Specific Enzymatic Dephosphorylation of the Retinoblastoma Protein

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The retinoblastoma gene product (RB) undergoes cell cycle-dependent phosphorylation and dephosphorylation. Pulse-chase experiments revealed that the change in RB gel electrophoretic migration which occurs near mitosis is due to enzymatic dephosphorylation (J. W. Ludlow, J. Shon, J. M. Pipas, D. M. Livingston, and J. A. DeCaprio, Cell 60:387-396, 1990). To determine the precise timing of RB dephosphorylation and whether a specific phosphatase is active in this process, we have utilized a nocodazole block and release protocol which allows a large population of cells to progress synchronously through mitosis. In such experiments, RB dephosphorylation began during anaphase and continued until complete dephosphorylation was apparent in the ensuing G₁ period. In addition, late mitotic cell extracts were capable of dephosphorylating RB in vitro. This RB-specific mitotic phosphatase activity was more active in anaphase extracts than in pro- or metaphase extracts, which is consistent with the results obtained in vivo. Okadaic acid and protein phosphatase inhibitors 1 and 2 inhibited this specific RB phosphatase activity. These results suggest a role for serine and threonine phosphoprotein phosphatase type 1 in the late mitotic dephosphorylation of RB.

The retinoblastoma susceptibility gene (*RB1*) is among the best-characterized tumor suppressor genes. The functional inactivation of both *RB1* alleles is linked to the development of retinoblastoma, indicating that it normally operates to control cell growth (7, 8, 13, 20, 26). *RB1* may control cell growth in a broad spectrum of tissues, since it is often mutated in a wide variety of human cancers, and the *RB1* product, RB, is expressed in most normal mammalian cell types tested.

RB undergoes cell cycle-dependent phosphorylation. During G₀/G₁ (6, 15, 17), terminal differentiation (21, 36), and senescence (41), RB is unphosphorylated or under phosphorylated. Before the onset of DNA synthesis, RB becomes phosphorylated on serine and threonine residues (6, 15, 17, 30, 34, 40). RB undergoes additional changes in its phosphorylation pattern as cells progress into S and again during G₂/M (15). RB becomes dephosphorylated near mitosis, but whether this process is truly intramitotic and how it is carried out are unclear (34).

At least one RB function, its ability to bind to simian virus 40 large T antigen (T), is controlled by its phosphorylation state (16). T binds only to underphosphorylated RB (33). T-RB complexes, formed in G₁, dissociate during S and G₂, when RB becomes phosphorylated, and reform when RB is dephosphorylated after the onset of mitosis (34). The phosphorylation of RB during the G₁/S transition and the observation that T-RB binding occurs only in G₁ suggest that underphosphorylated RB suppresses growth by contributing to a block to exit from G₁. This model further suggests that RB-mediated growth suppression can be overcome either by phosphorylation of RB or by T-RB complex formation (17, 33, 34). In keeping with this hypothesis, microinjection of purified, unphosphorylated RB prevented G₁ cells of a

continuous line from entering S (23). In this regard, dephosphorylation of RB, after the onset of mitosis, could lead to the recovery of its ability to interact with certain cellular proteins, which are, normally, targets of underphosphorylated RB binding (9, 24). This, in turn, might lead to entry into G₀ and G₁ and, later on, the decision to enter the next S phase.

In this report, we demonstrate that RB is dephosphorylated late in mitosis and the process begins during anaphase. Furthermore, in vitro analysis of RB dephosphorylation led to the detection of a mitosis-specific phosphatase activity. A large part of this activity is provided by a member of a known protein phosphatase family, PP1, with an established role in the normal regulation of eukaryotic cell division (19, 25).

MATERIALS AND METHODS

Nocodazole block and release. CV-1P cells were grown in Dulbecco modified Eagle medium (DMEM) (GIBCO) containing 10% newborn calf serum (NCS; GIBCO) in a 10% CO₂-containing atmosphere at 37°C. Thirty 90-mm tissue culture dishes were seeded with approximately 10⁶ cells each. Sixteen to 40 h later, hydroxyurea (Sigma) was added to each dish to a final concentration of 0.5 mM. Sixteen hours after hydroxyurea addition, the cells were trypsinized and replated at approximately 10⁶ cells per 90-mm plate in fresh, drug-free, medium. Eight hours later, a mitotic shake was performed by gentle pipeting to remove mitotic and other loosely adherent cells. The remaining adherent cells were refed with fresh medium containing nocodazole (Janssen) at 100 ng/ml for 2 h. Then, in a 37°C environment room, by using prewarmed equipment, plasticware, and media, a second mitotic shake was performed. The reproducibility of synchrony of exit from mitosis required that all manipulations be performed in the warm room. The cells were collected and centrifuged at 1,000 × g for 4 min at 37°C. The cells were washed in media B (DMEM, 10% NCS, 10

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mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES] [pH 7.4]) and recentrifuged at $1,000 \times g$ for 2 min. The cells were refed with media B and incubated in the 37°C environment room. At timed intervals, beginning at the start of the first wash with media B, the cells were collected, chilled on ice, and divided for various analyses. Chromatin morphology was analyzed in cells fixed in 3.7% formaldehyde in phosphate-buffered saline. The fixed cells were centrifuged onto glass slides, stained with 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes D-1306; 0.5 µg/ml) for 2 min at 4°C, and then observed by UV microscopy. At least 200 cells were counted at each time point in all assays.

Western blotting (immunoblotting). Cells were briefly centrifuged at $16,000 \times g$, washed once in ice-cold Tris-buffered saline (TBS; 20 mM Tris-HCl [pH 8.0], 120 mM NaCl) and lysed with ice-cold EBC buffer (20 mM Tris-HCl [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40) containing 4 mM NaF, 0.1 mM Na orthovanadate, and 10 µg (each) of aprotinin, leupeptin, β-glycerolphosphate, and phenylmethylsulfonyl fluoride per ml. Protein concentrations were determined with the Bio-Rad Protein Assay reagent. Approximately 140 µg of protein was separated in each lane of a sodium dodecyl sulfate (SDS)-polyacrylamide gel. After electrophoresis, the proteins were transferred to Immobilon-P (Millipore) according to the manufacturer's directions. The blot was preincubated in TBS containing 5% bovine serum albumin (BSA) and then incubated overnight in an anti-RB monoclonal antibody (RB-PMG3-245; PharMingen) diluted to 5 µg/ml in TBS-5% BSA. Where indicated, the blots were incubated for 2 h in the monoclonal antibody MPM-2, at a dilution of 1:1,000 in TBS-5% BSA. Similarly, where noted, blots were exposed to anti-human cyclin B rabbit serum for 2 h at a dilution of 1:4,000 in TBS-5% BSA. Blots were washed six times for 10 min each with TBS-0.5% BSA. They were then incubated for an additional 30 min in a second antibody, goat anti-mouse immunoglobulin G-alkaline phosphatase conjugate (Bethesda Research Laboratories) or goat anti-rabbit IgG-alkaline phosphatase conjugate (Bethesda Research Laboratories), at a dilution of 1:7,500 in TBS-5% BSA, washed six more times for 10 min each with TBS-0.5% BSA, and then developed with 5-bromo-4-chloro-3-indolyl-phosphate toluidinium-nitroblue tetrazolium (Promega).

In vitro phosphatase assay. Synchronization of CV-1P cultures for preparation of extracts for the in vitro phosphatase activity experiments was performed as described previously (17, 34). Enrichment of CV-1P cells for G₁ was achieved by incubating a culture in methionine-free DMEM containing 2% dialyzed calf serum (GIBCO) for 48 h. S- and G₂-phase cells were obtained after methionine starvation for 24 h followed by incubation for 16 h in complete DMEM containing 10% NCS and 0.5 mM hydroxyurea. S-phase cells were harvested 3 h after the removal of hydroxyurea. At that time, maximal DNA synthesis, assayed by [³H]thymidine incorporation, was noted (17, 34). Cells harvested 11 h after removal of hydroxyurea were enriched for G₂. Mitotic CV-1P cells were collected by mitotic shake following a 16-h incubation in DMEM-10% NCS supplemented with 0.4 µg of nocodazole per ml. Nocodazole-treated cells were washed three times for 8 min with warmed (37°C) DMEM with 10% NCS to advance them into late mitosis.

Synchronized populations of CV-1P cells were washed three times with ice-cold extraction buffer (40 mM HEPES (pH 7.4), 100 mM NaCl) (32). Approximately 2×10^6 cells were then lysed by 10 passages through a 28-gauge needle in 150 µl of extraction buffer containing 14 mM β-mercaptoethanol. The lysate was then incubated for 15 min at 37°C.

Following brief centrifugation at $14,000 \times g$, the supernate was removed and the protein concentration of the extract was measured (2). Aprotinin, phenylmethylsulfonyl fluoride, and leupeptin were then added to each supernatant to a final concentration of 10 µg/ml each. Aliquots (10 µg) of protein from each of the cell extracts were then each diluted to a final volume of 50 µl with extraction buffer containing protease inhibitors. These diluted extracts were then added to 15 µl of protein A-Sepharose beads (1:1 [vol/vol] in TBS) containing immunocomplexed ³²P-labeled RB. Following incubation at 30°C for 30 min, 30 µl of 3× SDS gel sample buffer was added, and this mixture was boiled for 5 min. The entire sample, consisting of the cell extract; immunocomplexed, labeled RB; protein A-Sepharose; and sample buffer was then loaded onto an 7.5% SDS-polyacrylamide gel (28) for separation of the proteins.

Where indicated, a mixture of the phosphatase inhibitors (sodium fluoride, sodium PP_i, sodium β-glycerolphosphate [each at 10 mM final concentration] and sodium orthovanadate [final concentration, 200 µM]) was added to the relevant M-phase extracts.

Radioisotopic labeling and immunoprecipitation. To standardize the quantity of ³²P-labeled RB immunocomplexed to protein A-Sepharose within each experiment, actively growing CV-1P cells at ~90% confluency in a 90-mm plastic dish were passaged 1:3 approximately 18 h before labeling. For ³²P-labeling, cells were maintained in serum-free, phosphate-free DMEM supplemented with ³²P_i (carrier free; NEN), as previously described (29, 33). Following radiolabeling, the culture was lysed and the clarified supernatant was immunoprecipitated with monoclonal antibody, RB-PMG3-245, rabbit anti-mouse immunoglobulin G (Cappel), and protein A-Sepharose. The final, washed immunoprecipitate was divided into eight aliquots. Each aliquot from such a harvest served as the standard quantity of substrate to be used in reaction mixtures within a given experiment, in which RB phosphatase activity was to be measured.

Preparation of serine ³²P-labeled phosphorylase a. Serine ³²P-labeled phosphorylase a was prepared by incubating phosphorylase a (Sigma) with phosphorylase kinase (Sigma) at 30°C for 15 min in buffer containing 10 mM Tris-HCl (pH 8.2), 10 mM sodium glycerolphosphate, 0.1 mM CaCl₂, 10 mM Mg acetate, and 0.2 mM [³²P]ATP (10).

RESULTS

RB dephosphorylation follows nocodazole block and release.

To study the precise timing of RB dephosphorylation and how it might be regulated, we adapted a protocol that allows cells to pass synchronously through the various phases of mitosis (27). Cultures of adherent CV-1P (monkey) cells were exposed to hydroxyurea. A mitotic shake was performed 8 h after initiating drug treatment to remove mitotic and loosely adherent cells. The remaining adherent cells were refed with medium containing nocodazole for 2 h, and the resulting cells, blocked in prometaphase, were collected by a second shake. They were washed free of nocodazole and refed with fresh medium to allow progression through mitosis and into G₁. Progression through mitosis was monitored by examining the chromosomal pattern of DAPI-stained cells. Remarkable synchrony in progression through mitosis occurred. Specifically, 94% of the cells in nocodazole contained condensed chromosomes, and the majority of them reached metaphase 30 min after release from the nocodazole block (Fig. 1 and 2). Forty minutes after release, most cells were in anaphase and telophase. Sixty minutes

FIG. 1. Western blot for RB from cells treated by nocodazole block and release. Lysates (140 μ g) prepared from CV-1P cells obtained from the first mitotic shake without added nocodazole (lane C), blocked by nocodazole from the second mitotic shake (lane 0'), or released from nocodazole for 20 (lane 20'), 40 (lane 40'), 90 (lane 90'), or 180 min (lane 180') were separated in a SDS-6% polyacrylamide gel, transferred to Immobilon-P (Millipore), and then blotted with the RB monoclonal antibody RB-PMG3-245 (PharMingen). The percentage of cells in a given phase of mitosis, determined by analyzing the chromosome morphology of cells stained with DAPI, is listed in the column below each lane. ND, not determined.

after nocodazole release, nearly all had reached G₁, as determined by the presence of decondensed chromatin and the completion of cytokinesis.

A Western blot for RB, performed on a lysate of CV-1P cells collected from the first mitotic shake, revealed several bands (Fig. 1, lane C). We have previously demonstrated that the fastest of them (pRB) contains little, if any, phosphate (33). By contrast, the slower migrating bands (pRB^{phos}) are multiply phosphorylated. The nocodazole-blocked cells from the second mitotic shake contained predominantly pRB^{phos} (Fig. 1 and 2, lane 0'), and for the first 30 min after nocodazole release, the RB gel migration pattern did not change (Fig. 1, lane 20', and Fig. 2, lanes 20' and 30'). However, 40 min after nocodazole release, when anaphase and telophase cells predominated, at least four forms of RB, including pRB, were apparent (Fig. 1 and 2, lane 40'). When the cells reached G₁, 90 min after release, only the fastest migrating RB doublet was apparent. A similar schedule for RB dephosphorylation was observed in the primary human fetal lung fibroblast strain WI38; a mink lung fibroblast line, Mv1Lu; as well as in an RB +/+ osteosarcoma cell line, U2OS (data not shown).

RB dephosphorylation follows cyclin B disappearance. We analyzed lysates of synchronized CV-1P cells for RB, MPM-2, and cyclin B to correlate RB dephosphorylation with other well-characterized late mitotic events. The monoclonal antibody MPM-2 recognizes several phosphoproteins ranging in size from 47 to 180 kDa, apparent only during mitosis (14). Vandr and Borisy (43) have shown that the immunofluorescent signal from cells stained for MPM-2 is negligible during G₂, peaks during metaphase, and rapidly decreases as cells progress through anaphase. When RB dephosphorylation and MPM-2 band intensity were compared in Western blots, the first evidence of RB dephosphorylation coincided with the first clear signs of diminishing MPM-2 reactivity (Fig. 2, lane 40', compare top and middle blots). The disappearance of MPM-2 reactivity during an-

FIG. 2. Western blot for RB, MPM-2, and cyclin B from cells treated by nocodazole block and release. Lysates (140 μ g) were prepared from cells blocked by nocodazole (lane 0'), and released from nocodazole for 20 (lane 20'), 30 (lane 30'), 40 (lane 40'), or 50 min (lane 50'). Proteins were separated in 6% (top panel) or 10% (middle and bottom panel) polyacrylamide-SDS gels before being transferred to Immobilon-P. The top panel was blotted with RB monoclonal antibody, the middle panel was blotted with the MPM-2 monoclonal antibody (14), and the bottom panel was blotted with a polyclonal rabbit antisera to human cyclin B (39).

aphase, as noted by Western blot analyses, coincides with the immunofluorescence results reported earlier (43).

It has been proposed that RB is a potential substrate for the MPF- p34^{cdc2} kinase (30, 31, 42). Cyclin B, in association with p34^{cdc2}, is required for the entry of cells into mitosis, and its destruction is required for exit from mitosis (37, 38). Immunofluorescence data suggest that cyclin B is rapidly degraded at the metaphase to anaphase transition (39). Cyclin B destruction is mediated by ubiquitination (22, 44). To compare the timing of cyclin B destruction with the dephosphorylation of RB, we utilized a polyclonal antiserum raised against human cyclin B (39). In a Western blot, this serum detected a 60-kDa band from CV-1P cells which comigrated with human cyclin B (data not shown). Cyclin B was present in the nocodazole-blocked CV-1P cells (Fig. 2, bottom blot, lane 0'). However, 20 min after release, there was a clear decrease in the intensity of the signal, followed by complete disappearance at 50 min (Fig. 2, bottom blot, lanes 20', 30', 40', and 50'). A comparison of the three blots in Fig. 1B suggests that cyclin B disappeared just prior to the onset of RB dephosphorylation and the beginning of the loss of MPM-2 reactivity.

In vitro assay for RB phosphatase activity. Given the finding of specific RB dephosphorylation intermediates, as well as the abrupt onset of dephosphorylation during anaphase, it seems likely that the process is catalyzed by a specific phosphatase(s). To address this question, an in vitro

FIG. 3. Late-mitotic-phase specificity of RB phosphatase activity. (A) Aliquots of ^{32}P -labeled RB immunocomplexed to protein A-Sepharose were incubated at 30°C for 30 min with 10 μg (50 μl) of extract prepared from cells enriched for G_1 (lane 1), S (lane 2), G_2 (lane 3), or late mitotic phase (lane 4) and then separated in a SDS-7.5% polyacrylamide gel. A reaction mixture in which OA (20 nM final concentration) was added to an aliquot of the late-mitotic-phase extract prior to incubation with the immunoprecipitated phosphorylated RB is shown in lane 5. (B) ^{32}P -labeled phosphorylase a (0.3 nM) was mixed with 10 μg each of extract from CV-1P cells enriched for G_1 (lane 1), S (lane 2), G_2 (lane 3), and late mitotic phase (lane 4) with (+OA) or without (-OA) 20 nM OA. A reaction mixture containing labeled phosphorylase a without added cell extract was analyzed in lane 5.

assay to characterize RB phosphatase activity was developed. Extracts of unlabeled G_1 , S, G_2 , or late-mitotic-phase CV-1P cells were mixed with ^{32}P -labeled RB immunoprecipitated from unsynchronized CV-1P cells. Only the late-mitotic-phase extract demonstrated specific RB dephosphorylation activity under the conditions employed (Fig. 3A, lane 4). In contrast, all extracts displayed phosphorylase a dephosphorylation activity (Fig. 3B). A similar experiment, performed with [^{35}S]methionine labeled RB as substrate, demonstrated a shift from the slower migrating, pRB^{phos} , to the fastest migrating, underphosphorylated, pRB species, suggesting that the disappearance of phosphorylated RB is the result of specific dephosphorylation and not degradation of the protein (data not shown). Furthermore, incubation of in vivo-synthesized ^{32}P -RB, isolated by immunoprecipitation, in buffer without added cell extract did not result in RB dephosphorylation, indicating that a cellular phosphatase was not coprecipitated with the labeled substrate (data not shown). Finally, as demonstrated in Fig. 3A, ^{32}P -labeled RB disappeared when incubated with the late-mitotic-phase extract, except when the reaction was carried out in the presence of a known specific phosphatase inhibitor, okadaic acid (OA) (compare lanes 4 and 5).

Sensitivity of late-mitotic-phase phosphatase to inhibitors.

Multiple serine and threonine protein phosphatases have been identified (11). Each can be distinguished by its sensitivity to certain inhibitors (4, 11, 12). OA inhibits protein phosphatase 1 (PP1) at nanomolar concentrations (e.g., 10 nM) and phosphoprotein phosphatase type 2A (PP2A) at subnanomolar concentrations (e.g., 0.5 nM). PP1 can be specifically inhibited by thermostable protein phosphatase inhibitors type 1 (I1) and 2 (I2), while members of the type 2 group (PP2A, PP2B, and PP2C) cannot. PP2A does not require divalent cations for activity, while PP2B and PP2C show an absolute requirement for Ca^{2+} and Mg^{2+} , respectively. As shown in Fig. 4, the in vitro RB phosphatase activity in late-mitotic-phase extracts was completely inhibited by ≥ 10 nM OA (Fig. 4A), but not by added EDTA or

FIG. 4. Sensitivity of late-mitotic-phase extracts to phosphatase inhibitors. (A) Aliquots of ^{32}P -labeled RB immunocomplexed to protein A-Sepharose were mixed with extraction buffer alone (lane 1) or with 10 μg of late-mitotic-phase extract containing no (lane 2), 2 nM (lane 3), 10 nM (lane 4), 20 nM (lane 5), 50 nM (lane 6), or 100 nM (lane 7) OA before incubation at 30°C for 30 min. (B) Aliquots of ^{32}P -labeled RB immunocomplexed to protein A-Sepharose were mixed with 10 μg of late-mitotic-phase extract (lane 1), extraction buffer alone (lane 2), or 10 μg of late-mitotic-phase extract containing 1 mM EDTA (lane 3) or 1 mM EGTA (lane 4) before incubation.

EGTA (Fig. 4B). These results suggest that the RB phosphatase activity in late mitotic extracts is, at least in part, the product of one or more PP1-like species.

The specificity of the in vitro phosphatase assay was further studied by comparing the abilities of purified PP1 and PP2A to dephosphorylate RB. Both enzymes were able to dephosphorylate the phosphorylase a substrate and, as predicted, purified PP1, but not PP2A, was inhibited by I1 (data not shown). In contrast, only purified PP1 dephosphorylated immunoprecipitated pRB^{phos} (Fig. 5).

The results from experiments shown in Fig. 4 and 5 suggest that PP1 is the major component of the RB phosphatase activity. Furthermore, the results of in vivo experiments shown in Fig. 1 and 2, demonstrate that RB is dephosphorylated in late mitosis. This could result from an increase in PP1 phosphatase activity during late mitosis. To determine whether the RB phosphatase activity increases as cells progress from early to late mitosis, we compared the inhibition profile of extracts prepared from pro- and metaphase cells (20 min after nocodazole release), to those from ana- and telophase extracts (45 min) with ^{32}P -labeled RB as substrate. OA at 10 nM inhibited nearly all phosphatase activity at both intervals (Table 1). However, OA at 0.5 nM completely inhibited all activity in the pro- and metaphase extract but only partially inhibited the ana- and telophase extract. Similarly, I1 (100 nM) and I2 (100 μM) inhibited most of the activity in both extracts. However, neither I1 nor I2, when studied at a 10-fold-reduced concentration (10 nM and 10 μM , respectively), inhibited the ana- and telophase extracts, but both could partially inhibit the phosphatase activity in the early extracts. These results are consistent with the interpretation that the abundance of PP1 activity

FIG. 5. Sensitivity of RB to purified PP1 and PP2A. A total of 0.3 U of purified PP1 (lane 1) or PP2A (lane 2) was added to aliquots of immunoprecipitated ^{32}P -labeled RB. Neither enzyme was added to the reaction mixture analyzed in lane 3. All reaction mixtures were incubated at 30°C for 30 min in buffers identical to those used in Fig. 3.

TABLE 1. Inhibitor profile of in vitro phosphatase activity in early- and late-mitotic-phase extracts

Mitotic extract	% Inhibition of phosphatase activity ^a					
	OA		I1		I2	
	10 nM	0.5 nM	100 nM	10 nM	100 μM	10 μM
Pro- and metaphase	83	100	60	26	74	32
Ana- and telophase	100	44	52	0	68	0

^a The in vitro phosphatase assay was performed with extracts prepared from cells treated with the nocodazole block and release protocol. The pro- and metaphase extracts were prepared from cells obtained 20 min after nocodazole release, and the ana- and telophase extracts were from cells obtained 45 min after nocodazole release. The assay was performed with the indicated final concentrations of OA, I1, and I2 present in the reaction mixture. ³²P-labeled RB was localized by autoradiography of the SDS-polyacrylamide gel with preflashed film, and densitometry was performed to quantitate changes in RB-labeled phosphate content. Percent inhibition of phosphatase activity was determined by measuring the percentage of phosphorylated RB remaining after incubation compared with that in the control reactions carried out in the absence of extract or inhibitor.

increased from early to late mitosis. This increase in PP1 phosphatase activity correlates with the RB dephosphorylation observed in vivo during anaphase (Fig. 1 and 2).

DISCUSSION

Using the nocodazole block and release protocol, we have found that RB dephosphorylation begins during anaphase. The nocodazole block and release protocol yielded a reproducibly synchronous passage of cells through mitosis. As shown in two different experiments (Fig. 1 and 2), approximately 65% of all cells were in anaphase or telophase 40 min after nocodazole release. A similar delay in initiating RB dephosphorylation was noted in the detached cells from the first mitotic shake that had never been exposed to nocodazole (data not shown). However, because the population of cells from the first mitotic shake was not as tightly synchronized as the drug-exposed population from the second mitotic shake, it was difficult to determine the precise point in mitosis when dephosphorylation began without the use of nocodazole. The nocodazole block and release protocol is useful for studying events in mid to late mitosis, such as cyclin B destruction (metaphase to anaphase) and loss of MPM-2 reactivity (anaphase). The loss of MPM-2 reactivity coincides with the dephosphorylation of RB and may also be due to the loss of phosphorylated epitopes by a phosphatase activated late in mitosis. These two events clearly follow the loss of cyclin B and places these events in late mitosis.

Using in vivo-synthesized and phosphorylated RB as substrate, we determined that extracts prepared from late-mitotic-phase cells contain a specific phosphatase activity. The results of inhibitor studies suggest that the major phosphatase activity was PP1 and that there was an apparent increase in PP1 activity as cells progressed from early to late mitosis. These results and the observation that purified PP1, and not PP2A, is able to dephosphorylate RB in vitro suggest that PP1 activity participates in the specific, orderly dephosphorylation of RB late in mitosis. It does not rule out the possibility that other phosphatases also participate in the RB dephosphorylation process.

These results do not address the question of how the relevant mitotic RB phosphatase(s) is specifically activated. It is possible that cyclical expression or activation of inhibitor 2 (5) or a change in the subcellular localization of PP1

(19) could account for the apparent activation of PP1 activity that results in the dephosphorylation of RB in anaphase.

Recent observations have led to some insight into how exit from mitosis is controlled (35). In particular, work from several laboratories suggests that the separation of sister chromatids is dependent on the activity of a PP1 species (1, 18, 25). In *Schizosaccharomyces pombe*, *Aspergillus nidulans*, and *Drosophila melanogaster*, the genetics imply that a PP1 species must become active to complete mitosis. What is not known is the nature of the relevant mitotic substrates for the PP1 activity. Their identity would, presumably, shed light on the mechanisms controlling certain late mitotic events. In this regard, RB may be among the first PP1 mitotic substrates identified, although it has also been suggested that lamins and vimentin are also specifically dephosphorylated by PP1 active during anaphase (3). Given this, it will be important to determine whether the dephosphorylation of RB actually contributes to one or more mitotic events.

The multimodal aspect of RB dephosphorylation (the initiation of RB dephosphorylation beginning in late mitosis and the last part of which likely occurs in G₁ [Fig. 1]) suggests that there may be several functional consequences of this process. If the phosphorylated intermediates in the dephosphorylation process, detected primarily during late mitosis, are also active, then one wonders whether these two RB species, partially dephosphorylated and unphosphorylated, have different functions. If so, the proper timing of dephosphorylation might contribute to orderly progression out of mitosis and into a normally controlled G₀ or G₁ period. Experiments aimed at determining whether there are significant differences in function among these species are in progress.

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