Regulated activity of PP2A–B55 δ is crucial for controlling entry into and exit from mitosis in *Xenopus* egg extracts

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Entry into mitosis depends on the activity of cyclin-dependent kinases (CDKs). Conversely, exit from mitosis occurs when mitotic cyclins are degraded, thereby extinguishing CDK activity. Exit from mitosis must also require mitotic phosphoproteins to revert to their interphase hypophosphorylated forms, but there is a controversy about which phosphatase(s) is/are responsible for dephosphorylating the CDK substrates. We find that PP2A associated with a B55 δ subunit is relatively specific for a model mitotic CDK substrate in *Xenopus* egg extracts. The phosphatase activity measured by this substrate is regulated during the cell cycle—high in interphase and suppressed during mitosis. Depletion of PP2A–B55 δ (in interphase) from ‘cycling’ frog egg extracts accelerated their entry into mitosis and kept them indefinitely in mitosis. When PP2A–B55 δ was depleted from mitotic extracts, however, exit from mitosis was hardly delayed, showing that other phosphatase(s) are also required for mitotic exit. Increasing the concentration of PP2A–B55 δ in extracts by adding recombinant enzyme inhibited the entry into mitosis. This form of PP2A seems to be a key regulator of entry into and exit from mitosis.

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Introduction

Entry into mitosis is brought about by the activation of protein kinases, of which it is generally agreed that the cyclin dependent kinases (CDKs) are primary, whereas others such as Polo and Aurora are somehow dependent on CDKs (Nigg, 2001). The activation of CDK itself depends on a network of protein kinases and phosphatases; in particular Wee1, an inhibitory kinase that acts on Cdk1, must be switched off and Cdc25, the phosphatase that antagonizes Wee1, must be turned on (Morgan, 2007). The activity of Wee1 and Cdc25

is regulated by phosphorylation, and it is well known that phosphatases such as PP2A tend to keep Cdk1 inhibited, whereas phosphatase inhibitors, such as okadaic acid, tend to activate Cdk1 (Tournabize *et al*, 1997). Exactly how this system is normally regulated such that cells do not enter mitosis while DNA replication is in progress is not so clear (Kumagai and Dunphy, 1995), whereas much work has been devoted to understanding how DNA damage is sensed and transmitted to this control system (Nyberg *et al*, 2002). Phosphorylation of Cdc25 by Chk1, downstream of ATM, seems to be crucial (Karlsson-Rosenthal and Millar, 2006). However, what normally keeps Wee1 active and inhibits Cdc25 during an undamaged S-phase, which other factors stop Cdk1 from turning on prematurely, is by no means well understood. Nor is it clear what changes at the end of G2 phase, to flip the switch to allow the entry into mitosis.

Our interest in phosphatases stems from a different concern, namely, the very end of mitosis, when cyclin levels have dropped, and the cell returns to interphase. There is an asymmetry between entering and leaving mitosis: at entry, Cdk1 is associated with the mitotic cyclins A and B, but phosphorylation on thr14 and tyr15 inhibits its kinase activity. Removal of these inhibitory phosphates by Cdc25 activates Cdk1. At the metaphase-to-anaphase transition, cyclins A and B are degraded, leaving Cdk1, which has no activity without a cyclin partner. There are reports that Cdk1 is re-phosphorylated and hence turned off by Wee1 at the end of mitosis, before cyclin degradation is complete, but it is not clear how important this is under normal conditions (D’Angiolella *et al*, 2007; Potapova *et al*, 2009). In any case, if it is true that phosphorylation of a set of mitotic phosphoproteins leads to metaphase, then phosphatases must be required to reverse or revert the state of these proteins to pass from M-phase back to G1 and keep them hypophosphorylated until next mitosis in the presence of low CDK activity. There is indeed good evidence to support this view. Three kinds of approaches have been used: genetic, biochemical and reverse genetic (Chen *et al*, 2007). Using histone H1 phosphorylated by Cdk1 and other canonical CDK substrates, Ferrigno *et al* (1993) identified one isoform of type-2A phosphatase (PP2A1) as the major phosphatase that could dephosphorylate such targets. In parallel, the *Drosophila* mutant *twins* and abnormal anaphase resolution (*aar*), which show mitotic arrest or anaphase phenotypes, proved to contain mutations in the regulatory B55 subunit of PP2A (Mayer-Jaekel *et al*, 1993). Notably, PP2A from flies containing the *twins* mutation shows reduced activity against substrates, such as caldesmon and histone H1 phosphorylated by Cdk1–cyclin B *in vitro* (Mayer-Jaekel *et al*, 1994). In *Aspergillus* and yeasts, however, it is type-1 protein phosphatase (PP1) mutants that were found to show mitotic-arrest phenotypes, as expected for defective CDK-target dephosphorylation; examples include BIM-G (Doonan and Morris,

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1989), dis2 (Ohkura *et al*, 1989) and glc7 (Hisamoto *et al*, 1994), although later, Cdc14 was identified as the key phosphatase responsible for the completion of mitosis in budding yeast (Visintin *et al*, 1998). Both PP1 and PP2A belong to the PPP phosphatase family, whose members are more or less sensitive to the naturally occurring algal toxins, okadaic acid and microcystin (Virshup and Shenolikar, 2009). Cdc14, however, belongs to a phosphatase family quite distant from the PPP family and is insensitive to okadaic acid (Queralt and Uhlmann, 2008). There is little or no evidence to suggest that Cdc14 is important for the exit from mitosis in higher eukaryotes. Trinkle-Mulcahy and Lamond (2006) recently reviewed this field.

Owing to its high specificity and membrane permeability, okadaic acid has been a powerful tool to analyze the roles of PPP family phosphatases in cells and cell extracts. The IC₅₀ values of okadaic acid are an order of magnitude higher for PP1 (15–50 nM) than PP2A (0.1–0.3 nM), allowing the drug to be used to distinguish between the roles of these two phosphatases in favourable situations. However, one has to be very cautious in evaluating its results when samples of high protein concentration are analyzed; for example, *Xenopus* egg extracts contain about 20 nM PP1 and about 150 nM PP2A, much higher concentrations than the IC₅₀ value for okadaic acid. Moreover, okadaic acid targets the catalytic subunits of these phosphatases. This makes it impossible to distinguish between the different isoforms of PP2A, for example, which probably have different substrate specificities. Another complexity is that several other PPP family members with similar IC₅₀ values have been identified (Prickett and Brautigan, 2006). These considerations make detailed interpretation of the (often confusing) effects of okadaic acid in relation to cell cycle control quite difficult. They may only indicate that protein phosphorylation is involved in the control of the process under investigation.

PP2A is composed of three subunits: catalytic C, scaffolding A and variable B subunits (Janssens *et al*, 2008). The substrate specificity and/or subcellular localization are regulated by its associated B subunits. The B subunit family has several different subfamilies (B55/B, B56/B', B'', B''' and so on) and multiple isoforms within them, and unfortunately there is no simple or memorable nomenclature by which to refer to the various types of holoenzyme. More than 20 different B subunits, including splicing variants have been identified in human transcripts so far (Janssens and Goris, 2001). *Drosophila* twins and budding yeast Cdc55, identified because of their involvements in cell cycle control, belong to the B55/B subfamily (Healy *et al*, 1991). The carboxyl terminus of PP2A's catalytic subunit is also subject to modification by phosphorylation and methylation, possibly contributing to the selection of B subunits (Mumby, 2007).

As CDK activity is regulated by multiple mechanisms to promote cell cycle progression, one or more antagonizing phosphatase(s) are probably regulated (in the opposite sense) during the cell cycle. We recently reported a protein phosphatase activity that dephosphorylated peptide substrates phosphorylated by CDKs, whose activity oscillated during the cell cycle (Mochida and Hunt, 2007). In this paper, we present evidence that this phosphatase corresponds to a particular trimeric form of PP2A, associated with the B55-delta regulatory subunit, and show that PP2A-B55 δ is important for the cell-cycle control in cooperation with CDKs.

Depletion of this form of PP2A strongly advances entry into mitosis in *Xenopus* egg extracts, and the depleted extracts are unable to exit mitosis, although cyclin B is degraded and Cdk1 activity is reduced back to basal levels. However, depletion of B55 from mitotic extracts hardly slowed the exit from mitosis in response to inhibition or removal of Cdk1, which implies that other protein phosphatase holoenzymes are also important in removing mitotic phosphoepitopes.

Results

A cell-cycle-regulated protein phosphatase activity suppresses mitotic phosphorylation during interphase

We first asked how the hypophosphorylated state of mitotic phosphoproteins is maintained during interphase. We used two different assays. In one, we observed the mobility of Apc3, a subunit of the anaphase-promoting complex/cyclosome APC/C, which undergoes a mobility shift on SDS-polyacrylamide gels when the extract enters mitosis owing to phosphorylation on multiple sites ((Peters *et al*, 1996; Pesin and Orr-Weaver, 2008)—see Figure 1A lanes 16–20). The second assay monitors a wider range of mitotic protein phosphorylations using a commercial antibody that specifically recognizes CDK target sequence (K/R)S*PX(K/R), where S* denotes phosphoserine. An interphase extract of *Xenopus* eggs remains in interphase if protein synthesis is inhibited (Murray and Kirschner, 1989; Murray *et al*, 1989); see Figure 1, lanes 1–5. Addition of okadaic acid to such an extract induced a mobility shift in Apc3, detectable after 20 min and complete by about 60 min (lanes 6–10), whereas addition of a bolus of indestructible cyclin B took 40–60 min to induce entry into M-phase (lanes 16–20). The mobility shift due to okadaic acid was delayed, but only partly suppressed, by addition of p27^{Kip1}, a specific CDK inhibitor protein (lanes 11–15). These results show that the hypophosphorylated state of mitotic phosphoproteins is maintained by a combination of low protein kinase activity (including CDK) and the activity of one or more okadaic acid-sensitive phosphatases. We note that there are examples of MPM-2 mitotic phosphoepitopes being added by CK2 (casein kinase 2), and removed by PP2A (Escargueil and Larsen, 2007).

We next confirmed our previous observations that the phosphatase activity against CDK substrates is regulated during the cell cycle. To this end, phosphatase activity was measured in the concentrated egg extract using a radiolabelled peptide substrate derived from ser 50 of Fizzy/Cdc20 protein phosphorylated by Cdk2–cyclin A and γ -[³²P]₄ATP (Mochida and Hunt, 2007). There was high phosphatase activity in interphase egg extract, which decreased sharply as the extract entered mitosis (induced by the addition of non-degradable cyclin B) as we reported previously (Figure 1B). U0126, a mitogen-activated-kinase kinase (MAPKK) inhibitor, did not affect the timing or the inhibition of phosphatase activity. The phosphatase activity reappeared when CDK was inactivated after either the normal degradation of cyclin or by addition of p27^{Kip1} (Mochida and Hunt, 2007, Figure 1C). Consistent with this result, Apc3 and a number of CDK target proteins were dephosphorylated after the addition of p27^{Kip1} (Figure 1D). These results show that phosphatase activity for CDK substrates is regulated during the cell cycle in a CDK-dependent manner. Moreover, this protein phosphatase seems to be important for maintaining the hypophosphorylated state

Figure 1 CDK substrate-directed protein phosphatase is regulated during cell cycle. (A) Interphase egg extract containing 0.1 mg/ml cycloheximide was supplemented with DMSO, okadaic acid (2.5 μ M), p27^{Kip1} (2 μ M) or cyclin Δ 90 (500 nM) as indicated. The extracts were incubated at 23°C. Aliquots were taken at 20 min intervals for immunoblotting with anti-Apc3 and anti-phospho CDK target antibodies. (B) Interphase egg extract was supplemented with cyclin Δ 90 (500 nM) in the presence or absence of U0126 (300 μ M). The extracts were incubated at 23°C and aliquots were taken for the Fizzy-Ser 50 phosphatase assay. Open triangles, + cyclin Δ 90; closed triangles, + cyclin Δ 90 + U0126. (C) CSF-arrested egg extract was supplemented with p27^{Kip1} (2 μ M), followed by incubation at 23°C. Histone H1 kinase and Fizzy-Ser 50 phosphatase activities were measured at each time point. Open squares, H1 kinase of mock extract; open circles, phosphatase activity of mock extract; closed squares, H1 kinase of p27^{Kip1}-added extract; closed circles, phosphatase activity of p27^{Kip1}-added extract. (D) Aliquots from each time point of (C) were blotted with anti-Apc3 and anti-phospho CDK target antibodies.

of potential mitotic Cdk1 targets during interphase as well as for the exit from mitosis, when mitotic phosphorylations return to their interphase status after the inactivation of Cdk1.

PP2A is the major phosphatase that dephosphorylates the CDK substrate peptide

It is crucial to see whether this phosphatase activity is required for proper cell-cycle control. To evaluate this point, we next set out to determine the identity of this phosphatase activity. Much is known about the okadaic acid sensitivity of PP2A family members, so we titrated okadaic acid into interphase extracts and measured the phosphatase activity. The half-maximal inhibition by okadaic acid was observed at around 200–300 nM (Figure 2A). Out of PPP phosphatase family members (which include PP1, PP2A, PP2B/calcineurin, PP4, PP5, PP6 and PP7), the known IC₅₀ values of PP2B

and PP7 for okadaic acid are much higher than 300 nM (4 μ M for PP2B and more than 1 μ M for PP7), so they were excluded as candidates.

During the analysis of phosphatase in egg extracts, we repeatedly found that simple dilution of egg extracts caused an increase in total okadaic acid-sensitive phosphatase activity by more than 50 times against a variety of proteins and peptide substrates, including phosphorylase a and histone H1 (Supplementary Figure 1). Passing egg extracts through ion-exchange column showed a similar effect (previously noted by Lee *et al* (1991)), suggesting that unrelated non-physiological phosphatases are activated artificially during a purification procedure. Therefore, we approached the identification of the interphase phosphatase activity using an immunodepletion technique, measuring residual activity in the unbound fractions. To this end, we raised rabbit polyclonal antibodies against the catalytic subunits of PP1, PP2A, PP4, PP5 and PP6. The concentration of each phosphatase in *Xenopus* egg extracts was estimated to be 20, 160, 20, 100 and 8 nM, respectively, by comparing immunoblot signals with that of dilution series of the corresponding recombinant proteins (data not shown). These antibodies were used to immunodeplete extracts, followed by phosphatase assays of the depleted extract. For PP2A, we had to use the 6F9 monoclonal antibody against the A-subunit of PP2A (Kremmer *et al*, 1997), because our anti-PP2A C-subunit antibodies did not work well for immunodepletion. As shown in Figure 2B, PP1, PP2A, PP5 and PP6 were specifically depleted from interphase egg extracts without affecting the remaining amounts of non-targeted phosphatases. Out of these, PP2A depletion removed the Fizzy-ser 50 phosphatase activity from an interphase extract at an efficiency similar to that for removal of the PP2A C-subunit (Figure 2C). PP1 depletion reproducibly removed a small portion of the activity, typically by 20–30%, whereas PP5 or PP6 depletions did not reduce phosphatase activity directed against this substrate at all. These results suggest that PP2A is the major phosphatase in interphase *Xenopus* egg extracts against the Fizzy phosphopeptide, but allow the possibility that PP1 might also have some role (see Discussion section). PP4 depletion was poor (50% depletion at most), so we could not evaluate the contribution of PP4 in this approach.

B55 δ is the major B subunit for PP2A that dephosphorylates the CDK substrate peptide

We next determined which form of PP2A holoenzyme was responsible for this interphase phosphatase activity, adopting the same strategy as we used for the catalytic subunits. We raised antibodies against each B subunit of PP2A (B55 α , B55 β , B55 δ , B'56 γ , B'56 ϵ and B''/PR48) and used them in immunodepletion experiments. Anti-B55 α and B55 β antibodies failed to detect or immunoprecipitate any endogenous protein at the expected molecular weights in egg extracts, probably because they are present at very low levels. The remaining four B subunits were successfully detected and depleted from interphase egg extracts by their corresponding antibodies, and these depletions did not affect the amount of the non-targeted subunits (Figure 3A). The phosphatase assay showed that 65% depletion of B55 δ removed about 65% of the phosphatase activity directed against the CDK-phosphorylated peptide, whereas the other depletions did not (Figure 3B). These results suggest that most of the

Figure 2 PP2A is the major phosphatase that dephosphorylates CDK substrates in interphase egg extracts. (A) Titration of okadaic acid on the Fizzy-Ser 50 phosphatase activity. Error bars denote the range from three independent experiments. The activities are expressed in percentage of phosphatase activity of okadaic acid-free extract. (B) Phosphatases were removed from interphase egg extract by immunodepletion. The depletion efficiency shown on the right side was estimated by comparing the signal intensities from immunoblotting a dilution series of mock-depleted extract. PP1, PP4, PP5 and PP6 were depleted using antibodies against their catalytic subunits. Depletion of PP2A was carried out with anti-A subunit monoclonal antibody 6F9 and confirmed with immunoblotting with anti-PP2A catalytic subunit antibody. (C) Fizzy-Ser 50 phosphatase activities were measured in each phosphatase-depleted extract shown in (B). The activities are expressed in percentages of the phosphatase activity of mock-depleted extract.

Figure 3 B55 δ is the major B subunit for PP2A that dephosphorylates CDK substrates in interphase. (A) B subunits of PP2A were removed from interphase egg extract by immunodepletion. The depletion efficiency shown on the right side was estimated by comparing the signal intensities from immunoblotting of a dilution series of mock-depleted extract. (B) The Fizzy-Ser 50 phosphatase activity of each B subunit-depleted extracts shown in (A). Activities are expressed as a percent of phosphatase activity of mock-depleted extract.

phosphatase activity against the model substrate comes from PP2A with a B55 δ subunit.

PP2A-B55 δ controls the proper timing of mitosis entry and is essential for protein dephosphorylations on exit from mitosis

To evaluate the role of the PP2A-B55 δ in cell-cycle control, we next depleted this complex from an interphase extract by at least 90% (Figure 4B). The control extract entered mitosis at around 28 min and returned to interphase seven minutes later, judging from the changes in mitotic phosphorylations and cyclin degradation (Figure 4C, upper panels). In the PP2A-B55 δ -depleted extract, however, mitotic phosphoryla-

tions occurred at 14 min with a lower amount of cyclin and the extract remained in a hyperphosphorylated state even after cyclin degradation (Figure 4C, middle panels). We do not understand why the activity of the APC/C is partially shut off, allowing the re-accumulation of cyclin B, under these conditions. To check whether the depletion of PP2A-B55 δ complex was truly the cause of these effects, we prepared recombinant PP2A heterotrimer complex with a hexahistidine-tagged rat B55 δ subunit (Figure 4A, see Materials and methods). The phosphatase activity of this recombinant complex was approximately the same as that of endogenous PP2A-B55 δ . The addition of the recombinant complex to the depleted extract returned it to a state similar to the original control extract in terms of the timing of mitotic phosphorylations/dephosphorylations and cyclin degradation (Figure 4C bottom panels). Figure 4D shows the quantitative histone H1 kinase activity in all three conditions. These results suggest that the timing of phosphorylations/dephosphorylations of mitotic phosphoproteins is controlled by the balance between PP2A-B55 δ and CDK activities, consistent with the results shown in Figure 1.

Adding extra PP2A-B55 δ delays mitotic progression in a dose-dependent manner

To further examine the relationship between PP2A-B55 δ and CDK, we next tested the effect of increasing the concentration of recombinant PP2A-B55 δ complex added to extract. Figure 5 shows that entry into mitosis was delayed when the concentration of PP2A-B55 δ was increased by 50%. A doubling in the level suppressed the entry into mitosis, but not the destruction of cyclin B (which was, however, greatly delayed compared with the control). When the phosphatase activity was more than twice normal, mitotic phosphorylations were abolished despite there being much more cyclin than in the control extract, and now cyclin degradation never occurred. The pattern of tyr 15 phosphorylation on Cdk1 also confirmed the timing of cell-cycle transition from interphase to mitosis. In the case of the threefold-enhanced concentration of PP2A-B55 δ , one can see partial attempts to enter mitosis in the

form of transient retardation of the mobility of Apc3, and transient increases in the pattern of mitotic phosphoproteins, but these flickerings never catch fire, so to speak. The phosphatase activity apparently overwhelms the mitotic kinases. We have not tried to add extra cyclin B or Cdk1 under these conditions, although such titrations might be revealing of the control mechanisms involved.

For the dephosphorylations on mitotic exit, PP2A-B55 δ is required throughout the preceding interphase but not after mitotic progression

We next asked whether PP2A-B55 δ was solely or directly responsible for dephosphorylating CDK substrates on mitotic

exit. If this were true, depletion of B55 δ in mitosis just before CDK inactivation should inhibit the dephosphorylation of mitotic phosphoproteins just as was seen in the experiment shown in Figure 4C, in which B55 δ was depleted in the preceding interphase. To allow enough time for depletion in mitosis, we induced entry into mitosis by adding full-length recombinant human GST-cyclinB1 protein to interphase egg extract in the presence of cycloheximide to inhibit translation. Under these conditions, the added cyclin that is degraded very slowly, maintains the mitotic state for several hours. We could subsequently trigger CDK inactivation by removing the residual added cyclin with anti-GST antibody-coupled beads. As shown in Figure 6A (lanes 1–6 and 13–18), removal of the added cyclin induced the dephosphorylations of Apc3 and CDK targets. When B55 δ was depleted before mitotic induction, the extract was considerably delayed in a hyper-phosphorylated state after removing cyclin (Figure 6A, lanes 7–12). However, if depletion of B55 δ was carried out after mitotic progression, only a very slight delay was observed in the timing of dephosphorylation of the mitotic phosphoproteins (Figure 6A, lanes 19–24). Furthermore, the effect of deleting B55 δ in the preceding interphase, namely, keeping extracts in a prolonged mitotic state, was only observed when mitotic cyclins were naturally degraded (as in Figure 4C) or when the cyclin that caused mitotic progression was removed (Figure 6A). In contrast, when CDK activity was inhibited by adding p27^{Kip1}, the effect of depletion of B55 δ in the preceding interphase was as weak as when B55 δ was depleted in mitosis (compare Figure 6B, lanes 1–5 and 11–15). We discuss the interpretation of these rather surprising results below.

Discussion

The main purpose of this study was to identify the protein phosphatase, whose activity (using the model substrate described below) appears after the transient wave of calcineurin activity that normally helps to release frog egg extracts from metaphase arrest (Mochida and Hunt, 2007). We previously observed, and here confirm, that this activity is high in interphase, and low in mitotic extracts. This behaviour helps to account for the very complete modification of certain

Figure 4 PP2A-B55 δ controls mitotic progression in *Xenopus* egg extracts. **(A)** Coomassie brilliant blue staining of the purified recombinant PP2A heterotrimer complex. A α and C α subunits were expressed from *Xenopus* cDNA clones, whereas B55 δ is a rat protein. Left lane shows molecular weight markers and their size in kDa (kilodaltons) **(B)** Immunoblots of mock, B55 δ -depleted and added-back extracts using anti-*Xenopus* B55 δ (top) and catalytic (bottom) subunits. Note that the amount of the recombinant rat B55 δ is equivalent to the endogenous protein but shows a weaker signal (right lane) because of weak reactivity of the rat protein with the anti-*Xenopus* B55 δ antibody used for blotting. **(C)** Cell-cycle progression of B55 δ -depleted extract. Fresh interphase extract was treated with protein-G Sepharose beads conjugated with unrelated rabbit serum or anti-B55 δ serum. To one half of a B55 δ -depleted extract, recombinant PP2A-B55 δ complex was added back to give the same level of phosphatase activity as the original extract. These extracts were incubated at 23°C to monitor progress through the cell cycle. Aliquots were taken at 7-min intervals for immunoblotting of Apc3, Cyclin B2 and CDK target phosphorylation. Numbers on the right side indicate molecular weight in kDa. **(D)** Histone H1 kinase activity of each extract shown in (C). Black squares, mock-depleted extract; red circles, B55 δ -depleted extract; blue triangles, B55 δ -depleted/added-back extract.

Figure 5 Additional PP2A-B55 δ changes the timing of mitotic progression in a dose-dependent manner. Fresh cycling extract was supplemented with 50% extra, 2, 3 or 5 times the amount of endogenous enzyme by adding recombinant PP2A-B55 δ (as measured by phosphatase activity). Aliquots were taken at 10-min intervals for immunoblotting of Apc3, cyclin B2, CDK target phosphorylation and Cdk1-tyr 15 phosphorylation.

mitotic phosphoproteins such as Apc3 in mitosis; not only are protein kinases activated but the antagonizing protein phosphatases are also inhibited.

Using specific antibodies against protein phosphatase catalytic and regulatory subunits, we have been able to ascribe the majority of the phosphatase responsible for dephosphorylating our model substrate, a peptide containing ser50 of Fizzy, to the particular subset of PP2A containing the B55 δ subunit. This form of PP2A represents about 30% of the total in *Xenopus* egg extracts. This identification is quite reassuring, because this B subunit has long been known to have a function in the control of mitosis from genetic experiments in budding yeast, in which the mutation is in Cdc55, and in *Drosophila*, in which the *twins* and *aar* mutations affect the equivalent protein. In budding yeast, however, the role of

PP2A-Cdc55 is thought to be primarily in promoting the entry into and preventing exit from mitosis (Minshull *et al*, 1996; Pal *et al*, 2008), and the evidence favours Cdc14 phosphatase as the major enzyme responsible for the return to interphase (Visintin *et al*, 1998; Queralt and Uhlmann, 2008). In fission yeast, the B55 *pab1* gene seems to be important for cell wall biosynthesis, and for neither entry into nor exit from mitosis (Kinoshita *et al*, 1996). There is a big difference, however, between the open mitosis found in higher eukaryotes, and the closed mitosis seen in yeast, which may explain these differences. In higher eukaryotes there is no evidence that Cdc14 plays any part in exit from mitosis (Saito *et al*, 2004; Berdugo *et al*, 2008; Hansen *et al*, 2008).

Entry into mitosis

The Wee1-Cdc25-Cdk1 inhibition/activation loop has long been known as a key circuit controlling the entry into mitosis, and this loop seems to be kept inhibited by a yet unidentified PP2A activity before mitosis (Picard *et al*, 1989; Yamashita *et al*, 1990; Trinkle-Mulcahy and Lamond, 2006; Pal *et al*, 2008). As we have identified PP2A-B55 δ as a major phosphatase activity against CDK substrates, which include Cdc25, PP2A-B55 δ may also suppress the activity of this loop by removing phosphates from phosphorylated Cdc25 and Wee1. We observed a strongly premature CDK activation in the B55 δ -depleted extract (Figure 4D), consistent with this interpretation. Other B55 isoforms, B55 α and B55 β , have also been reported to have anti-mitotic activity in the *Xenopus* system (Lee *et al*, 1991; Iwashita *et al*, 1997), implying that PP2A complexes share similar substrate specificity with other B55 isoforms but are regulated in different ways, either temporally and/or spatially. It is crucial to examine whether PP2A-B55 δ suppresses CDK target phosphorylation by directly dephosphorylating them, or by suppressing CDK activation, or by a combination of both effects.

Exit from mitosis

The role of PP2A-B55 δ seems more obvious, although its contribution is apparently indirect, as far as exit from mitosis is concerned, because the inactivation of Cdc25 is not supposed to be essential for the inactivation of CDKs; cyclin degradation, rather than re-phosphorylation of Cdk1 on inhibitory sites, is the crucial regulatory event. We found that CDK targets remained phosphorylated indefinitely in the B55 δ -depleted extract after the degradation of cyclin and the full inactivation of CDK (Figures 4C, D and 6A). This observation is consistent with the phenotype of *Drosophila twins* mutants (Mayer-Jaekel *et al*, 1993). The simplest interpretation is that PP2A-B55 δ is the phosphatase that directly dephosphorylates a broad range of CDK substrates on mitotic exit. This could be partly true because we reproducibly observed a slight delay in the dephosphorylation of mitotic substrates in extracts that had been depleted of B55 δ in mitosis (compare Figure 6A, lanes 13–18 and 19–24). However, PP2A-B55 δ seems more important in the preceding interphase for CDK substrates to be dephosphorylated following mitotic exit. Once the cell cycle progresses into mitosis, depletion of PP2A-B55 δ hardly affects mitotic exit at all (Figure 6A). This result suggests that PP2A-B55 δ is a regulator of other downstream phosphatase(s) that directly dephosphorylates CDK substrates on mitotic exit. As the

Figure 6 (A) PP2A-B55 δ is required throughout preceding interphase but not after mitotic entry for the dephosphorylations of CDK substrates. Interphase egg extracts supplemented with 0.1 mg/ml of cycloheximide were immunodepleted for B55 δ before (lanes 7–12) or after (lanes 19–24) mitotic induction by adding recombinant GST-cyclin B1. These extracts were then mixed on ice with anti-GST beads to remove the added cyclin, followed by incubation at 23°C to induce mitotic exit. The samples in lanes 1, 7, 13 and 19 were taken before cyclin depletion. Samples were collected at the indicated times after incubation at 23°C, analyzed by SDS-PAGE and blotted with the indicated antibodies. (B) Interphase egg extract was depleted for B55 δ (lanes 11–20) before adding GST-cyclin B1, then CDK activity was inhibited either by adding 2 μ M of p27^{Kip1} or by removing GST-cyclin B1 with anti-GST beads. Samples were processed as described in (A).

activity of PP2A-B55 δ is suppressed in mitosis (Mochida and Hunt, 2007 and Figure 1B and C), the phosphorylation(s) that is/are opposed by PP2A-B55 δ and which are critical for the massive dephosphorylation that occurs on mitotic exit would be catalyzed by some kinase which is active only in interphase but not in mitosis. An important question that remains is: which phosphatase(s) directly dephosphorylate(s) CDK substrates on mitotic exit? PP1 is a good candidate to account for the genetic evidence (Doonan and Morris, 1989; Ohkura *et al*, 1989; Hisamoto *et al*, 1994), and recent evidence from the Kornbluth laboratory (Wu *et al*, 2009) strongly suggests that PP1 has a role in mitotic substrate dephosphorylation, so it is possible that both PP1 and PP2A contribute to this dephosphorylation, whether in parallel and/or in tandem (i.e. with PP2A regulating PP1 or vice versa) remains to be determined. As we do not yet have specific substrates for other forms of PP2A, we cannot tell whether they are also inhibited during M-phase. Considering that several protein kinases are active during mitosis, and the multiplicity of mitotic phosphorylations, it is likely that multiple phosphatases contribute to exit from mitosis.

Our finding that CDK inhibition by p27^{Kip1} overrode the requirement for B55 δ for dephosphorylations on mitotic exit (Figure 6B) may perhaps be explained by the inhibition of Cdk2-cyclin E complexes by p27^{Kip1}. Cdk2-cyclin E has been reported to be relatively constant in activity throughout *Xenopus* embryonic cell cycles, such that the unusual inhibition of Cdk2-cyclin E by p27^{Kip1} might allow dephosphorylations to occur without B55 δ function. This is a testable idea.

Our experiments have not yet explained how the activity of PP2A-B55 δ is regulated. No changes were observed in either the protein level of B55 δ or in its binding to PP2A catalytic subunit throughout the cell cycle (Supplementary Figure 2A and B). PP2A-B55 δ is activated by the inhibition of Cdk1, implying that a phosphorylation step is involved in its inhibition. Zhao *et al* (2008) proposed Greatwall kinase in *Xenopus* as a phosphatase suppressor to account for its role in promoting the activation of MPF during oocyte maturation, as its kinase activity showed effects on the cell cycle similar to those observed on the addition of okadaic acid. Moreover, they recently showed that depletion of Gwl from mitotic egg

extract was enough to reactivate PP2A-B55 δ even in the presence of high mitotic CDK activity, strongly supporting their idea (PV Castilho *et al*, Manuscript Submitted).

In summary, we believe that we have provided strong evidence that the regulation of PP2A-B55 δ is of paramount importance for the regulation of the entry into and exit from mitosis, but for the moment, we do not have any molecular insights into the manner of its regulation. Until we understand how its activity is turned on and off, it will be difficult to provide a coherent account on how entry into mitosis is controlled, nor is it clear whether other forms of PP2A (and other protein phosphatases) are subject to similar regulation. Most likely, multiple phosphatases are required to undo the effects of multiple protein kinases. Moreover, if the switch mechanism depends on protein phosphorylation, there must be other phosphatases to allow the switch to be reversed.

Materials and methods

Interphase *Xenopus* egg extract

Interphase 'cycling' extracts were prepared according to the protocol described by Ohsumi *et al* (2006).

Reagents

Okadaic acid (potassium salt) was purchased from LC Laboratories. Recombinant p27^{Kip1} protein tagged with hexahistidine was expressed in *Escherichia coli* and purified using Ni-NTA agarose beads. Recombinant full-length human GST-cyclin B1 was expressed in Sf9 cells. Phosphorylase was a grateful gift from Dr Catherine Venien-Bryan.

Antibodies

Anti-Apc3 antibody was purchased from BD bioscience (anti-CDC27). Anti-Cdk1 phospho-Y15 and CDK target antibodies were purchased from New England Biolabs (#9111 and #2324, respectively). Anti-Cyclin B2 (clone #X121) and anti-Cyclin B1 (clone #V152) antibodies were made by Dr Julian Gannon. The 6F9 antibody was a generous gift from Drs Gernot Walter and Ralf Ruediger. To raise antibodies against PP1, PP4, PP5, PP6, B55 α , B55 β , B55 δ , B56 α , B56 γ , B56 ϵ and B''/PR48, recombinant proteins expressed in bacteria were injected into rabbits (BioGenex). These antisera were used for immunodepletion, whereas affinity-purified antibodies were used for immunoblot experiments. To strictly distinguish between very similar proteins such as PP2A, PP4 and PP6 in immunoblots, antibodies were purified in two steps. The crude antisera were first passed through columns containing the

family members, apart from the antigen under consideration, and then the flow-through fraction was adsorbed onto a second column containing the original antigenic protein. After elution using glycine–HCl (pH 2.5) the antibody from this column showed high specificity for its cognate antigen, and essentially no cross-reactivity with other phosphatase catalytic subunits.

Immunodepletion of phosphatases

Immunodepletion of *Xenopus* egg extracts were carried out using protein-G Sepharose beads (Sigma) covalently conjugated with the appropriate antibodies or antisera. We found it crucial to keep egg extracts as little diluted as possible when measuring physiological changes in the phosphatase activity. This is because the dilution of extracts results in a large increase in non-physiological phosphatase activity. For this reason, during the immunodepletion procedure, we tried to remove as much buffer as possible from the antibody-conjugated beads before mixing with egg extract. We followed Dr Gernot Walter's advice, and pierced the bottom of a plastic tube with a needle to make a slit that could remove the residual buffer by centrifugation just before mixing with extracts.

Recombinant PP2A–B55 δ holoenzyme

A fusion protein between GST and the α subunit of PP2A (*Xenopus laevis* cDNA) was expressed in bacteria, purified with GSH-Sepharose and subsequently cleaved using PreScission protease. The PP2A α subunit (*Xenopus laevis* cDNA) was expressed in HighFive cells using pFastBac baculovirus expression system (Invitrogen). We were unable to form complexes between *Xenopus* B55 δ and the PP2A A/C dimer, regardless of its tag (tags used: GST, MBP, FLAG and His tags), and were obliged to use rat B55 δ instead. His-tagged rat B55 δ was expressed in Sf9 cells. Infected cells were extracted in an extraction buffer (50 mM Tris–Cl, 150 mM NaCl, 3 mM dithiothreitol, 0.2% Tween-20, 1 mM EDTA, 1 mM EGTA, 5% glycerol, 5 mM benzamidine, 1 mM phenylmethanesulfonyl fluoride, 2 μ g/ml leupeptin, 2 μ g/ml chymostatin and 2 μ g/ml pepstatin A, pH 7.5) by rotating 30 min in a cold room, followed by

sonication. Purified GST– α subunit was mixed with cleared cell lysates expressing B55 δ and α subunits on ice. The complex was first purified on glutathione beads, followed by protease cleavage. Next, the eluted proteins were retrieved on Ni–NTA agarose beads to ensure all complexes contained a B subunit. Beads were washed and proteins were eluted in a buffer (20 mM Tris–Cl, 150 mM NaCl, 3 mM dithiothreitol, 0.01% Tween-20 and 0.2 M imidazole, pH 7.5), followed by three rounds of dialysis against (20 mM Tris–Cl, 150 mM NaCl, 1 mM dithiothreitol, pH 7.5).

The Fizzy–Ser50 phosphatase assay

Maltose binding protein with residues 38–62 of *Xenopus* Fizzy/Cdc20 at its C-terminus was prepared, and phosphorylated using Cdk2/cyclin A and γ -[32 P] γ -ATP as described by Mochida and Hunt (2007), and the radioactivity released after addition to extracts was measured by scintillation counting as described by Mackintosh and Moorhead (1999).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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