Role for protein phosphatases in the cell-cycle-regulated phosphorylation of stathmin

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Stathmin is a major cytosolic phosphoprotein that regulates microtubule dynamics during the assembly of the mitotic spindle. The activity of stathmin itself is regulated by changes in its state of phosphorylation during the transition from interphase to metaphase. For a better understanding of the regulation of stathmin activity during the cell cycle, we explored the mechanism(s) responsible for the decrease in the level of phosphorylation of stathmin as cells complete mitosis and enter a new G₁ phase. We show that stathmin mRNA and protein are expressed constitutively throughout the different phases of the cell cycle. This suggests that the non-phosphorylated stathmin that predominates during G₁ is not generated by degradation of phosphorylated stathmin in mitosis and synthesis of new

unphosphorylated stathmin as cells enter a new G₁ phase. This suggested that protein phosphatases might be responsible for dephosphorylating stathmin as cells enter a new cell cycle. Okadaic acid-mediated inhibition of protein phosphatases *in vivo* showed a major increase in the level of phosphorylation of stathmin. Dephosphorylation studies *in vitro* showed differential patterns of site-specific dephosphorylation of stathmin to protein phosphatase type 1, protein phosphatase type 2A and protein phosphatase type 2B. Thus stathmin might be a target for okadaic acid-sensitive protein phosphatase(s), and its activity in eukaryotic cells might be modulated by the sequential activity of specific protein kinases and phosphatases.

INTRODUCTION

Stathmin (also known as p17, p18, oncoprotein p18, p19, 19K, prosolin, metastasin and Lap 18) was identified on the basis of its increased phosphorylation in response to diverse extracellular signals or on the basis of its high level of expression in leukaemic cells [1,2]. Stathmin is highly expressed in a wide variety of cancers and its high level of expression seems to be necessary for the maintenance of the transformed phenotype [3–5]. The levels of expression and phosphorylation of stathmin are regulated in response to a variety of signals that affect the state of proliferation and/or differentiation of different cell types [1,6–8].

Stathmin seems to be involved in a number of different signalling pathways including the p34^{cdc2} kinase, mitogen-activated protein (MAP) kinase, cAMP-dependent protein kinase (PKA) and the Ca²⁺/calmodulin-dependent kinase IV/Gr signalling pathways [9–13]. There are numerous phosphoforms of stathmin that result from the phosphorylation of four different serine residues. Cdc2 kinase was shown to phosphorylate Ser-38 stoichiometrically and Ser-25 at a much lower stoichiometry [9,10,13]. In contrast, MAP kinase phosphorylates Ser-25 efficiently and Ser-38 at a much lower efficiency [11]. Ser-16 can be phosphorylated by Ca²⁺/calmodulin-dependent kinase IV/Gr and PKA, whereas Ser-63 can be phosphorylated only by PKA [10,12]. Phosphorylation at these four serine residues was shown to account for all 12 phosphoforms identified *in vivo* [10].

Stathmin was recently shown to increase the rate of transition of tubulin polymers from growth to shrinkage known as ‘Catastrophe’ [14]. Changes in the level of expression and the state of phosphorylation of stathmin are important in the regulation of the dynamics of microtubule polymerization,

especially during cell cycle progression [15]. Induced expression of wild-type stathmin results in depolymerization of microtubules in interphase [15]. The same study also showed that induced expression of Cdc2 kinase target-site-deficient mutants of stathmin results in the depolymerization of microtubules in interphase and failure to form the mitotic spindle [15]. These studies confirmed that stathmin promotes depolymerization of microtubules in interphase and phosphorylation at Cdc2 target sites early in mitosis eliminates the ability of stathmin to depolymerize microtubules, allowing the spindle to form [15]. Thus to be able to understand the role of stathmin in the cell cycle more thoroughly, it is important to determine how cells eliminate the phosphorylated form of stathmin that accumulates early in mitosis and regenerate the unphosphorylated form that predominates during the G₁ phase.

In the present study, we explored the mechanism(s) that regulate the level of expression and the state of phosphorylation of stathmin during the cell cycle. The roles of the different kinases in the phosphorylation of stathmin as cells progress through the division cycle had been thoroughly investigated in previous studies [9,15,16]. In contrast, the mechanisms responsible for the generation of the unphosphorylated stathmin in the post-mitotic stage have not received similar attention. We examined whether cells degrade the phosphorylated stathmin at the end of mitosis and resynthesize the unphosphorylated form before they enter the next G₁ phase. We also investigated the potential involvement of protein phosphatases in regulating the state of phosphorylation of stathmin during cell cycle progression. Our results demonstrate that the cell-cycle-dependent changes in the level of phosphorylation of stathmin are mediated by the relevant activities of protein kinases and protein

Abbreviations used: DTT, dithiothreitol; GST, glutathione S-transferase; MAP, mitogen-activated protein; PKA, cAMP-dependent protein kinase; PP1, protein phosphatase type 1; PP2A, protein phosphatase type 2A; PP2B, protein phosphatase type 2B.

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phosphatases without major changes in its rate of synthesis and degradation in the different phases of the cell cycle.

MATERIALS AND METHODS

Production of a polyclonal anti-stathmin antibody

Recombinant stathmin protein was generated with a glutathione S-transferase (GST)-based prokaryotic expression system [9]. Purified recombinant stathmin protein was coupled to keyhole limpet haemocyanin [17]. A rabbit was injected intradermally with 5 μ g of the conjugated protein and was boosted once every 2 weeks over a period of 3 months. At 2 weeks after the sixth booster injection, the rabbit was bled and serum was collected. IgG was purified from the immune serum by adsorption on protein A with a modification of the procedure described previously [18]. The antiserum was diluted 1:1 with PBS, and passed twice over a protein A-agarose column. After washing with PBS, IgG was eluted in 1 ml fractions with 0.1 M citrate buffer, pH 3.5, and neutralized with 2.0 M Tris/HCl, pH 10.5. The fractions containing IgG were identified by measuring the absorbance at 280 nm. The IgG-containing fractions were pooled and dialysed in PBS overnight at 4 °C. The specificity of the antibody was confirmed by Western blotting.

Analysis of stathmin protein synthesis during the cell cycle

Fractions of exponentially growing K562 cells were enriched with cells in different phases of the cell cycle by cell sorting on the basis of DNA content with Hoechst 33342 fluorescent dye as described [9]. Hoechst 33342 is a non-toxic vital stain specific for DNA and its fluorescence under UV is proportional to the DNA content of the cell [19]. K562 cells were incubated with this dye at 37 °C for 30 min followed by two washes in PBS with 0.05 % NaN₃ at 4 °C. The cells were sorted at 355 nm with a low-intensity UV laser (40 mW) in a Becton Dickinson FACStar flow cytometer.

The rate of synthesis of stathmin in the different phases of the cell cycle was evaluated by pulse labelling the G₀-G₁, S and G₂-M fractions followed by immunoprecipitation. Each fraction was incubated in 0.5 ml of methionine-free RPMI medium supplemented with 2 % (v/v) dialysed fetal calf serum and 300 μ Ci of [³⁵S]methionine for 30 min at 37 °C. Cells were washed several times with Tris/saline (140 mM NaCl/25 mM Tris base/7 mM Na₂HPO₄/5 mM KCl/5 mM dextrose/0.02 % Phenol Red) and lysed in NP40 lysis buffer containing 150 mM NaCl, 50 mM Tris/HCl, pH 8.0, 1 % (v/v) NP40, 2 μ g/ml leupeptin, 5 μ g/ml aprotinin, 1 μ g/ml pepstatin A, 5 μ g/ml PMSF, 1 mM benzamidine and 1 mM EDTA. The lysates were incubated on ice for 30 min and then passed several times through a 21-gauge needle. Lysates were centrifuged at 10 000 g and the incorporated counts were measured by precipitation with trichloroacetic acid [18]. The volumes of the lysates from the G₀-G₁, S and G₂-M phases that were used in the immunoprecipitation reaction were normalized on the basis of trichloroacetic acid-precipitable counts. Stathmin was immunoprecipitated by incubation with anti-stathmin antibody at 4 °C overnight. As a control, one of the lysates (G₀-G₁) was incubated with preimmune serum at 4 °C overnight. Immune complexes were adsorbed on protein A-agarose beads at 4 °C for 1.5 h. The agarose beads were spun gently and washed five times with lysis buffer. The beads were then resuspended in 2 $\times$ SDS sample buffer and boiled for 5 min; the denatured proteins were resolved by SDS/PAGE [12 % (w/v) gel]. The gel was fixed, dried and exposed to film for 3 days at -80 °C.

Analysis of stathmin mRNA accumulation during the cell cycle

Fractions of K562 cells enriched in G₀-G₁, S and G₂-M phases were washed with PBS and pelleted. Total RNA was isolated from each fraction by the guanidinium thiocyanate/phenol/chloroform extraction procedure [17]. Total cellular RNA (10 μ g) isolated from cells in G₀-G₁, S and G₂-M phases were analysed by Northern blotting [17]. As a control, the same filter was probed with β -actin, whose synthesis does not fluctuate during the cell cycle [20].

Effect of okadaic acid on the phosphorylation of stathmin *in vivo*

We examined the effects of inhibition of protein phosphatases on the phosphorylation of stathmin *in vivo* as described previously [21]. Approx. 3×10^6 K562 cells were washed and resuspended in 2 ml of phosphate-free RPMI media. The cell suspension was divided into three aliquots. Each aliquot was labelled metabolically *in vivo* with 500 μ Ci of [³²P]P_i for 1 h at 37 °C. The cells were incubated for an additional 15 min in different concentration of okadaic acid [0 nM (control), 200 nM and 1000 nM] [21]. The labelled cells were then harvested and washed thoroughly to remove unincorporated [³²P]P_i. The cell pellets were lysed in NP40 lysis buffer containing 150 mM NaCl, 50 mM Tris/HCl, pH 8.0, 1 % (v/v) NP40, 2 μ g/ml leupeptin, 5 μ g/ml aprotinin, 1 μ g/ml pepstatin A, 5 μ g/ml PMSF, 1 mM benzamidine and 1 mM EDTA supplemented with 20 mM β -glycerophosphate and 20 mM NaF to inhibit protein serine/threonine phosphatases [21]. Stathmin was immunoprecipitated and analysed by SDS/PAGE as described above.

Dephosphorylation of stathmin *in vitro* by protein phosphatase type 1 (PP1), protein phosphatase type 2A (PP2A) and protein phosphatase type 2B (PP2B)

We examined the activity *in vitro* of purified protein phosphatases on phosphorylated stathmin by using ³²P-labelled GST-stathmin fusion protein as a substrate [9]. The GST-stathmin recombinant was phosphorylated *in vitro* by either p34^{cdc2} kinase (New England Biolabs), MAP kinase (Upstate Biotechnology) or PKA (Sigma). Phosphorylation of recombinant GST-stathmin (100 μ g) with p34^{cdc2} kinase (100 units) and PKA (200 units) was performed in 50 mM Tris/HCl (pH 7.5)/10 mM MgCl₂/2 mM dithiothreitol (DTT)/1 mM EGTA/0.01 % (v/v) Brij 35 supplemented with 200 μ M ATP and [γ -³²P]ATP (specific radioactivity of 5000 d.p.m./pmol) at 30 °C for 1 h. Phosphorylation of GST-stathmin (100 μ g) by MAP kinase (4 μ g) was performed in a buffer containing 20 mM Mops, pH 7.2, 25 mM β -glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate and 1 mM DTT supplemented with 200 μ M ATP and [γ -³²P]ATP (specific radioactivity 5000 d.p.m./pmol) at 30 °C for 1 h. After 1 h, the incorporation of phosphate was confirmed by phosphocellulose assay ([22], p. 124). The reactions were stopped by heat-inactivating the kinase at 95 °C for 10 min followed by the addition of 2.5 vol. of 100 % -satd. (NH₄)₂SO₄ and leaving on ice for 30 min. The suspension was centrifuged for 10 min at 10 000 g. The supernatant was discarded and the radiolabelled precipitate was washed three or four times with 60 % -satd. (NH₄)₂SO₄ to remove unincorporated [γ -³²P]ATP. The precipitate was dissolved in a buffer containing 10 mM Tris/HCl, pH 7.4, 10 mM 2-mercaptoethanol and dialysed against the same buffer at 4 °C overnight. The ³²P-labelled GST-stathmin was used in subsequent phosphatase assays *in vitro*.

³²P-labelled GST-stathmin was dephosphorylated *in vitro* by

either PP1 (New England Biolabs), PP2A or PP2B. PP2A and PP2B phosphatases were prepared as described [23,24]. The specific activity of each phosphatase was determined with *p*-nitrophenyl phosphate as a substrate [25]: 1 μ g of the radio-labelled GST-stathmin was exposed to either PP1, PP2A or PP2B at two different concentrations (0.4 or 4 units). One unit of phosphatase activity is defined as the amount of enzyme required to hydrolyse 1 nmol of *p*-nitrophenyl phosphate in 1 min at 30 °C. PP1 and PP2A dephosphorylation assays were performed at 30 °C by incubating 32 P labelled GST-stathmin in 50 mM Tris/HCl (pH 7.0)/0.1 mM Na₂EDTA/5 mM DTT/0.01 % (v/v) Brij 35 supplemented with 1 mM MnCl₂ and 200 μ g/ml BSA without phosphatase (control) or with protein phosphatase (PP1 or PP2A) in the presence or absence of 50 nM okadaic acid, an inhibitor of PP1/PP2A [21]. PP2B dephosphorylation assays were performed at 30 °C by incubating 32 P-labelled GST-stathmin in 50 mM Tris/HCl (pH 7.0)/0.5 mM DTT/0.9 mM EGTA/1 mM MnCl₂ supplemented with 0.9 mM CaCl₂, 10 μ g/ml calmodulin and 200 μ g/ml BSA without PP2B (control) or with PP2B in the presence or absence of 150 μ M trifluoperazine, a more potent inhibitor of PP2B than okadaic acid [26]. After 1 h the phosphatases were heat-inactivated at 95 °C for 10 min. The reaction mixtures were then supplemented with 2 units of thrombin (Sigma) in thrombin cleavage buffer [2.5 mM CaCl₂/150 mM NaCl/50 mM Tris/HCl (pH 7.5)] and incubated for a further 1 h at 30 °C to cleave the GST-stathmin fusion protein. The thrombin reactions were terminated and the proteins were precipitated by adding trichloroacetic acid to a final concentration of 20 % (w/v) and incubation on ice for 20 min. The reaction mixtures were centrifuged for 10 min and the extent of dephosphorylation was determined by measuring the release of 32 P into the supernatant by using a scintillation counter. The protein precipitate was dissolved in 2 $\times$ SDS sample buffer and analysed by SDS/PAGE. The gel was dried and autoradiographed; the intensities of the individual bands were quantified on a phosphorimager. The percentage dephosphorylation was determined by subtracting the relative intensities of the fragments of phosphatase-treated stathmin from those of phosphorylated stathmin in the absence of phosphatase.

RESULTS

In the first experiment we analysed the rate of synthesis of stathmin in the different phases of the cell cycle. K562 erythroleukaemia cells were fractionated in different phases (G₀-G₁, S, G₂-M) by DNA fluorescence on a cell sorter. The purity of the enriched K562 fractions used in this experiment was 83.4 % for the G₀-G₁ fraction, 67.7 % for the S fraction and 78.7 % for the G₂-M fraction (Figures 1A and 1B). Each cell fraction was labelled metabolically with [35 S]methionine, and radiolabelled stathmin was immunoprecipitated with an anti-stathmin antibody. Densitometric scanning of the autoradiograph showed no significant differences in the rate of synthesis of stathmin in the different phases of the cell cycle (Figure 1C). We also examined the steady-state levels of stathmin mRNA in K562 cells during the different phases of the cell cycle. Exponentially growing K562 cells were divided into three fractions as above. The enriched fraction corresponding to the G₀-G₁, S and G₂-M phases exhibited 81.8 %, 67.3 % and 66.6 % purity respectively (Figures 2A and 2B). Total RNA was isolated from each fraction and the levels of stathmin and β -actin mRNA were assessed by a Northern blotting assay (Figure 2C). Densitometric scanning of the stathmin signals after correction for β -actin showed virtually no difference in the level of stathmin mRNA accumulation in cells in the different phases of the cell cycle.

Figure 1 Cell cycle analysis of stathmin protein synthesis in K562 cells

(A) Flow cytometric analysis showing the distribution of unfractionated K562 cells in the G₀-G₁, S and G₂-M phases of the cell cycle. (B) A similar flow cytometric analysis performed on the fractionated cells, aligned to show the purities of the different fractions. (C) An autoradiograph of immunoprecipitated radiolabelled stathmin from the different K562 fractions. Lane 1, labelled lysate; lane 2 immunoprecipitation of labelled G₀-G₁ lysate with preimmune serum; lanes 3, 4 and 5, immunoprecipitates from labelled lysates prepared from the G₀-G₁, S and G₂-M fractions respectively with anti-stathmin antibody. The positions of protein molecular mass standards (in kDa) are indicated at the left.

We then investigated the potential involvement of protein phosphatases in regulating the level of stathmin phosphorylation *in vivo*. We examined the effects of inhibiting cellular phosphatases by okadaic acid on the state of phosphorylation of stathmin in proliferating K562 cells. A progressive increase in the phosphorylation of stathmin was observed in the presence of increasing concentrations of okadaic acid (Figure 3, lanes 4-6). This inhibition of protein phosphatases by okadaic acid in intact cells resulted in a 33 % increase in the phosphorylation of stathmin at 200 nM and a 95 % increase at 1000 nM okadaic acid. This suggests that stathmin might be dephosphorylated *in vivo* by an okadaic acid-sensitive protein phosphatase.

We examined the ability of different okadaic acid-sensitive phosphatases to dephosphorylate the four different phosphoserine residues of stathmin (residues 16, 25, 38 and 63) by dephosphorylation assays *in vitro*. We analysed the effect of PP1, PP2A and PP2B on recombinant GST-stathmin phosphorylated *in vitro* at specific serine residues. p34^{cdc2} kinase was used to phosphorylate stathmin on Ser-38 (major) and Ser-25 (minor) [9,13], MAP kinase was used to phosphorylate stathmin on Ser-25 (major) and Ser-38 (minor) [11], and PKA was used to phosphorylate stathmin on Ser-63 (major) and Ser-16 (minor)

Figure 2 Cell cycle analysis of stathmin mRNA in K562 cells

(A) Flow cytometric analysis showing the distribution of unfractionated K562 cells in the G₀-G₁, S and G₂-M phases of the cell cycle. (B) A similar flow cytometric analysis performed on the fractionated cells, aligned to show the purities of the different fractions. (C) An autoradiograph of a Northern blot of total RNA isolated from the different K562 fractions shown above. Lanes 1, 2 and 3, the G₀-G₁, S and G₂-M fractions respectively. The same filter was sequentially hybridized to stathmin and β -actin.

[10]. The extent of dephosphorylation of stathmin was assessed by measuring the release of ^{32}P from the phosphorylated GST-stathmin after precipitation with trichloroacetic acid [18]. Figure 4 shows that stathmin can be partly dephosphorylated *in vitro* by PP1, PP2A and PP2B. However, there were significant differences in the efficiency of dephosphorylation of stathmin by the different phosphatases.

We also analysed the potential for site-specific dephosphorylation of stathmin by PP1, PP2A and PP2B (Figure 5). We took advantage of the presence of a single thrombin cleavage site between Ser-25 and Lys-29 in the stathmin amino acid sequence [27]. The digestion of GST-stathmin with thrombin releases the GST part of the fusion molecule and in addition it yields two fragments of stathmin that can be easily separated by SDS/PAGE: a 15 kDa fragment bearing Ser-38 and Ser-63, and a 3 kDa fragment bearing Ser-16 and Ser-25. This permits a detailed analysis of dephosphorylation of stathmin on different sites after cleavage with thrombin. Figure 5 is a representative SDS/PAGE gel showing the dephosphorylation of MAP kinase-phosphorylated GST-stathmin. MAP kinase, which preferentially phosphorylates Ser-25, was found to be easily dephosphorylated by PP1 (lanes 2 and 3), PP2A (lanes 6 and 7) and PP2B (lanes 10 and 11). The loss of ^{32}P signal in the presence of phosphatases (lanes 2, 3, 6, 7, 10 and 11) is a result of dephosphorylation rather than protein degradation because the same reactions did not show a loss of ^{32}P in the presence of

Figure 3 Effects of okadaic acid on the phosphorylation of stathmin *in vivo*

K562 cells were labelled with [^{32}P]P_i and incubated for 15 min in different concentrations of okadaic acid as indicated. Lanes 1, 2 and 3, labelled proteins extracted from K562 cells incubated with 0 nM (control), 200 nM and 1000 nM okadaic acid respectively; lanes 4, 5 and 6, immunoprecipitated radiolabelled stathmin from K562 cells incubated with 0 nM (control), 200 nM and 1000 nM okadaic acid respectively; lane 7, immunoprecipitation of labelled K562 cells with preimmune serum. The positions of protein molecular mass standards (in kDa) are indicated at the left of the autoradiograph. The bar graph is a quantification of the densitometric data.

phosphatase inhibitors (lanes 4, 8 and 12). Quantitative analysis of the data on a phosphorimager showed that the relative sensitivity of Ser-25 to phosphatases was in the following order:

Figure 4 Dephosphorylation of stathmin *in vitro* by PP1, PP2A and PP2B

The bar graph illustrates the effects of PP1, PP2A and PP2B on recombinant GST-stathmin phosphorylated by p34^{cdc2} kinase, MAP kinase or PKA. The extent of dephosphorylation of stathmin is expressed as the percentage of ^{32}P released after exposure to the different phosphatases. Hatched and filled bars show the ^{32}P released after exposure to 0.4 and 4.0 units of phosphatase respectively; open bars show the ^{32}P released after exposure to 0.4 unit of phosphatases in the presence of okadaic acid (for PP1 and PP2A) or trifluoperazine (for PP2B).

Figure 5 Site-specific dephosphorylation of MAP kinase-labelled stathmin by PP1, PP2A and PP2B

An autoradiograph of an SDS/PAGE gel that shows the products of thrombin cleavage of MAP kinase-labelled stathmin dephosphorylated by PP1, PP2A or PP2B. Lanes 1, 5 and 9, GST-stathmin in the absence of phosphatase; lanes 2 and 3, phosphorylated GST-stathmin exposed to 0.4 and 4.0 units of PP1 respectively; lane 4, GST-stathmin exposed to 0.4 unit of PP1 in the presence of okadaic acid; lanes 6 and 7, GST-stathmin exposed to 0.4 and 4.0 units of PP2A respectively; lane 8, phosphorylated GST-stathmin exposed to 0.4 unit of PP2A in the presence of okadaic acid; lanes 10 and 11, GST-stathmin exposed to 0.4 and 4.0 units of PP2B respectively; lane 12, phosphorylated GST-stathmin exposed to 0.4 unit of PP2B in the presence of trifluoperazine.

PP1 > PP2B > PP2A (Figure 6A). When GST-stathmin was phosphorylated with p34^{cdc2} kinase, the dephosphorylation of Ser-38 by all three phosphatases was less efficient (Figure 6D). More pronounced dephosphorylation of Ser-38 was observed at a higher concentration of phosphatases in the following order: PP2B > PP1 > PP2A (Figure 6D). In contrast, Ser-25 was more easily dephosphorylated by all phosphatases in the following order: PP1 > PP2B > PP2A (Figure 6C). When GST-stathmin was phosphorylated by PKA, PP1 was the most efficient in dephosphorylating Ser-16 and Ser-63, followed by PP2B and PP2A (Figures 6E and 6F). These observations demonstrate that distinct stathmin phosphoserine residues can be dephosphorylated at different efficiencies *in vitro* by the different okadaic acid-sensitive protein phosphatases.

DISCUSSION

A direct role for stathmin in the regulation of mitosis has previously been demonstrated by genetic studies in which both the inhibition of stathmin expression and the over-expression of the wild-type protein resulted in a mitotic block [9,15]. The more drastic mitotic block that resulted from the over-expression of mutants that cannot be phosphorylated suggested an important role for phosphorylation in regulating the mitotic activity of stathmin [15,16]. Although the protein kinases involved in the phosphorylation of stathmin during the different phases of the cell cycle have previously been the focus of numerous studies [9–13], the mechanism(s) of cell cycle regulated dephosphorylation of stathmin have not been investigated. There is considerable evidence in support of the hypothesis that the phosphorylation of stathmin in mitosis eliminates its microtubule-depolymerizing activity [15,16]. When stathmin is inactivated by phosphorylation, tubulin polymerizes and allows the formation of the microtubules that make up the mitotic spindles. However, to be able to complete the division cycle and enter a new G₁ phase, it is essential for the cell to depolymerize its microtubules and break up the mitotic spindles. Although a direct role for stathmin in the post-metaphase process of microtubule depolymerization has yet to be established, it would not be surprising if the cell used the same molecule to promote the depolymerization of the microtubules in the early and late stages of mitosis. Thus the regeneration of non-phosphorylated stathmin at the end of mitosis might be as important for completing cell division as the phosphorylation of stathmin by p34^{cdc2} kinase at the beginning of mitosis.

Our studies of the biosynthesis of the stathmin polypeptide and mRNA in proliferating K562 cells did not reveal significant

Figure 6 Site-specific dephosphorylation of p18 phosphoserines by PP1, PP2A and PP2B

The bar graphs summarize quantitative data from phosphorimager analyses of several autoradiographs such as that shown in Figure 5. Filled bars show the percentage dephosphorylation of GST-stathmin exposed to 0.4 unit of each of the respective phosphatases, and open bars show the percentage dephosphorylation of GST-stathmin exposed to 4.0 units of each phosphatase. (A) Dephosphorylation of Ser-25 phosphorylated by MAP kinase; (B) dephosphorylation of Ser-38 phosphorylated by MAP kinase; (C) dephosphorylation of Ser-25 phosphorylated by p34^{cdc2} kinase; (D) dephosphorylation of Ser-38 phosphorylated by p34^{cdc2} kinase; (E) dephosphorylation of Ser-16 phosphorylated by PKA; (F) dephosphorylation of Ser-63 phosphorylated by PKA.

fluctuations as cells progressed through the different phases of the cell cycle (Figures 1 and 2). Previous studies have also shown that the steady-state levels of stathmin do not change significantly during the different phases of the cell cycle [9–13]. In addition, the half-life of stathmin was previously estimated to be between 12 and 14 h [28]. These observations argue against a rapid degradation of the phosphoforms followed by synthesis of non-phosphorylated stathmin as the mechanism of regeneration of the non-phosphorylated protein at the end of mitosis. The observations described in the present paper are more compatible with a role for serine/threonine protein phosphatase(s) in the conversion of phosphorylated stathmin to its non-phosphorylated form. We used an inhibitor of protein phosphatases to investigate the potential role of phosphatases in the dephosphorylation of stathmin *in vivo*. Okadaic acid is a marine toxin that can readily enter eukaryotic cells and inhibit the PP1 and PP2A types of serine/threonine phosphatases [21]. The experiments described above demonstrate that the inhibition of phosphatases by okadaic acid results in a significant increase in the level of phosphorylated stathmin. This suggests that stathmin might be dephosphorylated *in vivo* by an okadaic acid-sensitive protein phosphatase, possibly by a member of the PP1 or PP2A family. However, we cannot rule out the possibility that the okadaic acid-mediated increase in stathmin phosphorylation might be an indirect effect mediated through the activation of kinases such as Cdc2 kinase. This is always a caveat in such *in vivo* experiments in which an inhibitor might affect a large number of phosphatases/kinases. If the dephosphorylation of stathmin is important for the depolymerization of the mitotic spindle and the exit from mitosis, this will predict that exposure of proliferating cells to okadaic acid might result in a mitotic arrest. In fact, the exposure of K562 cells or rat pituitary cells to okadaic acid has previously been shown to lead to an accumulation of cells in mitosis [29,30]. Although these observations are intriguing, they do not provide direct evidence that the okadaic acid-mediated increase in the level of phosphorylated stathmin is responsible for the mitotic arrest observed in this setting.

Even though the inhibition of protein phosphatases by okadaic acid has been widely used to investigate the role of protein phosphatases in the dephosphorylation of proteins *in vivo*, such an approach does not identify the specific phosphatase(s) that might be involved in this process. Okadaic acid can inhibit multiple different members of the serine/threonine family of protein phosphatases. Although the complete inhibition of PP2A can be achieved at sub-nanomolar concentrations of okadaic acid, 10–100-fold higher concentrations are required to inhibit PP1 [21]. Because both PP1 and PP2A are present in eukaryotic cells at concentrations approaching the micromolar range, 1 μ M okadaic acid is required for the total inhibition of these phosphatases in intact cells [21]. Thus the selective inhibition of PP1 and PP2A by okadaic acid is not possible in experiments *in vivo* [21]. PP2B can also be inhibited by okadaic acid but only at much higher micromolar concentrations (more than 5 μ M), which are very cytotoxic to eukaryotic cells [21].

We thus performed protein phosphatase assays to gain some insights into the nature of the phosphatases that are capable of dephosphorylating stathmin *in vitro*. These studies demonstrated that all three different classes of okadaic acid-sensitive phosphatase (PP1, PP2A and PP2B) are capable of dephosphorylating one or more of the stathmin phosphoserine residues. However, there were significant differences in the extent of dephosphorylation of the four different phosphoserine residues by the different phosphatases. For example, the PKA targets (Ser-16 and Ser-63) are efficiently dephosphorylated by PP1 and

less efficiently by PP2A and PP2B. In contrast, of the two other serine residues that are potential targets of p34^{cdc2} and/or MAP kinases, Ser-25 is dephosphorylated relatively efficiently by all three phosphatases, whereas Ser-38 is rather difficult to dephosphorylate and requires high concentrations of phosphatase to achieve a significant level of dephosphorylation.

These experiments do not allow us to determine which of the three phosphatases are more important for the dephosphorylation of stathmin *in vivo*. This is almost always true in such dephosphorylation studies *in vitro*. Whereas protein kinases have a high degree of specificity *in vitro*, protein phosphatases are generally more promiscuous in their activity *in vitro*. The specificity of phosphatase activity *in vivo* might be mediated by several different factors such as subcellular localization, targeting proteins, inhibitory proteins and regulatory proteins [31]. It would be extremely difficult to reproduce all these factors under the conditions of an assay *in vitro*. These experiments also do not allow us to determine whether the order of dephosphorylation of the four different phosphoserines might be important for their efficient dephosphorylation. Larsson et al. [16] showed previously that prior phosphorylation of Ser-25 and Ser-38 is essential for the efficient phosphorylation of Ser-16 and Ser-63. Thus it would not be surprising if the efficiency of dephosphorylation of some of the four phosphoserine residues depends on the state of phosphorylation of the other serine residues. Under the conditions described above, all four stathmin phosphoserine residues were more efficiently dephosphorylated by PP1 and PP2B than by PP2A *in vitro* (Figure 6). These observations, taken together with the increase in phosphorylation of stathmin by exposure to okadaic acid *in vivo* described above (Figure 3), suggest that a member of the PP1 family might be the phosphatase that is important in the regulation of the state of phosphorylation of stathmin *in vivo*. A potential role of the calcium/calmodulin-dependent phosphatase, PP2B, in the regulation of the level of stathmin phosphorylation is less clear at this stage.

Phosphorylation seems to have a crucial role in the regulation of the activity of several proteins that promote tubulin polymerization and stabilize microtubules. For many of these proteins, phosphatases might have an important role in reversing the effects of phosphorylation. For example, the phosphorylation of microtubule-associated protein 2 increases after the exposure of cultured neuronal cells to okadaic acid [32]. Microtubule-associated protein 2 can be dephosphorylated *in vitro* by PP1 and PP2A but not by PP2B [32]. In contrast, microtubule-associated protein 1B can be dephosphorylated *in vitro* by PP2A and PP2B but not by PP1 at sites that are phosphorylated by proline-directed kinases [33]. Tau, another microtubule-associated protein, can also be dephosphorylated *in vitro* by PP2A and PP2B [34]. It therefore seems that the activity of proteins such as stathmin that promote depolymerization of microtubules, and proteins such as the microtubule-associated proteins that stabilize microtubules, might all be regulated by phosphorylation and dephosphorylation. Because cell cycle progression is known to be regulated by the activity of cyclin-dependent kinases and phosphatases, it is not surprising that microtubule polymerization and depolymerization events that are crucial for the process of cell division are also controlled by a highly coordinated sequence of phosphorylation and dephosphorylation reactions.

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