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Distinct roles of PP1 and PP2A-like phosphatases in control of microtubule dynamics during mitosis

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Assembly of a mitotic spindle requires the accurate regulation of microtubule dynamics which is accomplished, at least in part, by phosphorylation–dephosphorylation reactions. Here we have investigated the role of serine-threonine phosphatases in the control of microtubule dynamics using specific inhibitors in *Xenopus* egg extracts. Type 2A phosphatases are required to maintain the short steady-state length of microtubules in mitosis by regulating the level of microtubule catastrophes, in part by controlling the the microtubule-destabilizing activity and phosphorylation of Op18/stathmin. Type 1 phosphatases are only required for control of microtubule dynamics during the transitions into and out of mitosis. Thus, although both type 2A and type 1 phosphatases are involved in the regulation of microtubule dynamics, they have distinct, non-overlapping roles.

Keywords: microtubule dynamics/mitosis/Op18/phosphatase/spindle

Introduction

Correct chromosome segregation during mitosis requires the assembly of a microtubule-based structure called the mitotic spindle. This assembly involves dramatic changes in microtubule turnover: from a relatively stable state in interphase, they become highly dynamic at the onset of mitosis, their half-life changing from ~5 min to 45 s (Zhai *et al.*, 1996). During assembly of the spindle, some mitotic microtubules appear to be preferentially stabilized around the chromosomes, and this contributes to generating the bipolar shape of the spindle. Thus, it is important to understand how microtubule dynamics are regulated both globally and locally around the chromosomes.

Changes in general microtubule stability can be described by examining the sum of the dynamic properties of individual microtubules. Microtubules either grow or shrink, the transition from one state to the other being unpredictable, a property known as dynamic instability. Thus, the behavior of a microtubule is defined by four parameters: the growth rate, the shrinkage rate and the frequencies of transitions between growth and shrinkage phases, called a catastrophe, and between shrinkage and growth phases, called a rescue (Walker *et al.*, 1988; Hyman and Karsenti, 1996). During the transition from interphase to mitosis, the primary effect on microtubule dynamics is an increase in the catastrophe rate (Belmont *et al.*, 1990; Verde *et al.*, 1992). However, little is known about direct effectors of microtubule dynamics or how they are regulated.

Changes in microtubule dynamics during the transitions into and out of mitosis are controlled by the activity of cdc2 kinase which phosphorylates a large variety of molecules either directly or indirectly (Belmont *et al.*, 1990; Verde *et al.*, 1990, 1992). It is likely that the control of microtubule dynamics during these cell cycle transitions is determined by the balance between cdc2 kinase and phosphatases that directly or indirectly oppose its action on target molecules. Such phosphorylation balances may also be involved in the stabilization of microtubules by chromosomes during spindle assembly.

Analysis of mutants in serine-threonine phosphatases indicates that they participate in ensuring correct chromosome segregation at mitosis. Some phenotypes suggest that they do so by regulating cell cycle control. For instance, type 2A phosphatase (PP2A) negatively regulates cdc2 kinase at the onset of mitosis in *Xenopus* (Felix *et al.*, 1990; Clarke *et al.*, 1993; Lee *et al.*, 1994; Lee, 1995), starfish (Picard *et al.*, 1989, 1991) and both in fission and budding yeasts (Kinoshita *et al.*, 1993; Lin and Arndt, 1995; Evans and Stark, 1997). Inhibition of type 1 phosphatases (PP1) arrests the cells at the metaphase–anaphase transition (Booher and Beach, 1989; Ohkura *et al.*, 1989; Fernandez *et al.*, 1992; Hisamoto *et al.*, 1994; Ishii *et al.*, 1996) because PP1 may be required to activate the anaphase-promoting complex (APC), required to degrade cyclin B (Ishii *et al.*, 1996). However, other phenotypes suggest that phosphatases are also involved in the control of structural events associated with mitosis. Mutants in PP2A genes show defects in spindle assembly and microtubule growth (Kinoshita *et al.*, 1993; Snaith *et al.*, 1996; Evans and Stark, 1997), as do some mutants in PP2A regulatory subunit genes (Gomes *et al.*, 1993; Kinoshita *et al.*, 1996). Mutants in PP1 genes in *Saccharomyces cerevisiae* (Black *et al.*, 1995), *Schizosaccharomyces pombe* (Ohkura *et al.*, 1988, 1989), *Aspergillus nidulans* (Doonan and Morris, 1989) and *Drosophila* (Axton *et al.*, 1990) show complex mitotic phenotypes with condensed chromosomes, abnormal spindles and chromosome separation malfunction. Since alterations in the phosphorylation state of proteins involved in spindle assembly and function can have effects on cell cycle progression and vice versa, the exact roles of phosphatases during mitosis are unclear.

We have addressed this problem using *Xenopus* egg extracts as they allow study of the role of phosphatases

in the regulation of microtubule dynamics and spindle assembly independently of their role in cell cycle progression. We have used two specific inhibitors of serine-threonine phosphatases. Okadaic acid at low concentrations inhibits PP2A but does not inhibit PP1 activity (for review, see Cohen, 1989; Shenolikar, 1994). Conversely, inhibitor 2 (I-2), a 23.8 kDa protein, specifically inhibits PP1 activity by binding to the catalytic subunit (Cohen, 1989; Shenolikar, 1994). We find that PP2A is required to maintain the short steady-state length of microtubules during mitosis, in part by regulating Op18/stathmin (Sobel, 1991), a recently identified molecule involved in the control of microtubule dynamics. PP1 has a different role, and is involved in control of microtubule dynamics during the transitions between interphase and mitosis.

Results

Extracts made from *Xenopus* eggs can be arrested in defined states of the cell cycle, either in interphase or in mitosis, and their position in the cell cycle monitored by measuring the activity of cdc2 kinase which is low in interphase and high in mitosis. Extracts can be arrested in mitosis in two ways. First, extracts made from metaphase II-arrested oocytes (CSF extracts) contain an activity which maintains cdc2 kinase in an active form. Second, a non-degradable version of cyclin B, cyclin Δ90, can be added to extracts in order to stabilize the cdc2 kinase activity. These latter extracts retain much of the mitotic state even though the APC and the machinery responsible for cyclin degradation are active (Murray *et al.*, 1989). In particular, mitotic spindles remain intact with a high microtubule turnover in such extracts, although anaphase A occurs (Holloway *et al.*, 1993; Surana *et al.*, 1993).

Spindle assembly can be induced in *Xenopus* extracts by adding sperm nuclei to a CSF extract that is then released into interphase in order to allow DNA replication and centrosome duplication. Then, the extract is sent back into metaphase by adding more CSF extract or cyclin Δ90. This results in the migration of centrosomes around the nucleus and spindle assembly in a succession of prophase and metaphase figures (Sawin and Mitchison, 1991; Shamu and Murray, 1992; Tournebize and Heald, 1996). Alternatively, spindles can be assembled by incubating sperm nuclei directly in CSF extracts. Microtubules grow from the sperm centrosome towards chromatin, forming half-spindles, then two half-spindles fuse, forming a bipolar spindle (Sawin and Mitchison, 1991; Tournebize and Heald, 1996). Microtubule dynamics can be examined separately by adding centrosomes in the absence of chromosomes. Under these conditions, one can see microtubule ends better because the microtubule density is lower. Additionally, one can determine the changes in microtubule dynamics in the absence of the influence of mitotic chromatin.

A PP2A-like activity keeps microtubules short during metaphase

In order to determine whether PP2A activity was involved in the regulation of microtubule length during metaphase, we tested the effect of okadaic acid on the morphology of spindles assembled in extracts. At 60 min after the initiation of spindle assembly, metaphase spindles were present. Then 0.4 μM okadaic acid was added and aliquots of the extract were fixed and observed. In the absence of the drug, microtubules were organized in a typical ellipsoidal shape, and chromosomes were aligned on the metaphase plate (Figure 1A). In contrast, bipolar spindles could not be found 20 min after addition of 0.4 μM okadaic acid, the microtubules were up to 100 μm long, with an average length of 77 μm, and the chromosomes were scattered throughout the structure (Figure 1A). Moreover, numerous free microtubules could be observed in the extract.

This effect of okadaic acid could reflect an indirect effect on cell cycle progression, for example by inducing the return to interphase, or a direct effect on microtubule dynamics. Indeed, this drug is known to activate cdc2 kinase in the presence of sub-threshold levels of cyclin (Felix *et al.*, 1990), but also to induce cyclin degradation after some time of incubation (Felix *et al.*, 1990; Lorca *et al.*, 1991), which results in final cdc2 kinase inactivation. In order to study PP2A function independently of any effect on cdc2 kinase activity, we repeated the experiment in the presence of cyclin Δ90. Because this cyclin construct is non-degradable, it produces a constitutively active kinase when it combines with the endogenous cdc2 catalytic subunit of the extract (Glotzer *et al.*, 1991). Under such conditions, cdc2 kinase activity remained high after addition of 0.4 μM okadaic acid. However, microtubules became long, and bipolar spindles disappeared from the extract, replaced by structures such as that shown in Figure 1A. We conclude that even in the presence of high cyclin B–cdc2 kinase activity, microtubules grow long after inhibition of PP2A activity. Moreover, this result also suggests that short microtubules are required to make a spindle. To test this further, we added 0.4 μM okadaic acid and sperm nuclei simultaneously to CSF extracts pre-incubated with cyclin Δ90. This resulted in the formation of asters with long microtubules (data not shown), but no bipolar spindles were assembled. Thus bipolar spindles may not assemble in the absence of PP2A activity because of the length of the microtubules formed under such conditions.

PP2A inhibition affects the catastrophe rate of microtubules

To understand how PP2A activity could control the steady-state length of microtubules during mitosis, we investigated how inhibition of PP2A affected the dynamics of individual microtubules by time-lapse video microscopy. Extracts pre-incubated with cyclin Δ90 were treated with 0.4 μM okadaic acid and supplemented with purified centrosomes and rhodamine–tubulin. Images of microtubules were recorded over time and the parameters of microtubule dynamics deduced from the analysis of the videos. Figure 1B shows images taken from two videos, in the presence and absence of 0.4 μM okadaic acid. Microtubules were longer in the presence of okadaic acid and we also noticed a significant increase in spontaneous assembly. Analysis of individual microtubules in several videos showed that inhibition of PP2A activity reduced the level of catastrophes by 1.5- to 3-fold, depending on the experiment (Table I). The average values from five independent experiments showed that okadaic acid addition decreased the catastrophe rate from 2.44 catastrophes/min to 1.22

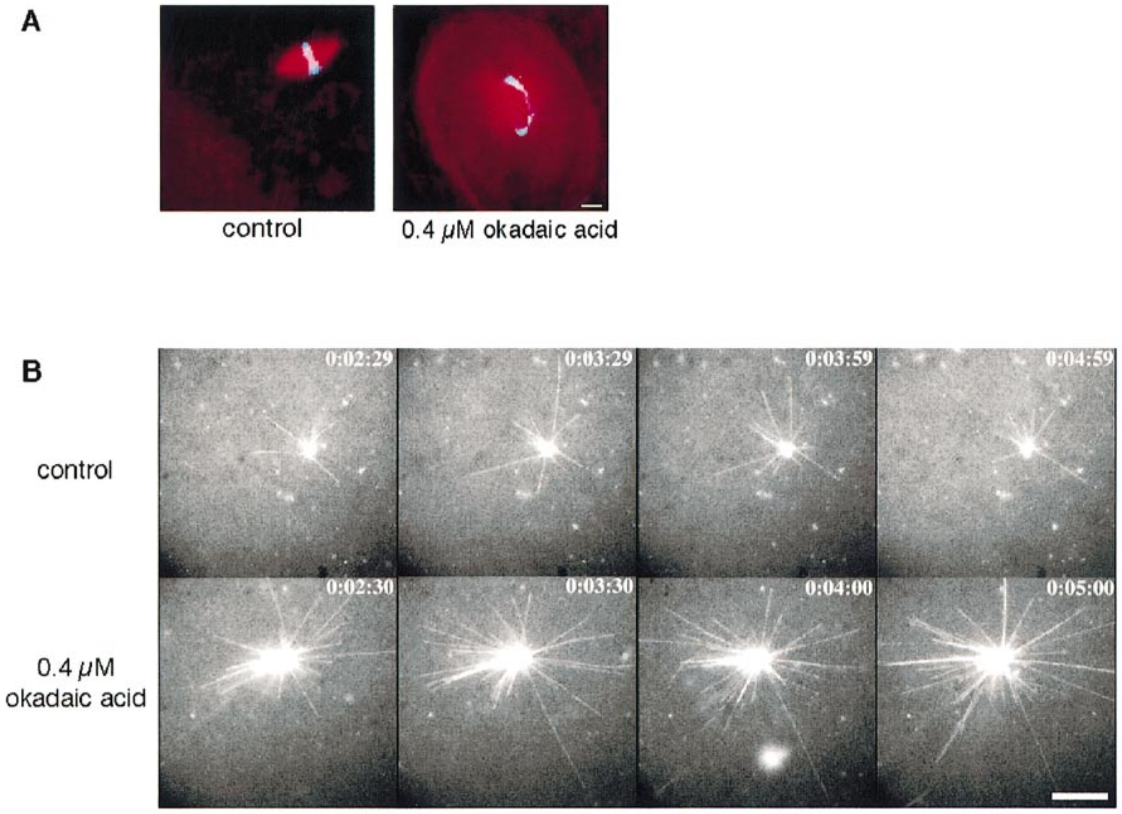

Fig. 1. (**A**) Addition of okadaic acid to spindles induces microtubule growth. Spindles assembled in cyclin Δ90 *Xenopus* egg extracts were mock treated (control) or treated with 0.4 μM okadaic acid for 20 min at 20°C. (**B**) Addition of 0.4 μM okadaic acid increases microtubule steady-state length. Images taken at the same recording time from videos in the presence (bottom) or absence (top) of 0.4 μM okadaic acid. Time is h:min:s. Bars are 10 μm.

Table I. Addition of okadaic acid to extracts reduces catastrophe frequencies

	Growth rate (μm/min)	Shrinkage rate (μm/min)	Catastrophe frequency (events/min)	Rescue frequency (events/min)	Calculated microtubule length (μm)
Control	11.44 ± 1.21 (105)	13.49 ± 1.90 (91)	2.44 ± 0.54 (91)	0.70 (29)	7.4
Okadaic acid	13.00 ± 1.24 (87)	16.37 ± 2.81 (58)	1.22 ± 0.21 (58)	1.40 (17)	34.4

Okadaic acid (0.4 μM) was added to cyclin Δ90 extracts. After 20 min incubation at 20°C, extracts were frozen and aliquots of these used for microtubule dynamics measurements for 5 min. Growth rate, shrinkage rate and catastrophe frequency were determined after measurements of microtubules over time. Rescue frequencies reported here are not statistically significant. Values are means ± standard error of the mean (number of total events measured). Average steady-state length <L> was calculated using the measured values.

catastrophes/min. Average growth and shrinkage rates were not significantly affected, being ~12 and 15 μm/min respectively. Rescues were 0.70 event/min in control and 1.40 events/min in the presence of okadaic acid. Due to a low number of events observed, this difference is not statistically significant. Okadaic acid addition had no effect on interphase microtubule dynamics (data not shown).

Verde *et al*. (1992) showed that the steady-state length of microtubules is related directly to the parameters of microtubule dynamics and that microtubule steady-state length can be calculated from the values of microtubule dynamics using a simple equation (see Materials and methods). Using this formula, we calculated the microtubule steady-state length <L> in the presence and absence of 0.4 μM okadaic acid. We assumed that the rescue frequency was not affected by treatment with okadaic acid, taking a value of 1.05 rescues/min, the average of the values measured with and without okadaic acid. We found an average microtubule length <L> of 7.4 μm in control and 34.4 μm in extracts treated with 0.4 μM okadaic acid (Table I). Thus, inhibition of PP2A results in a 5-fold increase in microtubule steady-state length <L> due to only a 2-fold reduction in the catastrophe rate.

PP2A regulates Op18/stathmin phosphorylation

We were interested in finding targets of PP2A that could regulate microtubule dynamics. Since Op18/stathmin is thought to destabilize microtubules in *Xenopus* egg extracts (Belmont and Mitchison, 1996), and since phosphorylation is known to regulate its activity (Marklund *et al.*, 1996; Horwitz *et al.*, 1997), we examined the role of PP2A in regulating Op18/stathmin phosphorylation. Okadaic acid was added to an extract containing cyclin Δ90 in order to inhibit PP2A, and [γ-^{32}P]ATP was added in order to label phosphorylated proteins. Op18/stathmin was then

immunopurified and the level of ^{32}P incorporation monitored. In untreated extracts, Op18/stathmin was poorly phosphorylated. Addition of increasing amounts of okadaic acid to the extract resulted in a gradual increase in the level of ^{32}P incorporated into Op18/stathmin protein (Figure 2B). Quantification of the level of ^{32}P incorporated showed that at 0.5 μM okadaic acid, Op18/stathmin contained four times more ^{32}P than the control. For comparison, addition of 3 μM I-2, which specifically inhibits PP1, only induced a 1.5-fold increase in the level of ^{32}P incorporated. Taken together, these observations show that PP2A activity maintains Op18/stathmin in a low phosphorylation state in metaphase extracts.

PP2A regulates microtubule dynamics by controlling the phosphorylation state of Op18/stathmin

Previous experiments had suggested that, when unphosphorylated, Op18/stathmin destabilizes microtubules (Marklund *et al.*, 1996; Horwitz *et al.*, 1997), while phosphorylated Op18/stathmin does not. Thus our experiments suggest that, normally, Op18/stathmin in extracts is dephosphorylated and active. PP2A inhibition promotes phosphorylation of OP18/stathmin, thus microtubules become more stable. If PP2A inhibition promoted microtubule growth by promoting hyperphosphorylation of Op18/stathmin, then a mutant Op18/stathmin which cannot be phosphorylated should overcome the effect of PP2A inhibition on microtubule steady-state length. In order to test this idea, we used purified Op18/stathmin protein in which the four known phosphorylated serines had been mutated to alanine. Okadaic acid (0.4 μM) was added together with purified Op18/stathmin to pre-assembled spindles that were then fixed and observed. Twenty minutes after addition of the mutant Op18/stathmin protein together with okadaic acid, most spindles were bipolar (Figure 3), but ~20% of them had short microtubules uniformly wrapped around chromatin without clear poles, forming round 'spindles' (data not shown). Addition of okadaic acid together with wild-type Op18/stathmin generated the same structures as okadaic acid alone (Figure 3). Thus, addition of unphosphorylatable Op18/stathmin rescues the effect of okadaic acid on microtubule growth while wild-type Op18/stathmin does not.

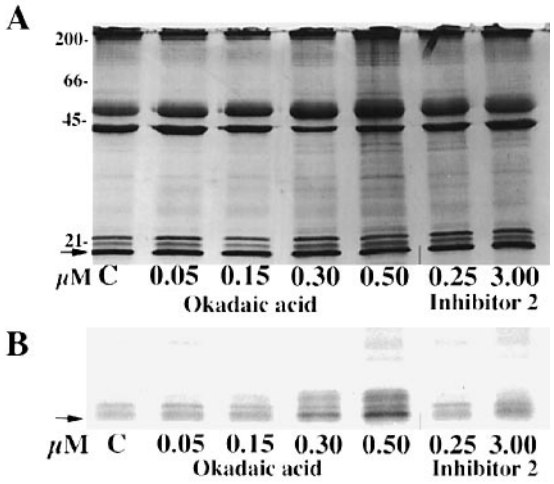

Fig. 2. The Op18/stathmin phosphorylation state is regulated by PP2A. Different concentrations of okadaic acid and I-2 were added to cyclin Δ90 extracts containing [γ-^{32}P]ATP. After 20 min, Op18/stathmin was immunoprecipitated and run on a 15% acrylamide gel. (**A**) Silver staining of the gel; (**B**) an autoradiogram. Phosphorylation of Op18/stathmin increased four times in the presence of 0.5 μM okadaic acid and 1.5 times in the presence of 3 μM I-2. The arrow points to Op18/stathmin, which resolves as a triplet. Molecular weight markers are indicated.

Op18/stathmin depletion decreases the catastrophe rate

The previous results strongly suggested that PP2A controls microtubule dynamics by regulating the phosphorylation of Op18/stathmin, but did not rule out the possibility that exogenously added mutant Op18/stathmin depolymerized microtubules independently of PP2A. If PP2A acted through Op18/stathmin to control the catastrophe rate of microtubules, then depletion of Op18/stathmin should decrease the catastrophe rate as well. We therefore depleted Op18/stathmin from extracts and measured microtubule dynamics. Results from four independent depletion experiments are shown in Figure 4. Upon removal of ~95% of Op18/stathmin, the catastrophe frequency decreased 1.5- to 5-fold, depending on the experiments. On average, catastrophes decreased from 1.46 events/min in control treated extracts to 0.86 events/min in Op18/stathmin-depleted extracts. Thus Op18/stathmin is required to

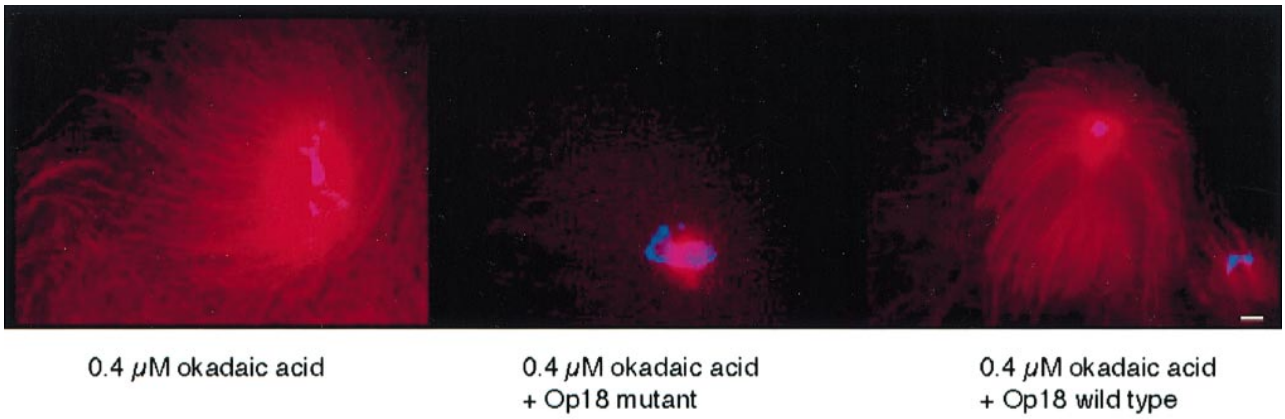

Fig. 3. Non-phosphorylatable Op18/stathmin induces microtubule depolymerization. Spindles were assembled in cyclin Δ90 extracts and okadaic acid was added alone (left panel), together with 250 μg/ml of human Op18/stathmin protein with its four phosphorylation sites mutated to alanine (middle panel) or with 250 μg/ml wild-type human Op18/stathmin (right panel). Spindles were fixed 20 min after incubation at 20°C. Bar is 10 μm.

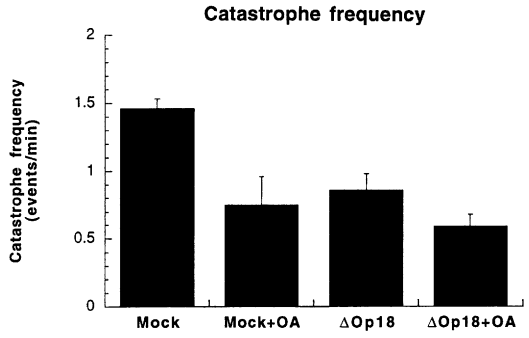

Fig. 4. Catastrophe frequency decreases after Op18/stathmin depletion and okadaic acid addition. Op18/stathmin was depleted from extracts containing cyclin Δ90, and microtubule dynamics were measured. Catastrophe frequency decreases after Op18/stathmin depletion (ΔOp18) and after 0.4 μM okadaic acid addition (Mock+OA). No significant decrease in catastrophe is observed after addition of 0.4 μM okadaic acid to Op18/stathmin-depleted extract (ΔOp18+OA). Values are means ± standard error of the mean.

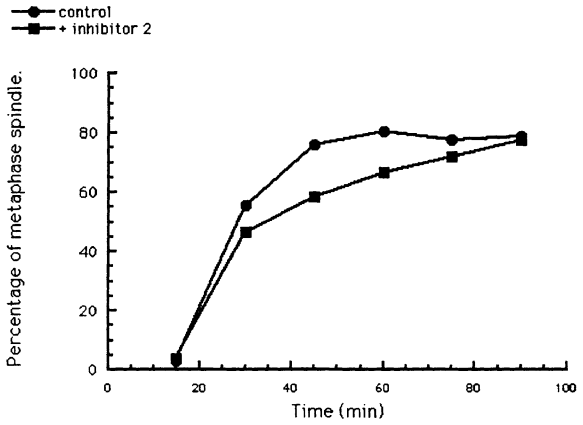

Fig. 5. PP1 is not required for spindle assembly. Spindle assembly was monitored in the presence or the absence of I-2. Extracts containing nuclei were released into interphase for 80 min before mitosis was triggered by addition of CSF extract together with 3 μM I-2 (1 μM I-2 was then added every 15 min). Spindles were fixed every 10 min and the percentage of spindles in metaphase scored. No statistical difference was observed upon PP1 inhibition compared with the control.

maintain a high level of catastrophes during metaphase. We also analyzed the effect of Op18/stathmin depletion on the growth and shrinkage rates of microtubules. These rates were not statistically changed by Op18/stathmin depletion. Using these values, we calculated the average length <L> of microtubules. We found that they are 7.9 μm long in mock-depleted extracts and >100 μm in Op18/stathmin-depleted extracts. These values show that Op18/stathmin is a key regulator of microtubule length *in vitro*.

In order to test whether Op18/stathmin could account for all the changes in catastrophes observed upon PP2A inhibition, we added 0.4 μM okadaic acid to Op18/stathmin- or mock-depleted extracts. The catastrophe frequency dropped from 0.85 to 0.59 in Op18/stathmin-depleted extracts. However, this difference was not found to be statistically significant (Figure 4). Growth and shrinkage rates were not significantly changed. The calculated microtubule length <L> after addition of 0.4 μM okadaic acid was 61.2 μm in mock-treated extracts and >100 μm in Op18/stathmin-depleted extracts. These results show that Op18/stathmin accounts for most of the catastrophes regulated by PP2A in *Xenopus* extracts. In conclusion, PP2A maintains the short steady-state length of microtubules by regulating the catastrophe rate, mainly through regulation of the activity of Op18/stathmin.

PP1 is required for the control of microtubule dynamics during the transitions into and out of mitosis

To examine the role of PP1 in the regulation of microtubule dynamics, we used a specific inhibitor of PP1, I-2, a 23.8 kDa protein which binds specifically to the catalytic site of PP1 to inhibit its activity. We added I-2 to interphasic and mitotic extracts and observed microtubule length and dynamics. Inhibition of PP1 had no effect on the parameters of microtubule dynamics in either interphase or metaphase (data not shown). To test whether PP1 is required for spindle assembly, extracts containing sperm nuclei were released into interphase and I-2 was added at the same time as CSF extracts. The kinetics of spindle assembly were statistically similar in the presence and absence of I-2 (Figure 5), and maintenance of spindle length was not affected over 60 min of observation (data not shown). These results suggested that once the extract contains a high level of cdc2 kinase activity, PP1 is not required to regulate microtubule dynamics and spindle assembly.

PP1 and cdc2 kinase act antagonistically to control the steady-state length of microtubules at the onset of mitosis

Although inhibition of PP1 had no effect on spindle assembly, we wondered whether it could participate in the regulation of microtubule dynamics during the early phase of mitosis when cdc2 kinase begins to act on microtubule dynamics, in prophase. To test this hypothesis, we made use of purified cdc2 kinase which, when added to an interphase extract, triggers mitotic microtubule dynamics (Verde *et al.*, 1990). We reasoned that if PP1 is opposing cdc2 kinase activity during the transition of microtubule dynamics from an interphase to a metaphase state, then inhibition of PP1 should speed up the rate at which cdc2 kinase phosphorylates substrates and microtubules become dynamic. Cdc2 kinase was added to interphase extracts with or without I-2. Extracts were complemented with centrosomes and rhodamine–tubulin, fixed 15 min later and spun down onto coverslips, and the length of microtubules were measured. As shown in Figure 6, microtubules are long in interphase extracts (25 μm) and become shorter upon addition of cdc2 kinase (Figure 6A). In the presence of PP1 inhibitor, 9–10 pmol/μl/min of cdc2 kinase activity were required to convert the average microtubule length to a mitotic state (Figure 6B and C) while 20 pmol/μl/min were necessary in the absence of inhibitor. This result suggests that PP1 and cdc2 kinase have opposing effects in controlling microtubule dynamics during the interphase to metaphase transition.

PP1 participates in the regulation of global changes in microtubule dynamics during the metaphase to anaphase transition

The previous results suggested that PP1 opposed cdc2 kinase during the onset of metaphase. We wondered

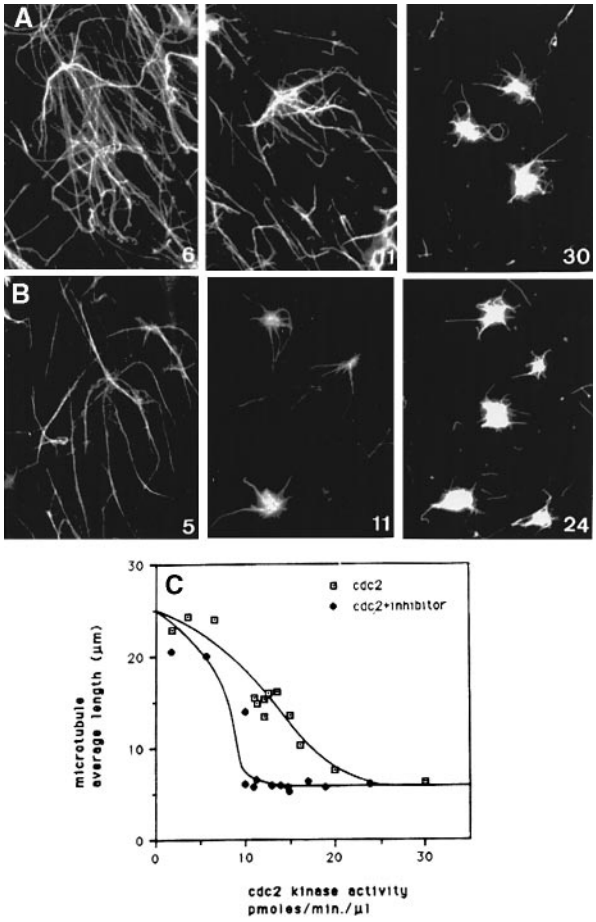

Fig. 6. PP1 opposes the effect of cdc2 kinase on microtubule length. Increasing amounts of purified starfish cdc2 kinase were added to interphase extract in the absence (**A**) or the presence (**B**) of I-2. Less cdc2 kinase is required to obtain mitotic microtubule length in the presence of I-2 (**C**). Numbers in the bottom right of the pictures indicate cdc2 kinase activity (pmol/μl/min).

whether PP1 was also required to increase the steady-state length of microtubules during the onset of anaphase. To measure these changes, we used a downstream effector of calcium in the cascade triggering cyclin degradation at anaphase onset: the calcium/calmodulin-dependent kinase II (CaMKII) (Lorca *et al.*, 1993; Morin *et al.*, 1994). We looked at cdc2 kinase activity after induction of anaphase in the presence and absence of I-2. In both cases, cdc2 kinase activity dropped to interphase levels by 30 min (Figure 7A) with identical kinetics, showing that, at least in *Xenopus* extracts, PP1 is not required for the inactivation of cdc2 kinase.

Microtubule dynamics were recorded at 15 min intervals after anaphase induction by CaMKII. In control extracts, chromosome to pole movement was completed 30 min after CAMKII addition (Figure 7B and see below). At this time, the catastrophe frequency decreased from 2 to 1 event/min, and the growth rate increased from 9.9 to 16.55 μm/min (Figure 7C and E). The shrinkage rate was not changed during this transition (Figure 7D). In contrast, in extracts treated with I-2, catastrophe frequency, growth and shrinkage rates were identical for the first 30 min after induction of anaphase (Figure 7C–E). We calculated the average length <L> of microtubules using the values measured and assuming a constant rescue rate of 1.05 events/min. In control extracts, the calculated microtubule length <L> was 8.7 μm in metaphase and >100 μm 30 min after anaphase induction. In the absence of PP1 activity, the calculated microtubule length <L> remained unchanged at ~5–8 μm (Figure 7F). Therefore, PP1 is required in anaphase to increase the steady-state length of microtubules by increasing the growth rate and decreasing the catastrophe rate.

PP1 is also required for spindle microtubule elongation and chromosome separation in anaphase

The previous results indicated that PP1 was required for microtubule elongation in anaphase in the absence of chromosomes. We wanted to examine what would happen to spindle microtubules. As shown in Figure 8A, under normal conditions, chromosomes start to separate and move towards the poles 10 min after calcium addition. By 25 min, anaphase is completed. When PP1 was inhibited, spindles gradually lost microtubules and the poles moved towards chromatin and eventually fell apart, forming half-spindles (Figure 8B). No clear chromosome segregation was observed. Similar observations were made using CaMKII to trigger anaphase. We quantified spindle length during anaphase with and without PP1. At the end of anaphase, spindles were 42 μm long in the control and 22 μm in the absence of PP1 activity (Figure 8C). We looked at chromosome separation under such conditions. As shown in Figure 8D, chromosomes moved towards poles in the control while they stayed in the middle of the spindle when I-2 was used. One hour after CaMKII addition, the microtubules had not returned to their interphase length and, in addition, chromatin was still condensed as previously reported (Axton *et al.*, 1990). These results show that PP1 is necessary both for spindle stability and elongation, and for chromatin decondensation during anaphase, and that this is not due to an effect of PP1 inhibition on the inactivation of cdc2 kinase.

Discussion

Since the initial demonstration that microtubule length is governed during metaphase by phosphorylation–dephosphorylation reactions under the control of cdc2 kinase (Verde *et al.*, 1990) and that this was mediated by an increase in catastrophe rate (Belmont *et al.*, 1990; Verde *et al.*, 1992), little progress has been made in the characterization of the biochemical pathways or signaling mechanisms involved. Here, we undertook a study of the role of type 1 and type 2A-like phosphatases in these processes during mitosis. We show that PP2A is required continuously in mitosis to control the short steady-state length of microtubules, primarily by regulating the phosphorylation state of the microtubule-destabilizing factor Op18/stathmin. In contrast, PP1 is required to stabilize microtubules during the transitions between interphase and mitosis. Thus these two phosphatases have distinct roles in the control of microtubule dynamics during mitotic progression.

We have characterized these activities using inhibitors of serine-threonine phosphatases. *In vitro*, the IC_{50} of okadaic acid is 0.2 nM for PP2A and 20 nM for PP1 (for review, see Cohen, 1989). These concentrations are much

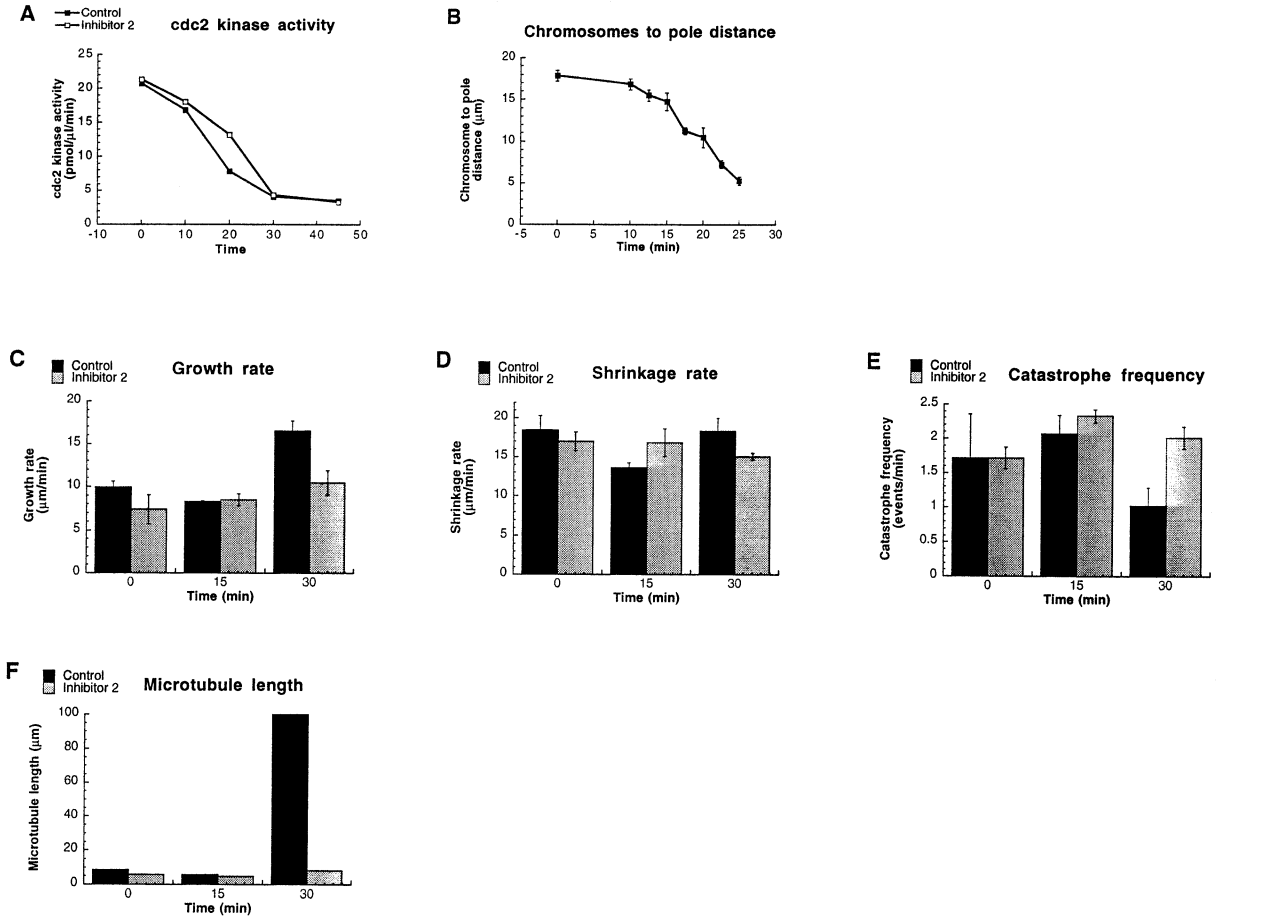

Fig. 7. PP1 is required for returning from metaphase to interphase microtubule dynamics. (**A**) Cdc2 kinase inactivation in anaphase induced by CaMKII is not affected by PP1 inhibition. (**B**) The distance from chromosomes to the pole in control extract decreases as chromosomes segregate. (C–E) PP1 prevents changes in microtubule dynamics in anaphase. In these experiments, CSF extracts were pre-incubated for 15 min with (gray bars) or without (black bars) 3 μM I-2 before anaphase was induced with CaMKII. Microtubule dynamics were recorded at 0, 15 and 30 min for 5–7 min. Growth rate (**C**), shrinkage rate (**D**) and catastrophe frequency (**E**) were determined after measurements of individual microtubules over time. Rescue frequency is omitted here because too few events were observed and values are not statistically significant. (**F**) Microtubule length <L> does not increase in the presence of I-2 during anaphase. Calculated microtubule lengths were determined using the measured values. Rescues were assumed to be 1.05 events/min. Data in (B–E) are means ± standard error of the mean.

lower than that used in our study, but it is known that the inhibitory activity of these inhibitors is altered by high protein concentration (Ingebritsen *et al.*, 1983) and our extracts have a protein concentration of ~60–80 mg/ml. We have measured phosphatase activities in concentrated extracts under the same conditions as used to assay microtubule dynamics and spindle assembly. We found that under these conditions, 0.4 μM okadaic acid is required to produce a 50% inhibition of PP2A-like activities, whereas 3 μM I-2 is required to inhibit PP1 fully. Low concentrations of okadaic acid may affect the activity of catalytic subunits other than the typical PP2A. Indeed, four phosphatases, PP3 (Honkanen *et al.*, 1991), PP4 (Brewis *et al.*, 1993) (previously named PPX), PP5 (Chen *et al.*, 1994) and PPV in *Drosophila* (Armstrong *et al.*, 1995), have been shown recently to be highly sensitive to okadaic acid and insensitive to I-2. Therefore, we cannot rule out the possibility that one of these is the inhibited phosphatase in our experiments. However, PP2A mutants in *Drosophila*, *S.pombe* and *S.cerevisiae* show abnormal microtubule morphology (Kinoshita *et al.*, 1993; Snaith *et al.*, 1996; Evans and Stark, 1997), with an extensive microtubule network similar to the one seen here. Mutants in PP2A regulatory subunits also display defects in microtubule organization (Gomes *et al.*, 1993; Kinoshita *et al.*, 1996). Furthermore, associated subunits of PP2A bind to microtubules and PP2A is associated with microtubules in tissue culture cells (Sontag *et al.*, 1995). This suggests that PP2A is indeed the phosphatase catalytic subunit involved in controlling microtubule length.

Previous results had also shown that inhibition of phosphatases using okadaic acid results in the formation of long microtubules. In starfish (Picard *et al.*, 1991), mouse oocytes (de Pennart *et al.*, 1993) and GH4 rat pituitary cells (Van Dolah and Ramsdell, 1992), spindles were shown to be destroyed and replaced by extremely long microtubule networks. This is consistent with our results but, in some of these previous experiments, it was not possible to know whether a type 1 or 2A phosphatase was inhibited. Interestingly, in clarified interphasic extracts from sea urchin, addition of high concentrations of okadaic acid (1–2.5 μM) resulted in a decrease in rescues and a slight increase in catastrophes (Gliksman *et al.*, 1992). However, these extracts had a lower protein concentration (20–25 mg/ml) and it is likely that both PP1 and PP2A were inhibited. The effects observed are probably the

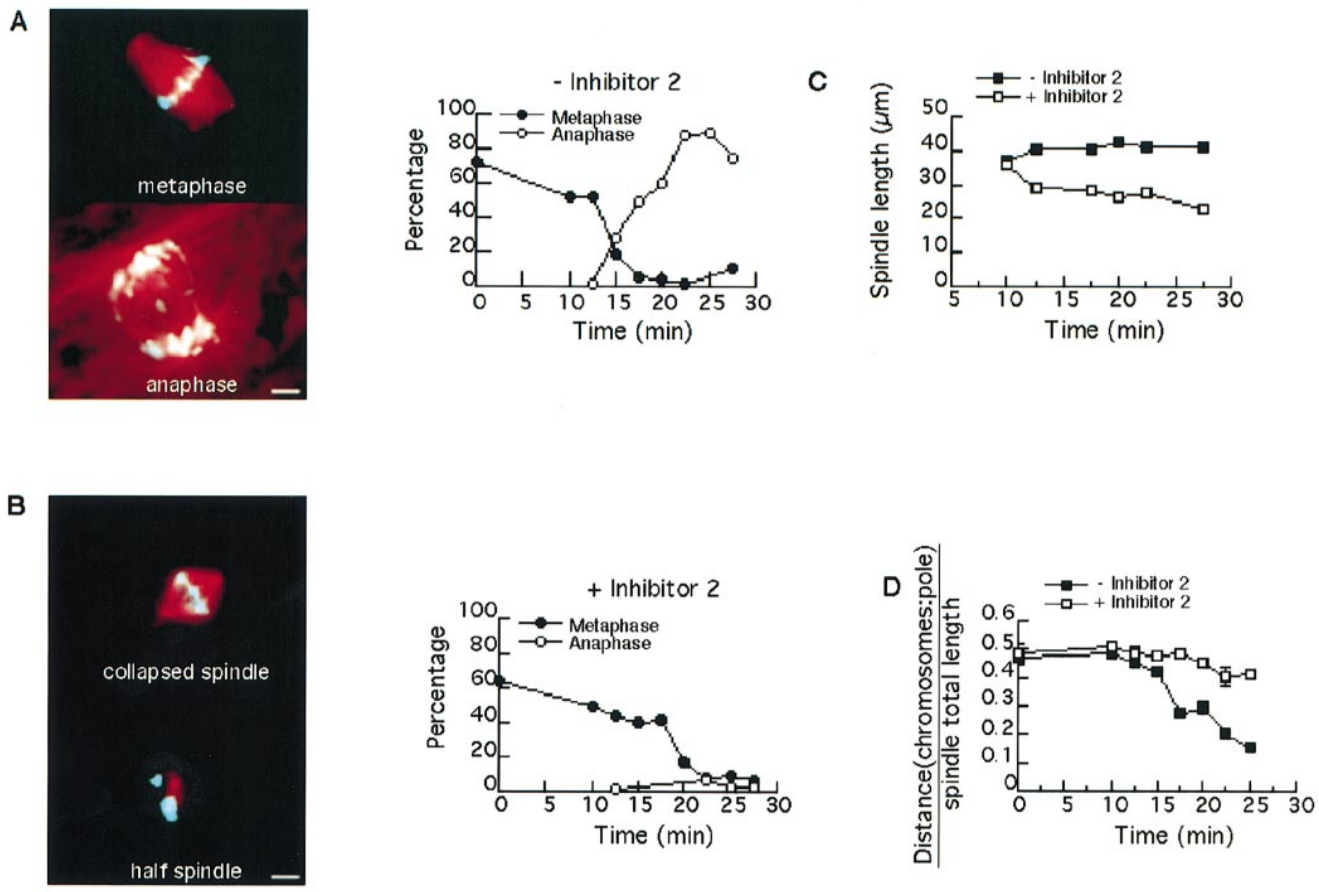

Fig. 8. PP1 is required for spindle stability and chromosome segregation in anaphase. Spindles were assembled and PP1 was inhibited for 15 min before anaphase was induced by CaMKII. Spindles were fixed at various time points. (**A**) Normal metaphase and anaphase spindles. (**B**) Collapsed and half-spindles observed in anaphase in the presence of I-2. Graphs show the percentage of spindles in metaphase (●) and anaphase (○) in the absence (A) or presence (B) of I-2 during anaphase. (**C**) Spindle length in the absence (■) or presence (□) of I-2. (**D**) Chromosome position in the spindle during anaphase in the absence (■) or presence (□) of I-2. Chromosome position is determined by the chromosome to pole distance divided by the pole to pole distance. Bar is 10 μm.

consequence of a complex regulation by many phosphatases which may act in many different ways. In clarified *Xenopus* interphase extracts, addition of high concentrations of okadaic acid results in a decrease in growth and shrinkage rates, while it has little effect on these rates in mitotic extracts (Parsons and Salmon, 1997). We also noticed that catastrophes were decreased in mitotic clarified extracts (R.Tournebize and R.Heald, unpublished observations). One possibility is that most of PP2A is depleted from clarified extracts (A.A.Hyman, unpublished observation), thus resulting in Op18/stathmin inactivation.

Because PP1 is the only phosphatase known to be inhibited efficiently by I-2, this small polypeptide can be used to determine what is regulated by PP1-like enzymes. Again, we determined that adding 3 μM I-2 followed by regular addition of 1 μM produced a complete inhibition of PP1 in our concentrated extracts. Our results show that PP1 has a particular role in the control of microtubule turnover in *Xenopus* extracts, during the switches between interphase and mitosis. In addition, PP1 is required for chromosome decondensation, confirming previous results in *Drosophila* (Axton *et al.*, 1990). However, PP1 does not seem to be required for cell cycle changes necessary for exit from mitosis in *Xenopus* egg extracts, while inhibition of PP1 in fungi causes a metaphase arrest or delay. Why should there be a difference between *Xenopus* and fungi? One major difference is the lack of checkpoint controls in *Xenopus* extracts under our conditions. Thus PP1 mutants may cause a metaphase delay in fungi by activating the spindle assembly checkpoint (for review, see Wells, 1996). Indeed, in *S.cerevisiae*, the PP1 encoded by the *GLC7* gene has been shown to regulate chromosome segregation (Francisco and Chan, 1994; Hisamoto *et al.*, 1994; Black *et al.*, 1995). Recent genetic data in *S.pombe* suggest that PP1 may regulate the APC and, therefore, exit from mitosis (Ishii *et al.*, 1996). However, since the APC regulates cyclin destruction and, therefore, cdc2 kinase inactivation, we would expect to see a mitotic delay in *Xenopus* if PP1 inhibition down-regulates the APC. As this is not observed in *Xenopus* extracts, it is possible that PP1 acts upstream of the APC through a spindle checkpoint rather than directly in cyclin degradation.

Regulation of microtubule dynamics and steady-state length during metaphase by PP2A and Op18/stathmin

Our results suggest that Op18/stathmin is a major downstream substrate of PP2A in the regulation of the steady-state length of microtubules. This protein regulates the length of microtubules both in *Xenopus* extracts (Belmont and Mitchison, 1996; this study) and in tissue culture cells

(Marklund *et al.*, 1996; Horwitz *et al.*, 1997), and this effect is regulated by phosphorylation. The phosphorylated form of Op18/stathmin loses its microtubule-destabilizing activity (Marklund *et al.*, 1996; Horwitz *et al.*, 1997). Our results in *Xenopus* confirm these observations. Moreover, they suggest that the phosphatase which dephosphorylates Op18/stathmin is PP2A. Although the kinase that opposes PP2A is unidentified, MAP kinase and the cdc2 kinase are likely candidates (Larsson *et al.*, 1995). This would make sense since PP2A opposes cdc2 kinase and some MAP kinase activities on many substrates like the brain microtubule-associated protein tau (Goedert *et al.*, 1992; Ferrigno *et al.*, 1993). Although it is formally possible that PP2A regulates Op18/stathmin by increasing MAP kinase activity (Shibuya *et al.*, 1992; Nebreda and Hunt, 1993), this is unlikely because, in extracts treated with cyclin Δ90, MAP kinase activity is already high (Shibuya *et al.*, 1992; Minshull *et al.*, 1994) although Op18/stathmin is poorly phosphorylated (Figure 3). The fact that Op18/stathmin is poorly phosphorylated in metaphase extracts means that it is active in these extracts. This finding is in apparent contradiction to results obtained *in vivo* where Op18/stathmin is phosphorylated during metaphase. This contradiction can be resolved by the observation that the phosphorylation of Op18 is stimulated by chromatin which is present at low levels in our extracts (Andersen *et al.*, 1997).

Catastrophes in mitosis are 8–10 times more frequent than in interphase. Following depletion of Op18/stathmin from a mitotic extract, the catastrophe rate drops to a rate only ~4–5 times more frequent than in interphase. Therefore, other microtubule-depolymerizing factors regulated independently of PP2A must account for the remaining catastrophes in mitosis. Such a factor can be XKCM1 (Walczak *et al.*, 1996) and other as yet unidentified catastrophe factors. In interphase, Op18/stathmin is also dephosphorylated (Brattsand *et al.*, 1994; Larsson *et al.*, 1995) and active, but catastrophes are low. This strongly suggests that although microtubules could be destabilized by the non-phosphorylated form of Op18/stathmin in interphase, they are not because of the presence of stabilizing factors like MAPs. We suggest that, during mitosis, the catastrophe rate decreases because Op18/stathmin remains active globally whereas MAPs do not stabilize microtubules efficiently.

We observed that PP2A inhibition resulted in an increase in microtubule spontaneous assembly in metaphase extracts. This could be due to regulation of the activity of microtubule nucleating sites. Alternatively, the high catastrophe rate observed in metaphase may normally prevent short microtubules from reaching a visible size. We were not able to determine whether the decrease in catastrophe rate produced by inhibition of PP2A may be sufficient to explain the increase in spontaneous assembly observed under these conditions.

In conclusion, PP2A controls the steady-state length of microtubules at least in part by controlling the activity of Op18/stathmin. Thus the level of stability of metaphase microtubules seems to depend upon a phosphorylation–dephosphorylation balance involving PP2A and cdc2 kinase acting directly or indirectly on Op18/stathmin. Subtle spatial regulation of this balance could participate in the regulation of microtubule dynamics and organization during spindle assembly and disassembly (Andersen *et al.*, 1997).

Regulation of microtubule dynamics by PP1 during the transitions from interphase to mitosis and back to interphase

We have shown that PP1 is required for reorganization of the microtubule network during the transitions between interphase and mitosis. It is hard to say whether PP1 is regulating microtubule-stabilizing or -destabilizing factors or both during entry into mitosis and during anaphase. One clue comes from the analysis of the dynamic parameters during exit from mitosis. We have shown that the growth rate increases during exit from mitosis, but that this increase in growth rate is blocked in the absence of PP1. Since MAPs are generally thought to stimulate microtubule growth, this strongly suggests that one of the roles of PP1 is to activate MAPs in order to stabilize microtubules during anaphase.

During metaphase, inhibiting PP1 has no effect on the stability of the mitotic spindle, whereas, during anaphase, it results in a highly unstable spindle. This instability manifests itself by a loss of microtubules from the spindles and a movement of the poles towards the chromatin. This is reminiscent of the effect of nocodazole, a drug which inhibits microtubule assembly (Ault *et al.*, 1991). It may seem strange that spindle microtubules only become unstable during anaphase following PP1 inhibition. However, two things must be considered. First, during metaphase, the chromosomes play an important role in stabilizing microtubules (Nicklas and Gordon, 1985; Zhang and Nicklas, 1995; Dogterom *et al.*, 1996; Heald *et al.*, 1996). This activity may be lost during anaphase and this may have to be substituted by a global PP1 activity acting to dephosphorylate factors required to stabilize microtubules again at the exit from metaphase. Secondly, there may be a large increase in length during anaphase B, which requires microtubule polymerization and stabilization. Thus, many mutants in PP1 probably manifest themselves in anaphase due to a lack of microtubule stabilization. In other words, PP1 may act to reverse the phosphorylation state of microtubule-stabilizing factors, like MAPs, that are inactivated by phosphorylation during metaphase.

Switches and steady-state balances

One of the main conclusions of our studies is that PP1 and PP2A have distinct functions in the control of microtubule dynamics. PP1 is required for the switches between mitosis and interphase while PP2A is required to maintain the short steady-state length of microtubules during mitosis. This illustrates two types of regulation involved in spindle assembly: global regulation during the transitions between cell cycle states and local regulation during spindle formation. Thus, PP2A seems to act as part of a phosphorylation balance that maintains the steady-state length of microtubules during metaphase. We imagine that the balance works in the following way. PP2A and its opposing kinase are both active with comparable activities. Subtle regulation of the activity of either of the enzymes would allow precise adjustment of the steady-state length of microtubules. Adjustment of such a balance would provide a means by which chromosomes locally

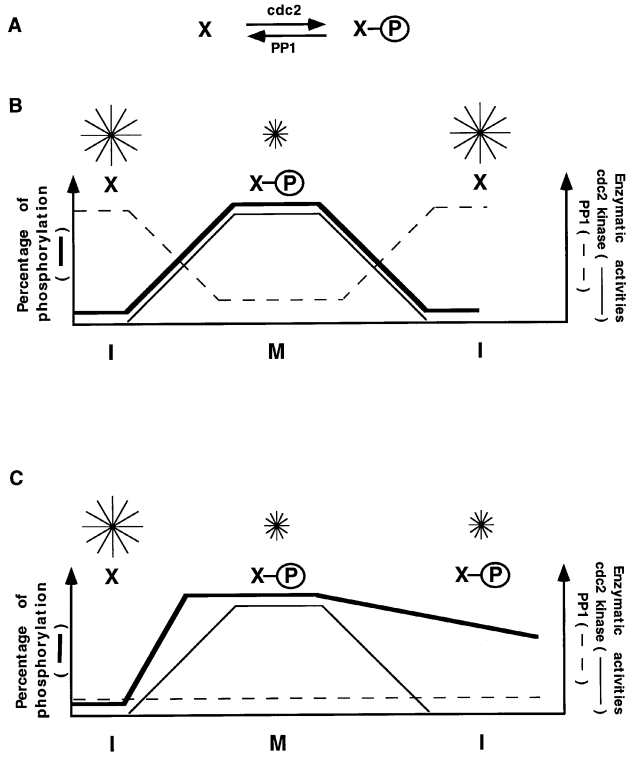

Fig. 9. Model for a switch regulation between cdc2 kinase and PP1. (**A**) A substrate X which stabilizes microtubules is regulated by cdc2 kinase and PP1. When phosphorylated (X-P), X is inactive, but it is active in its unphosphorylated form (X). (**B**) In a normal cell cycle, cdc2 kinase increases during mitosis (M) and decreases at anaphase. Concomitantly, PP1 activity decreases in mitosis and is high in interphase (I). In interphase, no cdc2 kinase is present and PP1 is active, therefore X is dephosphorylated, active and stabilizes microtubules (promoting a long steady-state length). In mitosis, less PP1 is active, cdc2 kinase activity is high and most of X is phosphorylated and inactive. Thus microtubules are short. (**C**) In the absence of PP1, at the interphase–metaphase transition, cdc2 kinase phosphorylates X more quickly, thus inactivating it, such that less cdc2 kinase activity is required to obtain a maximal effect. During the metaphase–anaphase transition, cdc2 kinase is inactivated. If PP1 is inactive, X stays in an inactive phosphorylated form for longer and microtubules stay shorter.

stabilize microtubules during spindle assembly (Karsenti, 1991; Hyman and Karsenti, 1996).

On the other hand, we believe that PP1 acts as a switch because the activities of PP1 and its opposing kinase, presumably cdc2, are different in interphase and mitosis. A model for the function of PP1 in the interphase–mitosis switch is shown in Figure 9. Figure 9A and B shows the normal situation. A substrate X is dephosphorylated and active and stabilizes microtubules in interphase. Conversely, it is phosphorylated and inactive in mitosis and does not stabilize microtubules so that they can undergo catastrophes and become shorter. At the entry of mitosis, cdc2 kinase activity increases and PP1 activity decreases, triggering phosphorylation and inactivation of X. Microtubules are no longer stabilized and become shorter. At the metaphase–anaphase transition, cdc2 activity declines and PP1 activity increases, dephosphorylating X and activating it. As a result, short microtubules in mitosis can be converted to long ones in interphase. Figure 9C shows what happens if PP1 activity is inhibited during the metaphase–anaphase transition. X will stay phosphorylated and inactive, with the consequence that microtubules will stay short during the progression into interphase. Evidence for such a model comes from our studies that show that PP1 opposes cdc2 at the onset of mitosis and in anaphase. It is well known that the activity of cdc2 fluctuates in the cell cycle, and it appears that the enzymatic activities of some PP1 catalytic subunits are inactivated by cdc2 phosphorylation during mitosis (Dohadwala *et al.*, 1994; Yamano *et al.*, 1994; Kwon *et al.*, 1997).

It is likely that PP1 and PP2A affect microtubule dynamics by acting on different substrates. Some may stabilize, others may destabilize microtubules. We have shown how one of these substrates, Op18/stathmin, is regulated by PP2A. Other microtubule regulators under the control of this enzyme and PP1 now need to be identified.

Materials and methods

Enzymatic assays

Unless specified, all reagents were from Sigma. Cdc2 kinase activity assays were performed as described by Felix *et al.* (1989). PP2A activities were measured using phosphorylated casein prepared as described by McGowan and Cohen (1988), and PP1 activities using phosphorylated phosphorylase (phosphatase assay kit, Gibco BRL). Phosphatase activities were measured in crude concentrated extracts similar to those used by Felix *et al.* (1989). We first characterized inhibitor concentrations necessary to inhibit phosphatases in crude 10 000 *g* extracts. Okadaic acid (Gibco BRL) was used to inhibit PP2A, and I-2 (a gift from Phil Cohen) used to inhibit PP1. Typically, after incubation of extracts with either okadaic acid or I-2 for 15 min at room temperature, 5 μl of substrate were added to 10 μl of extracts for 30–45 s. The reaction was stopped by adding 100 μl of 10 mM EDTA, EGTA, 100 mM NaF, β-glycerophosphate, 1 mg/ml bovine serum albumin (BSA), 0.1% β-mercaptoethanol (BME) and 100 μl of 10% trichloroacetic acid (TCA). Samples were incubated on ice for 10 min followed by centrifugation at 15 000 *g* for 10 min, and soluble ^{32}P released accounting for phosphatase activity were counted. Okadaic acid was titrated and found to inhibit 50% of PP2A at 0.4 μM. Using cyclin Δ90 (Glotzer *et al.*, 1991), cdc2 kinase activity was not modified by incubation with okadaic acid. It was found that 3 μM I-2 was sufficient to inhibit 50–60% of total phosphatase activity, showing that PP1 accounts for about half of the total serine-threonine phosphatase activity measurable with phosphorylated phosphorylase. Because I-2 can be inactivated by glycogen synthetase kinase phosphorylation, we added 1 μM I-2 every 15 min to the initial 3 μM used to inhibit PP1. Using such conditions, PP1 was constantly inhibited during 90 min.

Extract preparation and spindle assembly

Xenopus extracts were prepared as described in Murray (1991). Meiotic extracts (which we refer to as CSF extracts) and spindles were prepared using a two-step procedure (Shamu and Murray, 1992). Sperm nuclei were added to CSF extracts released into interphase by calcium. After DNA and centrosomes duplicated, some CSF extract was added back and spindles assembled for 60 min. When necessary, cyclin Δ90 was added 45 min after CSF extract addition for 45 min and then okadaic acid was added. To test the role of PP2A in controlling spindle assembly, sperm nuclei together with okadaic acid were added directly to extracts (Sawin and Mitchison, 1991) pre-incubated with cyclin Δ90. Okadaic acid was added at 0.4 μM final concentration and spindles fixed every 10 min. Maximum effect was observed 20 min after okadaic acid addition.

Anaphase was induced by adding 0.4 mM $CaCl_2$ (Shamu and Murray, 1992) or 0.1 vol. of a dominant active CaMKII (Morin *et al.*, 1994) subcloned in pCYCΔ13T (Glotzer *et al.*, 1991) and transcribed in reticulocyte lysates (TNT-coupled reticulocyte lysate systems, Promega). During anaphase, I-2 was first added to 3 μM to CSF extracts for 15 min. Then 1 μM I-2 and $CaCl_2$/CaMKII were added to induce anaphase, and spindles were observed during 30–45 min. After anaphase induction,1 μM I-2 was added every 15 min.

Purified human Op18/stathmin, either wild-type or with all four phosphorylatable serines mutated to alanine [kind gift of Andre Sobel,

10 mg/ml in phosphate-buffered saline (PBS)], was added to pre-assembled spindles at the same time as 0.4 μM okadaic acid. Microtubule growth was dependent on the concentration of mutated Op18/stathmin added. Images of spindles were taken on a Zeiss Axioskop microscope with either a Sony SSC-M370CE black and white camera or a color Coolview camera (Photonic Science). Images were then processed further with Adobe Photoshop.

Immunodepletions

Peptide antibodies to Op18 were prepared according to Belmont and Mitchison (1996). Immunodepletions were done as previously described (Walczak *et al.*, 1996). Typically, 400 μg of antibody were bound to 60 μl of Affiprep protein A beads (Bio Rad) for 1 h at 4°C . After washing, 200 μl of cyclin Δ90 extracts were added to packed beads. Extracts were depleted for 1 h at 4°C on a rotating wheel. Depleted extracts were then used for microtubule dynamics. Ninety-five percent depletion was usually achieved by this procedure, as determined by Western blotting. Mock-treated extracts were depleted using the same amount of pure rabbit IgG (Dianova). Treatment of the extract with the beads affected microtubule polymerization, explaining a decrease in catastrophe frequency between untreated extracts and mock-depleted extracts.

Op18 phosphorylation

Op18 was phosphorylated in a 55 μl extract containing cyclin Δ90, 1 μCi [γ-^{32}P]ATP/μl extract and different concentrations of okadaic acid or I-2. After 15 min, phosphorylation was stopped by adding 1 vol. of 2× stabilization buffer (SB: 50 mM NaF, 40 mM β-glycerophosphate, 10 mM EDTA, 10 mM sodium pyrophosphate, pH 7.2), plus 1 μM microcystin, 10 μg/ml pepstatin, leupeptin, chymostatin containing 7 μl of Affiprep protein A beads coated with anti-Op18/stathmin antibodies. Op18/stathmin was depleted for 30 min at 4°C. Beads were washed once with 2× SB, then twice with PBS, 100 mM NaCl, 0.1% Triton X-100, and resuspended in 40 μl of sample buffer. Twenty-five microlitres were loaded onto a 15% gel and analyzed by silver staining and PhosphorImager (Molecular Dynamics).

Microtubule dynamics

Microtubule dynamics were measured in 10 000 *g* extracts. Typically, 10 μl of extract were complemented with 1 μl of purified human centrosomes (2×10^8 centrosomes/ml), 0.42 μl of rhodamine–tubulin (Hyman *et al.*, 1991) [10 mg/ml, diluted to 2.8 mg/ml in BRB80 (80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.8) and spun for 5 min in an Airfuge at 30 p.s.i. at 4°C to remove aggregates], 0.5 μl of saturated hemoglobin (bovine, Sigma) (dissolved in water, spun in a microfuge for 3 min to pellet undissolved hemoglobin), 0.33 μl of antifade [5 μl of catalase (10 mg/ml in BRB80–50% glycerol); 5 μl of glucose oxidase (10 mg/ml in BRB80–50% glycerol); and 5 μl of glucose (1 M made in water)]. All mixing was done on ice. Then 2.2 μl of this extract mix were spotted onto a slide and gently covered by a 22×22 mm coverslip (Fisher Scientific). All glass used was washed with ethanol and water. Images were recorded using a Zeiss Axioskop, a 100× Apochromat lens NA1.4 and a long pass rhodamine filter (Chromatech). Images were taken using an 8-bit black and white camera (Sony SSC-M370CE), processed using an Argus 10 image processor (Hamamatsu) and stored on a Macintosh using the public domain NIH Image program (developed at the US National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). Recordings were done for no more than 5 min. Images were recorded every 2 s. Special care was taken to avoid photobleaching and photodamage by reducing the illumination time using a computer-controlled shutter (Uniblitz, Vincent Associates).

For experiments using okadaic acid, extracts were frozen 20 min after addition of 0.4 μM okadaic acid and used for microtubule dynamics. Extracts depleted of Op18/stathmin with or without okadaic acid addition were treated similarly. For experiments investigating the role of PP1 in anaphase, 3 μM I-2 was added to extracts at room temperature for 15 min. Extracts were complemented with 0.1 vol. of CaMKII, an additional 1 μM I-2, rhodamine–tubulin, hemoglobin and antifade, and kept at room temperature. Then 1 μM I-2 and centrosomes were added every 15 min after triggering anaphase. Recordings were done for 5 min at 0, 15, 30 and 45 min after CaMKII addition.

Data analysis

Analysis of microtubule dynamics was done using a home-made Microsoft Excel macro. Differences between experiments were evaluated using Student's *t*-test; *t*-tests were performed within single experiments to assess significant variations. Different experiments were then averaged, and a *t*-test (assuming the variance unknown) and paired *t*-test were performed. Values from experiments where n was <5 were discarded. Differences reported were significant at least at the 5% level of confidence, except for the catastrophe frequency after I-2 addition in anaphase and okadaic acid addition to cyclin Δ90 extracts which are significant at the 7% level. This reflects variations between different experiments. Only few rescues could be observed such that proper statistics could be done.

Microtubule steady-state length was calculated using the following equation described in Verde *et al.* (1992):

$$\langle L \rangle = \frac{V_g F_{res} - V_s F_{cat}}{F_{cat} + F_{res}}$$

where V_g, V_s, F_{cat} and F_{res} are growth rate, shrinkage rate, catastrophe frequency and rescue frequency respectively. As a rescue frequency, we used the averaged value (1.05 events/min) of the rescue frequencies from the okadaic acid addition experiment. If rescues had increased in any of the experiments, we should have noticed. In the absence of any increase, we assumed that the rescue frequency was not changed and used a value of 1.05 events/min in all microtubule length calculations.

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