Role for cyclin-dependent kinase 2 in mitosis exit

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Mitosis requires cyclin-dependent kinase (cdk) 1-cyclin B activity [1]. Exit from mitosis depends on the inactivation of the complex by the degradation of cyclin B [2]. Cdk2 is also active during mitosis [3, 4]. In *Xenopus* egg extracts, cdk2 is primarily in complex with cyclin E, which is stable [5]. At the end of mitosis, downregulation of cdk2-cyclin E activity is accompanied by inhibitory phosphorylation of cdk2 [6]. Here, we show that cdk2-cyclin E activity maintains cdk1-cyclin B during mitosis. At mitosis exit, cdk2 is inactivated prior to cdk1. The loss of cdk2 activity follows and depends upon an increase in protein kinase A (PKA) activity. Prematurely inactivating cdk2 advances the time of cyclin B degradation and cdk1 inactivation. Blocking PKA, instead, stabilizes cdk2 activity and inhibits cyclin B degradation and cdk1 inactivation. The stabilization of cdk1-cyclin B is also induced by a mutant cdk2-cyclin E complex that is resistant to inhibitory phosphorylation. P21-Cip1, which inhibits both wild-type and mutant cdk2-cyclin E, reverses mitotic arrest under either condition. Our findings indicate that the proteolysis-independent downregulation of cdk2 activity at the end of mitosis depends on PKA and is required to activate the proteolysis cascade that leads to mitosis exit.

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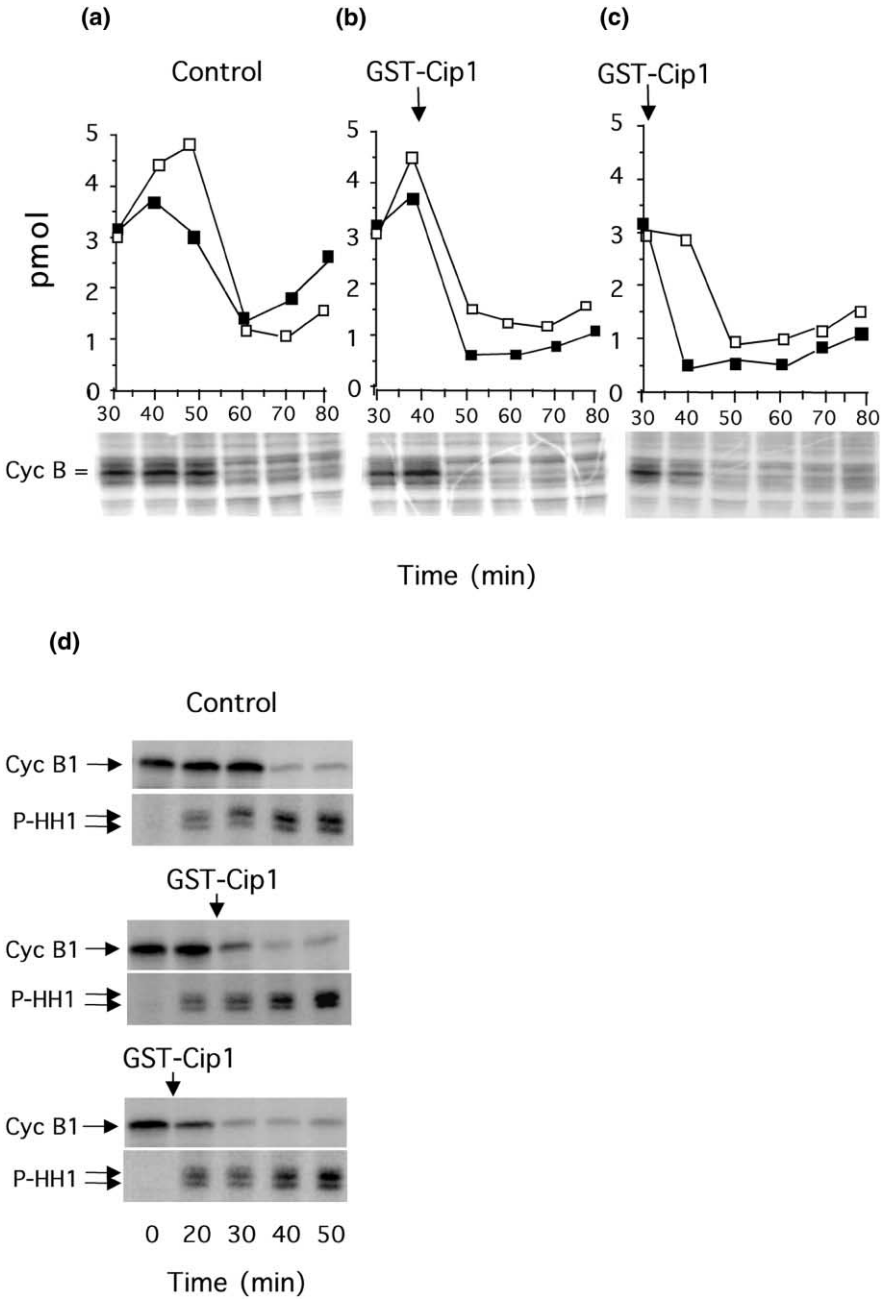
Results and discussion

Activity of the cyclin-dependent kinase (cdk) 1-cyclin B complex, also called M phase-promoting factor (MPF), is crucial for mitosis. MPF is required for spindle assembly and chromosome condensation, whereas MPF downregulation by ubiquitin-mediated proteolysis of cyclin B is needed to complete mitosis [1, 2]. Sister chromatid separation also requires ubiquitin-mediated proteolysis of securins, which inhibit anaphase. The degradation of securins and mitotic cyclins relies upon the cell cycle-regulated activity of the ubiquitin ligase anaphase-promoting complex/cyclosome (APC/C). This appears to be stimulated by cdk1-dependent phosphorylation [2, 7–9]. In addition, at least two WD-40 repeat-containing proteins bind APC/C and are required for its action [2]. In *Xenopus* egg extracts, the Cdc20/Fizzy homolog is required for APC/C-mediated ubiquitination of both anaphase inhibitors and mitotic cyclins [10]. In yeast and somatic vertebrate cells, APC/C-mediated ubiquitination of mitotic cyclins also requires Hct1/Cdh1 [2]. How the cell cycle-dependent timing of APC/C activation is determined, however, is still not completely elucidated. The activity of cdk2 also increases in mitosis [3, 4]. In cycling *Xenopus* egg extracts, cdk2 is primarily found in a complex with cyclin E, and its activity is required for cdk1 activation at the onset of mitosis [5, 11]. At the end of mitosis, cdk2 activity is downregulated by inhibitory phosphorylation of cdk2 rather than degradation of cyclin E, which is stable [5, 6, 11, 12]. We used this system to ask whether cdk2 had a role in regulating mitosis exit. Oscillations between mitosis and interphase during the extracts' cell cycle are indicated by fluctuations in MPF activity and cyclin B concentration. MPF activity increases during mitosis and falls at the end of mitosis as a result of cyclin B degradation. We added the cdk inhibitor p21/Cip1/Waf1 (in the form of a glutathione-S-transferase fusion protein; GST-Cip1) to the extracts after MPF activation. We used a concentration of GST-Cip1 (100 nM) that preferentially inhibits cdk2-cyclin E and cdk1-cyclin A rather than MPF in egg extracts as previously demonstrated [11, 13; see below]. We monitored MPF, measured as total histone H1 kinase activity in the extracts; cdk2 activity, measured as histone H1 kinase activity in anti-cdk2 immunoprecipitates; and the stability of cyclin B during incubation at 23°C. In the control extract, total histone H1 kinase activity and cyclin B abundance increased until 50 min and fell at 60 min (Figure 1a, the addition of GST at 40 or 30 min did not affect cell cycle kinetics; data not shown). Cdk2 activity was higher during mitosis (Figure 1a, 30–40 min) and declined thereafter, consistent with previous reports [4]. Interestingly, the addition of GST-Cip1 at 40 or 30 min (Figure

Figure 1

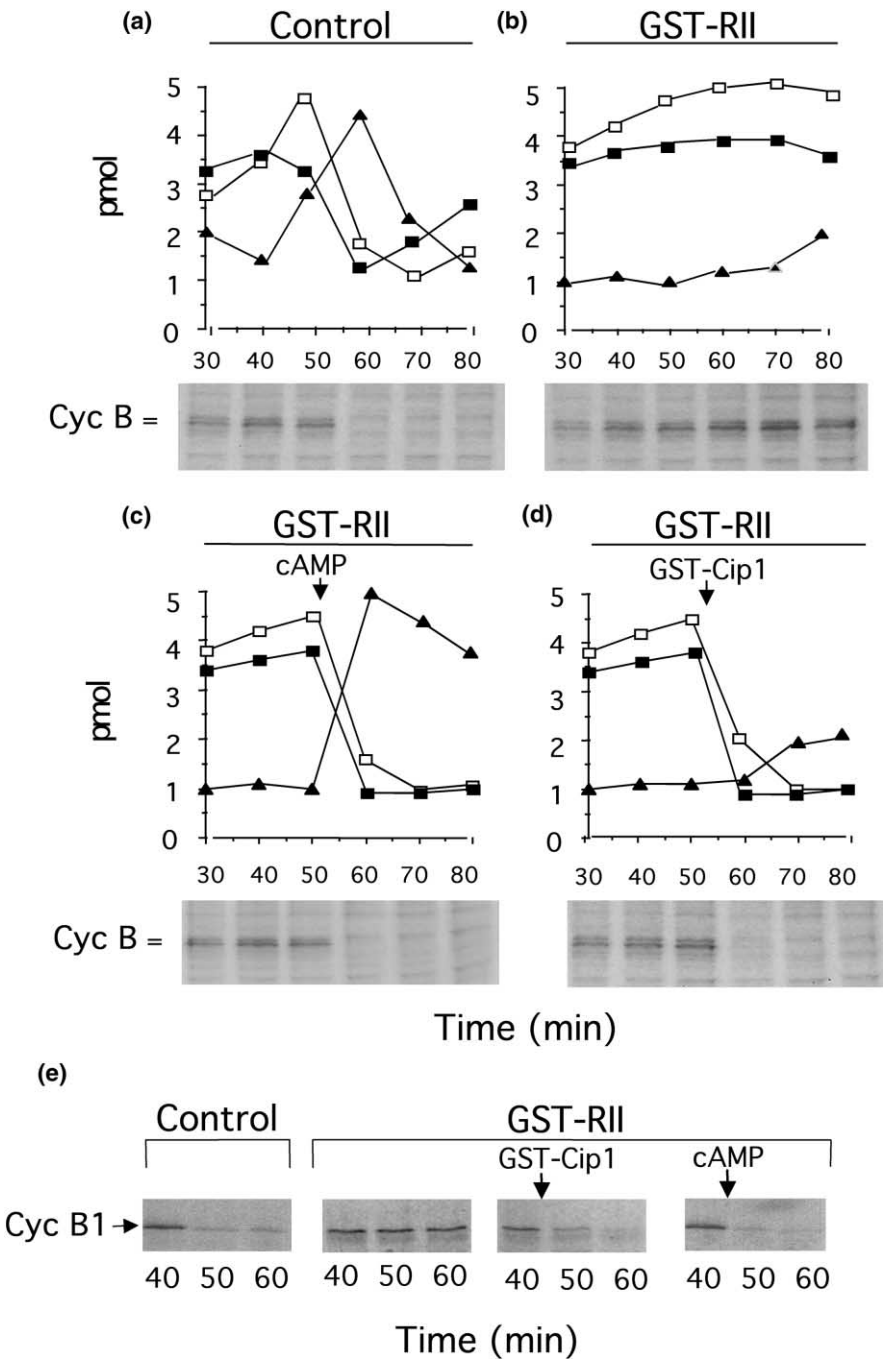


The inhibition of cdk2 by p21/Cip1/Waf1 induces premature cyclin degradation. Portions of a cycling *Xenopus* egg extract, containing [^{35}S]methionine (400 μCi/ml), were incubated at 23°C. **(a)** The control extract, GST (100 nM) was added at 30 min. **(b)** GST-Cip1 fusion protein (100 nM) was added at 40 min. **(c)** GST-Cip1 fusion protein (100 nM) was added at 30 min. Samples were withdrawn at 10 min intervals from 30 to 80 min of incubation. (Upper panels) MPF (open squares; from 1 μl extract sample) and cdk2 (filled squares; from 15 μl extract sample) activities. (Lower panels) Autoradiographs of [^{35}S]methionine extracts' proteins separated on a 12% SDS-PAGE (the positions of the cyclin Bs are indicated). The cell cycle progression was not significantly affected by the addition of GST. **(d)** An interphase extract was obtained from eggs preincubated in cycloheximide (CHX; 0.2 mg/ml) for 30 min before activation [13]. The extract was preincubated at 23°C for 40 min after activation, in the continuous presence of CHX. Pretreatment of the eggs with CHX yields interphase extracts devoid of cyclin A and B [13]. At time 0 of incubation, Δ90cyclin B1 (10 μg/ml) and reticulocyte lysate (5% of extract volume) containing [^{35}S]methionine full-length *Xenopus* cyclin B1 were added, and the extract was divided into three portions. The portions were treated with either GST (100 nM; added at 15 min; Control), or GST-Cip1 (100nM; added at 20 or 15 min). Aliquots were withdrawn at the indicated time points and either mixed with SDS sample buffer and separated by a 12% SDS-PAGE or assayed for histone H1 kinase activity of MPF. Autoradiographs of the remaining [^{35}S]methionine full-length cyclin B1 (Cyc B1) and of the phosphorylated histone H1 (P-HH1) are shown. The timing of cyclin degradation and MPF activation were not significantly affected by the addition of GST. Metaphase levels of histone H1 kinase activity were reached 20–30 min after the addition of Δ90cyclin B1. The data shown are representative of four independent experiments.

1b,c) not only inhibited cdk2 activity but also induced premature cyclin degradation and MPF inactivation.

We then asked if GST-Cip1 prematurely activated cyclin degradation by inhibiting cdk2-cyclin E rather than cdk1-cyclin A. Eggs were incubated with cycloheximide (CHX) for 30 min before activation. This treatment yields interphase extracts that are depleted of cyclin A and B [11, 13] and therefore lack cdk1 activity that was confirmed by anti-cyclin A and anti-cyclin B2 immunoprecipitation/kinase assays (data not shown). The extracts retain cyclin E, which is stable, and cdk2-cyclin E activity [11, 13]. To induce cdk1 activation, we added nondegradable Δ90cyclin B1 [11]. Subsequent activation of the cyclin degradation pathway was monitored with [^{35}S]methionine full-length cyclin B1, which was added along with Δ90cyclin B1. Figure 1d shows that full-length cyclin B1 was stable until 30 min in the control extract and was degraded thereafter (Cyc B1). The addition of GST-Cip1 after 20 or 15 min of incubation did not inhibit further cdk1 activation, which was sustained by nondegradable Δ90cyclin B1 (Figure 1d; P-HH1); instead, it induced premature degradation of full-length cyclin B1 (Figure 1d; Cyc B1). These data indicate that cdk2 helps maintain cdk1-cyclin B dur-

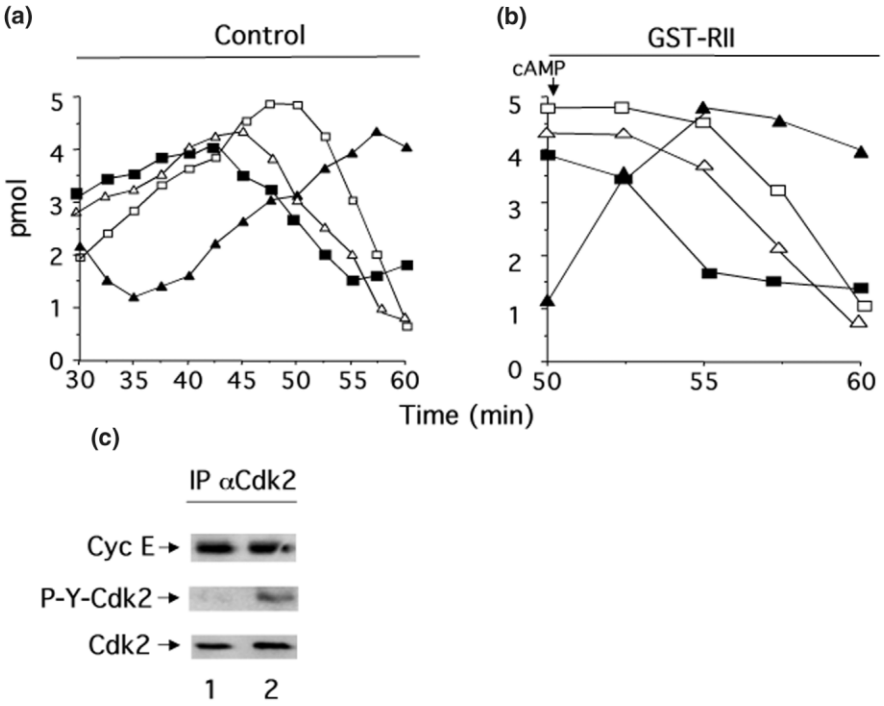
Figure 2



Cdk2 blocks cell cycle progression in PKA-inhibited mitotic-arrested extracts. Portions of a cycling *Xenopus* egg extract, containing [^{35}S]methionine (400 μCi/ml), were incubated at 23°C. **(a)** The control, GST protein (80 μg/ml) was added at time 0. **(b)** GST-RII fusion protein (80 μg/ml) was added at time 0. **(c)** GST-RII fusion protein (80 μg/ml) was added at time 0, and cAMP (600 nM) was added after 50 min of incubation. **(d)** GST-RII fusion protein (80 μg/ml) was added at time 0, and GST-Cip1 (100 nM) was added after 50 min of incubation. Samples were withdrawn at 10 min intervals from 30 to 80 min of incubation. (Upper panels) MPF (open squares; from 1 μl extract sample), cdk2 (filled squares; from 15 μl extract sample), and PKA (filled triangles; from 1 μl extract sample) activities. (Lower panels) An autoradiograph of [^{35}S]methionine extracts' proteins separated on a 12% SDS-PAGE (the positions of the cyclin Bs are indicated). The data shown are representative of at least six independent experiments. **(e)** Interphase extracts were obtained from CHX-treated eggs as described in Figure 1d. At time 0 of incubation, the following additions were made to extracts samples: (Control) Δ90cyclin B1 (10 μg/ml); (GST-RII) Δ90cyclin B1 (10 μg/ml) + GST-RII (80 μg/ml); (GST-RII + GST-Cip1) Δ90cyclin B1 + GST-RII, then GST-Cip1 (100 nM) was added after 40 min; and (GST-RII + cAMP) Δ90cyclin B1 + GST-RII, then cAMP (600 nM) was added after 40 min. Cyclin degradation was monitored by adding reticulocyte lysate (5% of extract volume), containing [^{35}S]methionine full-length *Xenopus* cyclin B1, to all samples after 40 min of incubation. Aliquots were withdrawn immediately, 10 and 20 min after the addition of the labeled cyclin. An autoradiograph of the remaining labeled full-length cyclin B1 is shown. The data shown are representative of three independent experiments.

ing mitosis, blocking the cyclin degradation pathway. We previously showed that the activity of the cAMP-PKA pathway oscillates during the extracts' cell cycle; it is at a minimum at the onset of mitosis and peaks at the transition from mitosis to interphase [14]. The inhibition of PKA arrests the cell cycle in mitosis with high MPF activity and stable cyclins A and B. The reactivation of PKA restores cyclin degradation and the transition into interphase [15]. Extending the time between activation and inactivation of MPF by blocking PKA provided us a means of studying whether cdk2 was in the pathway between PKA and mitotic cyclin degradation. We asked if PKA promoted degradation of mitotic cyclins and MPF inactivation by downregulating cdk2 activity. We inhibited PKA with excess recombinant rat PKA regulatory subunit type IIβ in the form of a glutathione-S-transferase fusion protein (GST-RII) [15]. We then measured total histone H1 kinase, cdk2, and PKA activities as a function of time of incubation of the extract at 23°C. In the control extract (containing GST), total histone H1 kinase and cdk2 activi-

Figure 3

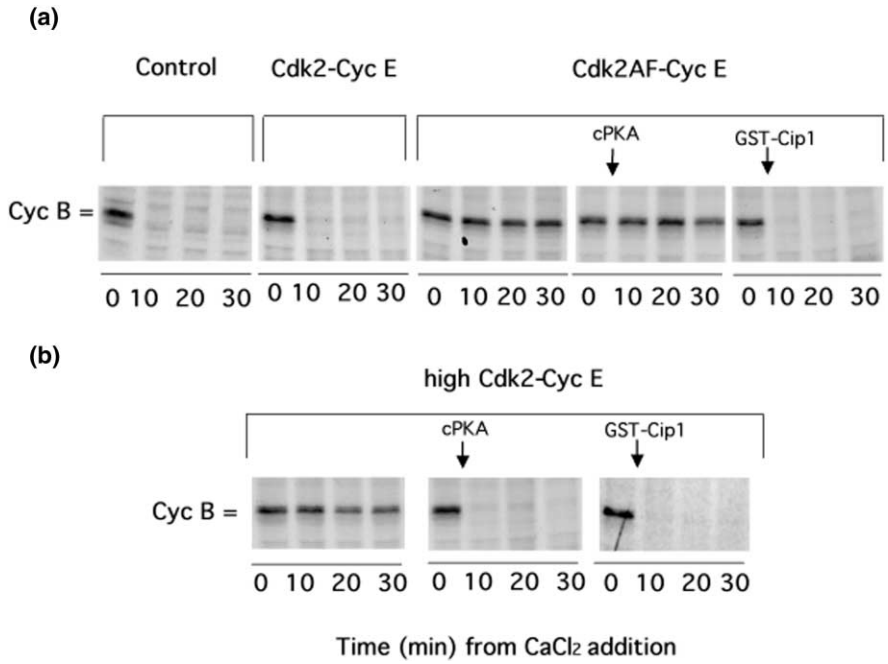


Cdk2, PKA, and cyclin B2- and cyclin A-associated kinase activities during mitosis exit. The control of cdk2 tyrosine 15 phosphorylation by PKA. **(a)** Cdk2, PKA, and cyclin B2- and cyclin A-associated kinase activities from samples of a cycling extract taken at 2.5 min intervals (from 30 to 60 min) during incubation at 23°C. **(b)** Cdk2, PKA, and cyclin B2- and cyclin A-associated kinase activities from samples of a GST-RII-treated extract taken at 2.5 min intervals after PKA reactivation (at 50 min). (Open squares) Cyclin B2-associated kinase activity (from 1.5 μl extract). (Open triangles) Cyclin A-associated kinase activity (from 3 μl extract). (Filled squares) Cdk2 activity (from 15 μl extract). (Filled triangles) PKA activity (from 1 μl extract). **(c)** Cdk2 was immunoprecipitated from triplicate samples (50 μl each) of a GST-RII- (lane 1) and a GST-RII + cAMP-treated (lane 2) extract incubated for 70 min at 23°C (GST-RII added at time 0; cAMP added at 50 min). Samples were separated on a 12% SDS-PAGE and blotted. The blots were probed with an anti-cyclin E antibody (Cyc E), an anti-cdk1/cdk2-tyrosine 15 phospho-specific antibody (P-Y-Cdk2), and an anti-cdk2 antibody (Cdk2). The data shown are representative of three independent experiments.

ties oscillated with kinetics similar to those shown in Figure 1 (Figure 2a). PKA activity rose as cdk2 and total histone H1 kinase activities declined. GST-RII blocked PKA, stabilized high cdk2 and total histone H1 kinase activities, and prevented the degradation of cyclin B (Figure 2b). The reactivation of PKA with cAMP induced a rapid decline in cdk2 and total histone H1 kinase activities and cyclin B concentration (Figure 2c) [15]. To know if PKA acted through cdk2, we asked if the inactivation of cdk2 could reverse mitotic arrest induced by GST-RII. Accordingly, GST-Cip1 (100 nM) was added at 50 min of incubation to a portion of a GST-RII-treated extract. Figure 2d shows that GST-Cip1 overcame the effects of PKA inhibition, restoring mitosis exit. These results were confirmed by the morphological examination of sperm nuclei (50/μl) added to similar extracts. PKA inhibition fixed sperm chromatin in a condensed state. GST-Cip1-induced chromatin decondensation occurred 10–20 min after the addition of GST-Cip1 (data not shown). To show that cdk2 activity, rather than cdk1-cyclin A activity, was responsible for mitotic arrest in PKA-inhibited extracts, we prepared cyclin A-depleted extracts from CHX-treated eggs (Figure 2e). Nondegradable Δ90cyclin B1 was added to activate cdk1, as described for Figure 1d. Where indicated, PKA was blocked with GST-RII. Full-length cyclin B1, added 40 min after Δ90cyclin B1, was degraded in control extracts (containing GST) but was stable in extracts treated with GST-RII (Figure 2e; Control and GST-RII). Timely degradation of full-length cyclin B was restored in GST-RII-treated extracts by GST-Cip1 or cAMP (Figure 2e; GST-RII + GST-Cip1 or cAMP). Taken together, these data indicate that PKA inactivates cdk2, allowing mitosis exit.

To dissect the pathway initiated by PKA, we compared in detail the kinetics of cdk2, PKA, and cyclin B2- and cyclin A-associated kinase activities at the end of mitosis. Cdk2 and cyclin A-associated kinase activities peaked and declined before cyclin B2-associated activity reached its maximum, consistent with previous results (Figure 3a) [4, 16]. The activity of cdk2 declined slightly prior to cyclin A-associated activity, at approximately the time as PKA activation (Figure 3a). The four protein kinases were also assayed in a GST-RII-treated extract immediately after PKA reactivation with cAMP (Figure 3b). Under these conditions, PKA activity rose more abruptly, while cdk2 activity declined significantly more rapidly than cyclin A- or B2-associated activity. The loss of cyclin A- and cyclin B2-associated activities paralleled the loss of cyclin A and B2 protein (data not shown). These kinetics show that cdk2 inactivation precedes inactivation of cdk1 following PKA activation. Whereas cyclins A and B are degraded at the end of mitosis, the abundance of cyclin E and its association with cdk2 do not vary during the cell cycle [5]. Cdk2 activity appears primarily regulated by changes in cdk2 tyrosine 15 phosphorylation [6, 12]. We immunoprecipitated cdk2 from samples of a GST-RII- and GST-RII + cAMP-treated extract prepared as in Figure 2b,c

Figure 4



Cdk2AF-cyclin E delays cyclin degradation and is resistant to PKA. Excess wild-type cdk2-cyclin E delays cyclin degradation but is sensitive to PKA. **(a)** CSF-arrested extracts were preincubated with [^{35}S]methionine (400 μCi/ml) for 20 min before the addition of CHX (0.2 mg/ml). Then, buffer (control) or wild-type cdk2-cyclin E (Cdk2-Cyc E) or mutant cdk2AF-cyclin E (Cdk2AF-Cyc E) were added at levels equal to the endogenous cdk2 activity, and preincubation was prolonged for 10 min. $CaCl_2$ (400 μM) was then added to all samples at time 0. Purified PKA catalytic subunit (cPKA, 0.08 U/μl; Sigma) or GST-Cip1 (300 nM) were immediately added to portions of the cdk2AF-cyclin E-treated samples. Aliquots were taken at the indicated time points after the addition of $CaCl_2$, separated on SDS-PAGE, and autoradiographed. The positions of cyclins B are indicated. **(b)** Extracts were incubated as in (**a**), but CSF-arrested extracts were treated with 3- to 4-fold higher concentrations of wild-type cdk2-cyclin E (high Cdk2-Cyc E). $CaCl_2$ (400 μM) was added to all samples at time 0. Where indicated, cPKA (0.08 U/μl; Sigma) or GST-Cip (300 nM) were immediately added to portions of the wild-type cdk2-cyclin E-treated extract. The data shown are representative of four independent experiments.

at 70 min incubation (Figure 3c; lanes 1 and 2, respectively; cell cycle kinetics were also performed as in Figure 2b,c). Samples were separated on SDS-PAGE and immunoblotted with an anti-cyclin E antibody, an anti-cdk2 antibody, and with an antibody that specifically recognizes the cdk2 phosphotyrosine 15 epitope. The amounts of cyclin E and cdk2 were comparable in the immunoprecipitations from the two samples (Figure 3c; IP αCdk2), showing comparable binding of cdk2 to cyclin E (Figure 3c; Cyc E and Cdk2; lanes 1 and 2). The two samples, however, differed significantly in their content of cdk2-phosphotyrosine 15. The GST-RII-treated extract contained little phosphorylated, inactive cdk2 (Figure 3c; P-Y-Cdk2, lane 1) compared to the same extract after PKA reactivation (Figure 3c; P-Y-Cdk2, lane 2). We conclude that the cAMP-PKA pathway downregulates cdk2 activity via cdk2 tyrosine 15 phosphorylation.

Our findings suggest that mitosis exit requires a PKA-dependent downregulation of cdk2 activity via tyrosine 15 phosphorylation. If this is indeed the case, a cdk2 variant that cannot be inhibited by phosphorylation should delay mitosis exit despite active PKA. To assay this, we used extracts derived from nonactivated mature eggs that are in a stable, cytostatic actor (CSF)-dependent, M phase arrest [17]. In these extracts, M phase arrest can be reversed by the addition of calcium, which inactivates CSF. We compared the effects of a recombinant cdk2-cyclin E complex containing such a variant cdk2 (cdk2AF; thr 14 > ala, and tyr 15 > phe) [18] with wild-type cdk2-cyclin E complex in the exit from M phase after the inactivation of CSF with $CaCl_2$. A CSF-arrested extract was preincubated for 20 min with [^{35}S]methionine. Portions were then mixed with equivalent amounts of wild-type cdk2-cyclin E or cdk2AF-cyclin E, equal to the endogenous level of cdk2 activity. After an additional 10 min of preincubation, $CaCl_2$ was added to all portions to inactivate CSF and induce cyclin degradation. The rate of cyclin B loss was equivalent in the control and in extracts treated with wild-type cdk2-cyclin E-treated (Figure 4a; Control and Cdk2-Cyc E). Consistent with our hypothesis, cdk2AF-cyclin E blocked cyclin B degradation (Figure 4a; Cdk2AF-Cyc E). The block was resistant to the addition of the purified PKA catalytic subunit (cPKA, 0.08 U/μl; Figure 4a; Cdk2AF-Cyc E + cPKA) but was completely overcome by GST-Cip1, which directly inhibits cdk2AF-cyclin E (Figure 4a; Cdk2AF-Cyc E + GST-Cip1). The addition of wild-type cdk2-cyclin E at a concentration 3- to 4-fold higher than the endogenous cdk2 activity also retarded cyclin B degradation (Figure 4b; high Cdk2-Cyc E). In this case, however, cPKA (0.08 U/μl) restored rapid degradation of cyclin B (Figure 4b; high Cdk2-Cyc E + cPKA), as did GST-Cip1 (Figure 4b; high Cdk2-Cyc E + GST-Cip1). To confirm the relevance of the control of cdk2 activity via inhibitory phosphorylation, we examined the effects of adding an excess

of the dual specificity phosphatase Cdc25A that activates cdk2 [18]. We found that this treatment also retarded cyclin B degradation. This was reversed by the addition of cPKA or GST-Cip1(see Supplementary material available with this article online). An excess of wild-type cdk2-cyclin E also significantly delayed anaphase onset, which was overcome by stimulation of the PKA pathway (see Supplementary material).

Cdk2 presumably blocks anaphase onset and cyclin degradation by inhibiting the APC/C-proteasome pathway. It has been recently proposed that, while cdk-dependent phosphorylation of APC/C is a prerequisite for APC/C activity, the phosphorylation of Cdc20/Fizzy reduces its ability to stimulate APC/C [19]. Cdk2 may contribute to phosphorylations that negatively control Cdc20/Fizzy in egg extracts. Unlike embryos or the egg extracts described above, somatic cells contain cdk2-cyclin A in addition to cdk2-cyclin E [3]. It is possible that both cdk2 complexes block the APC/C-proteasome pathway during mitosis. In *Xenopus*, little is known about the regulation of the kinase(s) and phosphatase(s) that directly control the phosphorylation of cdk2 [12]. Our data indicate that PKA antagonizes the phosphatase pathway that dephosphorylates cdk2 at inhibitory sites (see Supplementary material) [14]. We find that sustained cdk2 activity inhibits cyclin degradation in CSF extracts after the addition of calcium (Figure 4). This is in agreement with previous findings involving cdk2 in the establishment of meiotic metaphase II arrest by CSF [20]. However, we also find that, once CSF arrest is established, the sole inhibition of cdk2 cannot induce cyclin degradation in the absence of calcium (data not shown), as previously reported [11, 21]. Hence, the release from CSF arrest requires the downregulation of cdk2 activity along with other calcium-dependent effects [6, 22]. It has been reported that the in vitro phosphorylation of APC/C by PKA inhibits its ubiquitin ligase activity [23]. Our data suggest that the relevant action of PKA in egg extracts is unlikely to be mediated by direct effects of the kinase on APC/C. Our findings rather indicate that PKA is responsible for the proteolysis-independent downregulation of cdk2 activity and that this event is required at the end of mitosis to activate the proteolysis cascade that leads to mitosis exit. We propose that cdk2 delays mitosis exit after cdk1 activation and that mitosis exit requires a PKA-dependent downregulation of cdk2 activity.

Supplementary material

Supplementary material including additional methodological details and two figures showing that excess Cdc25A delays cyclin degradation and excess cdk2-cyclin E delays the onset of anaphase is available at http://images.cellpress.com/supmat/supmatin.htm.

Acknowledgements

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