sites dissociates c-abl from the C domain of pRb (Knudsen and Wang, 1996) and prevents the binding of LXCXE proteins (Harbour et al., 1999). It also contributes to the conformational change that permits S567 phosphorylation and disruption of E2F binding (Harbour et al., 1999). Whether any of these steps is required for inactivating pRb's suppression of G0 to G1 transition remains to be determined. However, a more likely scenario is that cyclin C/cdk3-mediated phosphorylation at S807/811 in G0-arrested cells produces effects specific for G0 exit. In contrast, when cells are actively cycling, D-cyclin/cdk4 phosphorylation of these sites promotes S phase entry and cyclin C/cdk3 would not be needed again for pRb inactivation unless cells reenter G0. In further support of this model is the fact that S807/811 phosphorylation occurs within 3 hr of stimulating G0-arrested T lymphocytes with anti-CD3/anti-CD28 (Lea et al., 2003).

The importance of pRb as a cdk3 target in G0 exit is supported by our cotransfection experiments in SAOS-2 cells but is more directly confirmed by examination of $Rb^{-/-}$ 3T3 cells. Unlike wild-type cells, $Rb^{-/-}$ 3T3 cells were not stimulated to exit G0 by ectopic expression of cdk3. pRb's suppression of G0 exit is further supported by the observation that a significantly lower proportion of $Rb^{-/-}$ 3T3 cells could be arrested in G0 than wild-type 3T3 cells. Thus, even though p107 is overexpressed in $Rb^{-/-}$ 3T3 cells (Hurford et al., 1997), it cannot fully compensate for pRb's absence. These observations are also consistent with the demonstration that acute, Cre-mediated inactivation of Rb in G0 arrested fibroblasts stimulates G0 exit (Sage et al., 2003). However, this is not meant to imply that other pocket proteins such p130 do not also play a role in the G0/G1 transition (Canhoto et al., 2000; Mayol et al., 1995, 1996; Moberg et al., 1996; Sardet et al., 1995), and we are investigating the ability of cyclin C/cdk3 to phosphorylate p130, too. Also, it is important to note that S807/811 may not be the only pRb sites that must undergo phosphorylation in order for cells to exit G0. For example, our MS analysis also revealed phosphorylation of S249 in response to cyclin C overexpression (data not shown). Thus, S807/811 phosphorylation may be necessary but not sufficient for G0 exit, and a more thorough mapping is currently underway which may reveal additional relevant phosphorylation sites.

Finally, it is curious that despite the robust ability of human cyclin C to rescue G1 cyclin-deficient yeast, functional analyses of the nominal yeast homolog have consistently identified its associated CTD kinase activity, an effect that appears to have little to do with cell cycle regulation. A possible explanation would be that SRB11/SSN8 is simply not able to model mammalian cyclin C. For example, *S. cerevisiae* SSN8 cannot rescue triple CLN-deficient *S. cerevisiae*, and human cyclin C cannot complement an *ssn8* mutant (Kuchin et al., 1995). Thus, SRB11/SSN8 and eukaryotic cyclin C are not functionally interchangeable even though human cyclin C is clearly active in yeast (Lew et al., 1991). Whether this is due to the presence of unique pathways in eukaryotes or to the possibility that SRB11/SSN8 is not a functional ortholog of cyclin C remains to be established.

Experimental Procedures

Cell Culture

All cells were grown in Dulbecco's Modified Eagle Medium (MDEM) (GIBCO) containing 10% fetal bovine serum (FBS), 10 units/ml penicillin (Sigma) and 0.1 μg/ml streptomycin (Sigma). For synchronization studies, cells were incubated in medium containing 0.1% FBS (3T3 cells) or no FBS (T98G and SAOS-2 cells) for 72 hr, then stimulated with medium containing 15% FBS. For some experiments, cells were metabolically radiolabeled by incubation in phosphate-free medium for 1 hr followed by 0.42 mCi/ml [^{32}P]orthophosphate (285 Ci/mg, PerkinElmer Life Sciences, Inc., Boston, MA) for 3 hr. 3T3 cells from $Rb^{+/+}$ and $Rb^{-/-}$ mice in the same strain backgrounds (C57Bl/6 × 129Sv) were provided by Dr. W. Kaelin (Dana-Farber Cancer Institute).

Plasmids and Transfections

cDNAs encoding HA-tagged human Rb, human CD19, and eGFP in pcDNA3, and cyclin D1 in pSG5, were provided by Dr. J. De Caprio (Dana-Farber Cancer Institute, Boston, MA). HA-tagged cdk2, dominant-negative cdk2, dominant-negative cdk3, and dominant-negative cdk4 in pCMV-Bam-neo were provided by Dr. S van den Heuvel (Massachusetts General Hospital, Boston, MA). Human wild-type cdk3 (a gift of Dr. M. Meyerson, Dana-Farber Cancer Institute, Boston, MA) was constructed by inserting the cDNA into vector, pRcCMV. Human cyclin C cDNA was a gift from Dr. Steven Reed (Scripps Research Institute, La Jolla, CA). Sequences encoding the FLAG epitope were introduced at the C-terminus of cyclin C using standard techniques (Zhang et al., 1994). FLAG-tagged cyclin C cDNA was cloned into pcDNA3, pCMV-Bam-neo, and pMT21-neo. HA-tagged Rb cDNAs were mutagenized using the QuikChange XL Site-Directed Mutagenesis system (Stratagene, La Jolla, CA). COS cell transfections were performed using Fugene 6 Transfection Reagent (Roche Diagnostic Corp., Indianapolis, IN) as recommended by the manufacturer, and analyzed 48 hr later. SAOS-2, T98G, and 3T3 cell transfections were performed using Lipofectamine Plus Reagent (Invitrogen Corp., Carlsbad, CA) and analyzed 48–60 hr after transfection.

RNAi

To generate shRNA targeting cyclin C in SAOS-2 cells, complementary single strand oligonucleotides targeting cyclin C mRNA at nucleotides 267–287 (GenBank accession number gi: 7382485), 5′GATCC CGTTATTGCCACTGCTACGGTTCAAGAGACCGTAGCAGTGGCAA TAACTTTTTTGGAAA3′ and 5′AGCTTTTCCAAAAAAGTTATTGCCA CTGCTACGGTCTCTTGAACCGTAGCAGTGGCAATAACGG3′, were annealed and cloned into pSilencer-2.0-U6 (Ambion, Inc, Austin, TX). Plasmids were introduced into cells using Lipofectamine Plus Reagent. To suppress cyclin C expression in G0-arrested T98G cells, double-strand RNA oligonucleotides targeting cyclin C mRNA at nucleotides 267–287 (GenBank accession number gi: 7382485),

were synthesized and introduced into cells using Oligofectamine (Invitrogen Corp., Carlsbad, CA). To target cdk3, double-stranded RNA oligonucleotides targeting cdk3 mRNA at nucleotides 254–274 or 350–370 (GenBank accession number gi: 4557438), were synthesized and also introduced into cells using oligofectamine.

Immune Precipitation

Except where indicated, antibodies were coupled to protein A or Protein A/G Plus agarose beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) by equilibrating 400 μl bead suspension with an equal volume of 50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 5 mM EDTA and 0.1% NP-40 for 10 min at RT. Antibody (50 μg) was added with buffer to a final volume of 1 ml and mixed gently at 4°C overnight. Beads were washed three times with equilibration buffer, once with 0.1 M sodium borate [pH 9.0], resuspended in 1 ml of 40 mM dimethylpimelimidate dihydrochloride in 0.1 M sodium borate [pH 9.0], and gently mixed for 1 hr at RT. Beads were washed twice with 40 mM ethanolamine in 0.1 M sodium borate [pH 8.0], resuspended in 1 ml of 40 mM ethanolamine in 0.1 M sodium borate [pH 8.0], and mixed gently at RT for 2 hr. Finally, beads were washed once with NP-40 lysis buffer (150 mM NaCl, 1.0% NP-40, 50 mM Tris-HCl [pH 8.0]), twice with NET.N buffer (100 mM NaCl, 0.5% NP-40, 20 mM Tris-HCl [pH 8.0]), once with PBS, and then stored in PBS containing 0.1% sodium azide at 4°C.

Cells were lysed after washing with cold PBS by incubation in cold NP-40 lysis buffer with protease inhibitor cocktail set I (Calbiochem, San Diego, CA), 1 mM NaF, 0.5 mM Na_3VO_4, and 10 mM β-glycerophosphate for 10 min on ice before collection by scraping. The lysate was rotated for an additional 30 min at 4°C and clarified by centrifugation. Lysates containing 0.1 to 1.0 mg protein were precleared using conjugated normal rabbit, mouse or goat IgG at 4°C for 30 min. Immune precipitations were then performed by incubating lysates overnight at 4°C with 2 to 2.5 μg of primary conjugated antibodies. These included: rabbit anti-HA (clone Y-11), rabbit antihuman Rb (C-15), rabbit antihuman cyclin C (T-19), rabbit antihuman cyclin D1 (M-20), rabbit antihuman cyclin D3 (C-16), rabbit antihuman cdk3 (Y-20), and goat antihuman cdk8 (C-19) (all from Santa Cruz Biotechnology, Inc.); rabbit antihuman cdk3 (Biosource, Camarillo, CA); rabbit antihuman cdk3 (NeoMarker, Fremont, CA); murine antihuman Rb (clone G3 245, IgG_1, BD Biosciences PharMingen, San Diego, CA); and murine anti-FLAG M2 MAb (Sigma, St. Louis, MO). Immune complexes were collected by centrifugation, washed twice with NP-40 lysis buffer, twice with NET.N buffer, and once with PBS. Complexes were eluted by boiling beads in 1.5× SDS-PAGE sample loading buffer.

Cell Cycle Analysis

To analyze DNA content, cells were suspended in chilled 80% ethanol and kept at 4°C for at least 1 hour. Fixed cells were treated with 500 μg/ml RNase A in PBS at 37°C for 45 minutes, and then stained with 69 μM propidium iodide in 38 mM sodium citrate at RT for 30 min. DNA content was determined by flow cytometry using FACScan (Becton Dickinson, Hialeah, Fla.), and data were analyzed with Cell Quest software (Becton Dickinson). For experiments involving transiently transfected cells, cells were fixed in 3% paraformaldehyde/2% glucose in PBS on ice for 10 min and GFP^+ cells were gated during FACS analysis.

For combined RNA and DNA analysis, cells were trypsinized, washed twice with Buffer I (1% FBS in PBS), resuspended at 0.5−1.0 × 106 cells in 1 ml of 10 μg/ml Hoechst stain in Buffer I, and incubated for 40 min at 37°C. An equal volume of 0.5 μg/ml Pyronin Y in Buffer I was added directly and incubation continued for an additional 20 min. Cells were washed once with Buffer II (1% FBS and 0.01% sodium azide in PBS, [pH 7.4]), resuspended in 200 μl of Buffer II, and incubated with 20 μl APC-conjugated antihuman CD19 (Pharmingen) for 30 min at 4°C. (CD19 staining was only used for SAOS-2 cell transfections, not T98G or 3T3 cell transfections.) Cells were washed once with Buffer I and analyzed using a Becton Dickinson LSR 3 Laser (HE-NE 633 nm for APC, ARGON 488 nm for Pyronin Y, and HE-CAD 325 nm for Hoechst stain). Gates were set using empty vector-transfected SAOS-2 cells stained with APC-conjugated anti-CD19 in the absence of Pyronin Y and Hoechst staining, cells stained with Hoechst in the absence of APC-anti-CD19 and Pyronin Y, and cells stained with Pyronin Y in the absence of APC-anti-CD19 and Hoechst dye.

Immunoblotting

Whole-cell lysates (20–100 μg protein) or immune precipitates were separated by SDS-PAGE and electrophoretically transferred to PVDF membranes (Millipore, Billerica, MA). After blocking in 10% nonfat dry milk for 90 min at RT, they were incubated with the following antibodies in 2% nonfat dry milk for 2 hr at RT or overnight at 4°C: rabbit antihuman cyclin C (T-19) at 1:1000; rabbit antihuman cyclin D3 (C-16) at 1:1000; rabbit antiphosphorylated Rb at S807/811 (Cell Signaling Technology, Beverly, MA) at 1:1000; rabbit antihuman cdk3 (Y-20) at 1:1000; murine antihuman Rb (clone G3 245) at 1:1000; murine anti-HA (clone 12CA5) at 1:200; murine anti-GST (clone DG122) at 1:200; murine antitubulin (Sigma) at 1:4000 or murine anti-FLAG M2 at 8 μg/ml. Blots were then incubated with HRP-conjugated sheep antimouse Ig or donkey antirabbit Ig at 1:2000 in 2% nonfat dry milk for 90 min at RT. Blots were developed using Enhanced Chemiluminescence Reagent Plus (DuPont/New England Nuclear, Boston, MA). In some cases, 300 μg of whole-cell lysate protein was incubated with 3 μl λ-phosphatase (New England Biolabs, Beverly, MA) in 150 μl of the manufacturer's recommended buffer for 30 min at 30°C with or without phosphatase inhibitors (1 mM NaF, 0.5 mM Na_3VO_4, and 10 mM β-glycerophosphate).

In Vitro Kinase Assays

To assay cyclin C-associated kinase activity, 1 mg protein from an NP-40 lysate was incubated with 2.5 μg rabbit antihuman cyclin C (T-19) conjugated to protein A beads for 2 hr at 4°C. Beads were washed twice with NP-40 lysis buffer, twice with NET.N buffer, and twice with kinase buffer (50 mM Tris HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM DTT). Beads were resuspended in 20 μl of kinase buffer containing 13 μM ATP or 20 μM ATP (for assays analyzed using phosphospecific Rb antibodies), 1 μl [γ-^{33}P]ATP (3000 Ci/mmol, PerkinElmer Life Sciences), 0.45 μg GST-Rb(379–928), GST, or a peptide containing tandem repeats of the RNA polymerase II C-terminal domain substrate site (Rickert et al., 1996). the reaction was incubated for 30 min at 30°C with occasional mixing. (Expression and purification of GST-Rb(379–928) and GST were performed as described (Smith and Johnson, 1988) using plasmids pGSTRb-Rb (379–928) provided by Dr. W. Kaelin (Dana-Farber Cancer Institute, Boston, MA) and pGEX-2T (Amersham Biosciences Corp., Piscataway, NJ). The reaction was stopped by adding 10 μl of 4× SDS-PAGE sample buffer and boiling. Samples were resolved by SDS-PAGE and electrophoretically transferred to PVDF membranes. Membranes were exposed to BioMax MR X-ray film (Kodak, Rochester, NY) to visualize phosphorylated proteins followed by immunoblotting.

Cyclin D1-associated kinase activity was assayed as described (Matsushime et al., 1994) with minor modifications. Cells were lysed in 50 mM HEPES [pH 7.5], 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol and 0.1% Tween 20, and immune precipitations were performed using 200 μg of protein and 15 μg murine antihuman cyclin D1 (clone DCS-11, NeoMarkers/Lab Vision Corp., Fremont, CA). Immune precipitates were washed four times with lysis buffer, and twice with cyclin D1 kinase buffer (50 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 2.5 mM EGTA, and 1 mM DTT). Beads were resuspended in 15 μl cyclin D1 kinase buffer supplemented with 13 μM ATP, 1 μl [γ-^{33}P]ATP (3000 Ci/mmol, PerkinElmer Life Sciences), 0.45 μg GST-Rb(379–928) or GST, and incubated for 30 min at 30°C with occasional mixing.

Cyclin E-associated kinase activity was assayed as described (Geng et al., 1999) with minor modifications. Immune precipitations were performed using 200 μg of protein from NP-40 lysates and 15 μl of murine antihuman cyclin E (clone HE-111) conjugated to agarose beads (Santa Cruz Biotechnology, Inc.). Kinase assays were performed as described for cyclin C precipitates.

RT-PCR

For RNA preparation, SAOS-2 cells were cotransfected with human CD19 and the indicated plasmids. Transfectants were isolated using antihuman CD19 magnetic beads (Dynabeads, Dynal Biotech, Lake Success, NY) and RNA was extracted using the RNeasy Mini Kit

(Qiagen). cDNA was synthesized from 5 ng of total RNA by using oligo-dT_{16} (Applied Biosystems, Foster City, CA) and Sensiscript Reverse Transcriptase (Qiagen). PCR was performed for 27–30 cycles by using Taq PCR Master Mix (Qiagen). The followed primers were employed: GAPDH, positions 221–240 and 459–480 (GenBank accession number gi: 182976); c-*fos*, positions 100–120 and 444–467 (GenBank accession number gi: 182734); TK, positions 216–237 and 396–417 (GenBank accession number gi: 339708); DHFR, positions 640–659 and 820–839 (GenBank accession number gi: 7262376); E2F-1, positions 414–435 and 686–706 (GenBank accession number gi: 181917); and cyclin E, positions 341–360 and 642–660 (GenBank accession number gi: 181248).

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