Stathmin Inhibition Enhances Okadaic Acid-induced Mitotic Arrest

A POTENTIAL ROLE FOR STATHMIN IN MITOTIC EXIT*

Received for publication, December 26, 2000, and in revised form, April 17, 2001 Published, JBC Papers in Press, June 19, 2001 DOI 10.1074/jbc.M011654200

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Stathmin is a microtubule-destructing phosphoprotein that plays a critical role in the regulation of mitosis. The microtubule-depolymerizing activity of stathmin is lost upon phosphorylation in mitosis. Although the role of phosphorylation of stathmin by p34\(^{cd2}\) kinase in the assembly of the mitotic spindle is well established, the role of dephosphorylation of stathmin in mitosis is unknown. In this study, we tested the hypothesis that dephosphorylation of stathmin may be critically important for the depolymerization of the mitotic spindle and the exit from mitosis. We compared the effects of okadaic acid, a specific inhibitor of serine/threonine protein phosphatases, on different parameters of mitotic progression in the presence or absence of stathmin deficiency. Because okadaic acid prevents dephosphorylation of stathmin and results in accumulation of the inactive phosphorylated form, exposure to okadaic acid would be expected to have a more profound effect on mitosis than in the presence of relative stathmin deficiency. We found that inhibition of stathmin expression results in increased sensitivity to the antimitotic effect of okadaic acid. This was reflected by increased growth inhibition associated with mitotic arrest. A vast majority of the stathmin-inhibited cells were arrested in late metaphase/anaphase and had severe mitotic spindle abnormalities. Exposure to okadaic acid also resulted in a bigger ratio of polymerized/unpolymerized tubulin in stathmin-inhibited cells relative to control cells. Because the only difference between the control and the stathmin-inhibited cells is the deficiency of stathmin in the latter, the increased susceptibility of the stathmin-inhibited cells to okadaic acid-induced mitotic arrest implies a role for stathmin in the later stages of mitosis.

Stathmin is a microtubule regulatory phosphoprotein that plays an important role in the assembly of the mitotic spindle (1, 2). It is a major cellular substrate for p34\(^{cd2}\) kinase, mitogen-activated protein kinase, and other kinases that mediate different cell signals (3–7). The level of phosphorylation of stathmin is increased as cells enter the G\(_2\)/M phases of the cell cycle (3), and its phosphorylation on multiple sites is required for orderly progression through the cell cycle (8). The unphosphorylated form of stathmin that predominates during interphase promotes depolymerization of microtubules and phosphorylation of stathmin at cdc2 target sites early in mitosis eliminates the ability of stathmin to depolymerize microtubules allowing the mitotic spindle to form (1). Thus, the microtubule-depolymerizing activity of stathmin that is regulated by changes in its level of phosphorylation plays a critical role in the regulation of the dynamic instability of microtubules during the different phases of the cell cycle (1, 9).

We had previously investigated the mechanism(s) responsible for the dephosphorylation of stathmin as cells complete mitosis and enter a new division cycle (10). We used okadaic acid, a well known inhibitor of protein phosphatases, to investigate the potential role of phosphatases in the regulation of the level of stathmin phosphorylation during cell cycle progression (10). Okadaic acid is a marine toxic that readily enters eukaryotic cells and selectively inhibits PP1 and PP2A types of serine/threonine phosphatases (11, 12). We showed that inhibition of phosphatases by okadaic acid in K562 leukemic cells results in a marked increase in the level of phosphorylation of stathmin (10). We also demonstrated that stathmin mRNA expression and protein synthesis do not change significantly in the different phases of the cell cycle (10). Based on these observations, we proposed that the nonphosphorylated form of stathmin, which predominates during G\(_1\), is generated by dephosphorylation of stathmin by an okadaic acid-sensitive protein phosphatase (10). Although these experiments suggested that stathmin might be a target for okadaic acid-sensitive protein phosphatases in vivo, they did not provide direct evidence for a physiologic role of dephosphorylation of stathmin in the regulation of mitosis. The experiments described in this report were designed to investigate a potential role for stathmin in the depolymerization of the mitotic spindle and the exit from mitosis.

EXPERIMENTAL PROCEDURES

Cell Lines—K562 erythroleukemic cell lines that were used in this study were previously generated by stable transfections using an amplifiable expression vector containing the complete transcription unit for a mutant dihydrofolate reductase (DHFR) that has low affinity for methotrexate (3). The control cells, K562.DHFR, were transfected with an expression construct without stathmin cDNA (3). Pools of stathmin-inhibited cell lines were generated by transfecting the expression construct containing the full-length stathmin cDNA in the antisense orientation (3). The antisense stathmin cDNA sequences were co-amplified with the DHFR sequences by exposing the transfected cells to 1 or 50 \(\mu\)M methotrexate (MTX) (3). The two stathmin-inhibited cell lines were previously designated as K562.DHFR.p18\(^{-}\)-1 \(\mu\)M MTX and K562.DHFR.p18\(^{-}\)-50 \(\mu\)M MTX (3, 13). In this study, we used pools of stably transfected cells rather than unique clonal isolates to eliminate the possibility of clonal variations. These cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 100 units/ml penicillin and 100 \(\mu\)g/ml penicillin.

*This work was supported by National Institutes of Health Grant HL54184. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: DHFR, dihydrofolate reductase; MTX, methotrexate; PBS, phosphate-buffered saline; PIPES, 1,4-piperazine-dithanesulfonic acid; DAPI, 4',6'-diamidino-2-phenylindole dihydrochloride.
stathmin inhibition. These graphs illustrate the effects of different concentrations of okadaic acid on the relative growth rates of K562.C, K562.AS1, and K562.AS2 as indicated. Viable cells were counted daily for a period of 5 consecutive days.

**FIG. 1.** Effects of okadaic acid on the growth rate of K562 cells with different degrees of stathmin inhibition. The control and stathmin-inhibited cell lines were seeded at a density of 2 × 10^5 cells/ml in the presence and absence of okadaic acid (Sigma) at different concentrations of okadaic acid as indicated. The cells were stained with trypan blue, and viable cell counts were determined daily over a period of 5 days using a hemocytometer.

**Cell Cycle Analyses**—We used propidium iodide staining of fixed whole cells to analyze the distribution of cells in the different phases of the cell cycle (14). The control and stathmin-inhibited cell lines were incubated for 24 h with different concentrations of okadaic acid as indicated. Approximately 1 × 10^6 cells were harvested, washed, and fixed in 0.5% paraformaldehyde for 30 min. The fixed cells were permeabilized in 0.1% Triton X-100 for 3 min, washed in phosphate-buffered saline (PBS), and resuspended in 1 ml of propidium iodide solution (PBS containing 0.05 mg/ml propidium iodide, 0.1% sodium citrate, and 1 mg/ml RNase). The cells were incubated for 15 min at 4 °C. DNA content was analyzed within 2 h using a Becton Dickinson FACStar Plus flow cytometer at 488 nm single laser excitation. The cell cycle distribution was analyzed using Lysis II software.

**Evaluation of Mitotic Index**—The control and stathmin-inhibited cell lines were treated with different concentrations of okadaic acid for 24 h as indicated. The cells were harvested, washed, and cytocentrifuged onto glass slides. The cells were fixed in methanol for 5–7 min, air-dried, and then stained with Wright-Giemsa for 10 min. The mitotic cells were counted and expressed as a percentage of total cells with a minimum of 300 cells counted per slide in different fields.

**Immunofluorescent Staining**—The control and stathmin-inhibited cells were exposed to different concentrations of okadaic acid (0, 5, and 10 nM). After 24 h of incubation with okadaic acid, the cells were washed in PBS, extracted in microtubule-stabilizing buffer (0.1 M PIPES, pH 6.9, 2 mM glycerol, 5 mM MgCl₂, 2 mM EGTA, 0.2% Triton X-100, 5 μM Taxol, and 10 μg/ml RNase) and cytocentrifuged onto polylysine-coated slides at 500 rpm for 4 min. Cells were fixed in 4% paraformaldehyde, 0.5% glutaraldehyde for 15 min. The fixed cells were rinsed in three changes of PBS (5 min each). The unretracted aldehyde groups were reduced by two changes of NaBH₄ (5 min each). The cells were rinsed three times in PBS (5 min each) and incubated in 3% bovine serum albumin for 1 h. After three rinses in PBS, cells were incubated with fluorescein-conjugated rabbit anti-mouse IgG (1:100) and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma) for 15 min and rinsed with two changes of PBS. The cells were mounted in aqueous dry mounting medium and analyzed by epifluorescence.

**Biochemical Analyses of Microtubule Polymerization in Vivo**—Control and stathmin-inhibited K562 cell lines were treated with 10 nM okadaic acid and harvested after 24 h. The amount of polymerized and soluble forms of tubulin in untreated or okadaic acid-treated cell lines were quantified using a modification of the procedure described previously (1, 15). An equal number of cells (1 × 10⁶) from untreated or okadaic acid-treated control and stathmin-inhibited cell lines were lysed in microtubule-stabilizing lysis buffer containing 0.1 M PIPES, pH 6.9, 2 mM glycerol, 5 mM MgCl₂, 2 mM EGTA, 0.5% Triton X-100, 4 μM Taxol, and 5 μg/ml leupeptin. Taxol was included in the lysis buffer to stabilize the microtubules during extraction and to maintain their stability during subsequent processing. The cell lysates were subjected to high speed centrifugation at 40,000 × g for 30 min at 22 °C to sediment the polymeric tubulin (16, 17). Supernatants containing soluble tubulin were separated from the pellets containing the polymerized tubulin. The pellets containing the polymerized tubulin were solubilized in microtubule-stabilizing buffer and subjected to ultrasonic waves for 10 min to ensure complete disruption of the microtubules. The tubulin content in the pellets and supernatants was then analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with an anti-α-tubulin antibody (clone B512, Sigma). The bound antibodies were visualized by chemiluminescence. To measure the total level of tubulin in untreated or okadaic acid-treated cells and stathmin-inhibited cells, the cells were lysed in a buffer containing 25 mM Tris, pH 7.4, 0.4 M NaCl, 1% Nonidet P-40, 0.5% SDS, 0.1% deoxycholate, and 5 μg of leupeptin (18). The protein concentrations were measured by the Bio-Rad DC assay, and equal amounts (100 μg) of the protein extracts were used. The total level of tubulin was then analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with an anti-α-tubulin antibody as described above. This assay was performed more than three times with essentially identical results.

**RESULTS**

We tested the hypothesis that dephosphorylation of stathmin may be critical for depolymerization of the mitotic spindle and exit from mitosis. We compared the effects of okadaic acid on mitotic progression in cells expressing different levels of stathmin. Because okadaic acid prevents dephosphorylation of stathmin and results in accumulation of the inactive phosphorylated form (10), exposure to okadaic acid should have a more profound antimitotic effect in the presence of relative stathmin deficiency. We had previously generated several stable K562 erythroleukemia cell lines that express different levels of stathmin RNA (3). The cell line in which stathmin expression was moderately inhibited by antisense RNA was previously referred to as K562.DHFR.p18(−) [1 μM MTX], and the cell line in which stathmin expression was maximally inhibited was referred to as K562.DHFR.p18(−) [50 μM MTX] (3, 13). A control cell line, K562.DHFR, was also established by transfecting the same expression plasmid without stathmin cDNA sequences (3, 13). In this report, we refer to the K562.DHFR.p18(−) [1 μM MTX] cell line as K562.AS1, the K562.DHFR.p18(−) [50 μM MTX] cell line as K562.AS2, and the control K562.DHFR cell line as K562.C. We compared the effects of okadaic acid on different parameters of mitotic progression in the control and the two stathmin-inhibited K562 cell lines.

**Effects of Inhibition of Dephosphorylation on Cell Proliferation**—We first examined the effects of okadaic acid on the proliferation of K562 leukemic cells in the presence and absence of stathmin inhibition. Fig. 1 illustrates the effects of different concentrations of okadaic acid on the rate of proliferation of K562.C, K562.AS1, and K562.AS2 cells. Exposure of K562.C and K562.AS1 cells to 5 nM okadaic acid had very little effect on their rate of growth, whereas the growth of K562.AS2 cells was moderately decreased (Fig. 1). When the control K562.C cells were exposed to 10 nM okadaic acid, they proliferated at a moderately decreased rate (Fig. 1). However, exposure of stathmin-inhibited cells to the same concentration of okadaic acid resulted in a very profound growth inhibition of K562.AS1 cells and the near complete loss of proliferation of...
K562.AS2 cells (Fig. 1). The doubling time of the three cell lines was calculated in the presence and absence of exposure to okadaic acid (Table I). Exposure to 10 nM okadaic acid resulted in 22.2% prolongation of the doubling time of the control K562.C cells. Exposure of the stathmin-inhibited cells to the same concentration of okadaic acid resulted in a 45.5% prolongation of the doubling time of K562.AS1 cells and nearly 100% prolongation in the K562.AS2 cells (Table I). Thus, inhibition of stathmin expression is associated with increased sensitivity to the antiproliferative effects of okadaic acid.

**Effects of Inhibition of Dephosphorylation on Cell Cycle Progression**—We also examined the effects of okadaic acid on cell cycle progression of K562 cells in the presence and absence of stathmin inhibition. The cell cycle distribution of control and stathmin-inhibited cells that were exposed to different concentrations of okadaic acid were analyzed by propidium iodide staining and flow cytometry. Fig. 2 illustrates the effects of different concentrations of okadaic acid on the cell cycle profile of K562.C, K562.AS1, and K562.AS2 cell lines. As expected, exposure of all three cell lines to increasing concentrations of okadaic acid resulted in a dose-dependent accumulation of cells in the G2/M phases and a corresponding decrease in the proportion of cells in the G0/G1 phases of the cell cycle (Fig. 2). However, the magnitude of the okadaic acid-induced G2/M accumulation was markedly different in the different cell lines. Exposure of K562.C and K562.AS1 cells to 5 nM okadaic acid resulted in a modest increase in the fraction of cells in the G2/M phases (4 and 5.5%, respectively) (Fig. 2). In contrast, the fraction of K562.AS2 cells in the G2/M phases was increased by 18% (Fig. 2). When the cells were exposed to 10 nM okadaic acid, the G2/M fraction of the control K562.C cells was increased by 22%, whereas the G2/M fractions of K562.AS1 and K562.AS2 cells were increased by 43.8 and 47.7%, respectively (Fig. 2). Thus, inhibition of stathmin expression results in a dose-dependent increase in the sensitivity to okadaic acid-induced mitotic arrest.

**Effects of Inhibition of Dephosphorylation on the Mitotic Index**—Because it is not possible to distinguish between accumulation in the G2 phase and accumulation in the M phase by propidium iodide staining, we analyzed the effects of okadaic acid on the mitotic index of control and stathmin-inhibited cells. Fig. 3 illustrates the effects of different concentrations of okadaic acid on the mitotic index of K562.C, K562.AS1, and K562.AS2 cell lines. When the control K562.C cells were exposed to different concentrations of okadaic acid, the proportion of mitotic cells increased to 20.2% at 5 nM and 24.8% at 10 nM okadaic acid (Fig. 3). In contrast, when the stathmin-inhibited K562.AS1 cells were exposed to the same concentrations of okadaic acid, the proportion of mitotic cells increased to 39.7% at 5 nM and 51.3% at 10 nM okadaic acid (Fig. 3). Similarly, when K562.AS2 cells were exposed to okadaic acid, the proportion of mitotic cells was increased to 39.7% at 5 nM and 61.8% at 10 nM okadaic acid (Fig. 3). Thus, stathmin inhibition accentuates the okadaic acid-induced mitotic arrest. Analyses of the data presented in Figs. 2 and 3 make it possible to deduce the fraction of cells arrested in mitosis and the fraction of cells arrested in the G2 phase of the cell cycle. A comparison of the relative fraction of cells arrested in mitosis as assessed by DNA content and morphology in the three cell lines following exposure to okadaic acid is shown in Table II. When the control K562.C cells were exposed to okadaic acid, more cells were found to be arrested in G2/M by DNA content than the cells arrested in M by morphology. In contrast, when the stathmin-inhibited cells were exposed to the same concentrations of okadaic acid, the fraction of cells arrested in G2/M by DNA content and the cells arrested in M by morphology were similar. This suggests that when stathmin-inhibited cells are exposed to okadaic acid essentially all cells that are in the G2/M fractions are arrested in M, whereas exposure of control cells to okadaic acid results in accumulation of cells in the G2 phase and the M phase.

**Potential Role for Stathmin in Mitotic Exit**

| Cell lines   | 0 nM OA | 5 nM OA | 10 nM OA |
|--------------|---------|---------|----------|
| K562.C       | 22.6    | 23.1    | 26.1     |
| K562.AS1     | 26.7    | 27.3    | 37.5     |
| K562.AS2     | 27.3    | 30.0    | 54.5     |

**FIG. 2.** Effects of okadaic acid on cell cycle progression of K562 cells with different degrees of stathmin inhibition. Control K562.C and stathmin-inhibited K562.AS1 and K562.AS2 cell lines were grown in the absence (0 nM) or presence of different concentrations of okadaic acid (OA) for 24 h as indicated. The cells were harvested, and DNA content was analyzed by flow cytometry. The fraction of cells arrested in the G0/G1 and the G2/M phases is indicated.

**FIG. 3.** Effects of okadaic acid on the mitotic index of K562 cells with different degrees of stathmin inhibition. Control K562.C and stathmin-inhibited K562.AS1 and K562.AS2 cell lines were grown in the absence (0 nM) or presence of different concentrations of okadaic acid (OA) for 24 h as indicated. The number of mitotic cells is expressed as percentiles of total number of cells examined with a minimum of at least 300 cells counted in different fields.
the majority of the mitotic cells showed normal bipolar organization of the mitotic spindle and were blocked in a metaphase-like early mitotic stage. However, there were fewer cells in late mitosis in K562.AS1 (23.2%) and K562.AS2 (19.6%) than in the control K562.C cells (39.8%). When the concentration of okadaic acid was increased to 10 nM, there was a dramatic increase in the number of cells with aberrant mitosis. The fraction of cells with abnormal mitotic spindles in the control K562.C cells was 36%. In contrast, the fraction of cells with abnormal mitotic spindles in stathmin-inhibited K562.AS1 and K562.AS2 cells markedly increased to 72 and 85.7%, respectively (Fig. 4). Both the control and stathmin-inhibited cells showed a wide range of mitotic spindle abnormalities after okadaic acid treatment (Fig. 5). In many instances, the microtubules of bipolar spindles were disorganized with scattered chromosomes as shown in Fig. 5, A and B. In other instances, although the spindles retained their bipolar organization, they were disrupted (Fig. 5, C and D). In some cells, the spindles had a unipolar organization (Fig. 5, E and F). A striking phenotype that was more commonly seen in okadaic acid-treated stathmin-inhibited cells was the presence of multipolar spindles with three or more poles (Fig. 5, G and I). These multipolar spindles were frequently observed with chromosomes that were either aggregated in multiple metaphase plates (Fig. 5, G and H) or randomly scattered between spindle poles (Fig. 5, I and J). The frequencies of the different types of spindle abnormalities are summarized in Table III. Although okadaic acid treatment elicited the same type of spindle abnormalities in all three cell lines, the incidence of the severe spindle abnormalities increased with increased inhibition of stathmin expression.

Effects of Inhibition of Dephosphorylation on Microtubule Polymerization in Vivo—We wanted to determine whether the increased mitotic aberrations observed in stathmin-inhibited cells after okadaic acid treatment are a result of perturbations in the dynamic equilibrium of microtubules that make up the mitotic spindles. Because phosphorylation of stathmin eliminates its microtubule-depolymerizing activity, preventing its dephosphorylation by okadaic acid treatment should be expected to result in a shift of the equilibrium between polymerized and unpolymerized tubulin in favor of the polymerized form. We used two different approaches to test this hypothesis. We first analyzed by immunofluorescence microscopy the effects of stathmin inhibition on the morphology of the microtubule network in the presence and absence of okadaic acid (Fig. 6). The K562.AS2 stathmin-inhibited cells displayed a more prominent microtubule network than the K562.C and the K562.AS1 cells (Fig. 6A). When the cells were exposed to okadaic acid, this increase in density of microtubules became more pronounced in both the K562.AS1 and K562.AS2 stathmin-inhibited cell lines than in the control K562.C cells (Fig. 6B). Although these observations are compatible with increased polymerization of microtubules in okadaic acid-treated stathmin-inhibited cells, they are difficult to quantify. Therefore, we used a second approach that is more quantitative in nature to compare the levels of polymerized and unpolymerized tubulin in untreated or okadaic acid-treated control and stathmin-inhibited cells. We used a biochemical assay that allows the isolation of the in vivo assembled microtubules in their polymerized state and the unpolymerized tubulin as a soluble protein (1, 15). The two forms of tubulin were separated by ultracentrifugation, and the levels of polymerized and unpolymerized forms of tubulin were quantified by immunoblotting with an anti-tubulin antibody (Fig. 7). Immunoblot analyses of the levels of polymerized and unpolymerized tubulin in the absence of okadaic acid showed no significant differences in the ratio of polymerized to unpolymerized tubulin in stathmin-inhibited K562.AS1 cells relative to the control K562.C cells (Fig. 7, A and B). In contrast, the K562.AS2 cells showed a 2-fold increase in the ratio of polymerized to unpolymerized tubulin relative to the control K562.C cells (Fig. 7, A and B). When the cells were exposed to okadaic acid, the ratio of polymerized to unpolymerized tubulin in K562.AS1 cells remained essentially unchanged, whereas the ratio of polymerized to unpolymerized tubulin was further increased (from 2- to 5-fold) in K562.AS2 cells relative to the control K562.C cells (Fig. 7, A and B). This demonstrates that stathmin inhibition results in increased polymerization of microtubules that is further enhanced by exposure to okadaic acid. These findings are supportive of the morphological data shown in Fig. 6. In another experiment, we compared the total level of tubulin (i.e. polymerized and unpolymerized) in untreated and okadaic acid-treated control and stathmin-inhibited cells. Immunoblot analyses of tubulin levels in control and stathmin-inhibited cell lines showed no signifi-
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Representative micrographs illustrate the different spindle abnormalities that were seen following treatment with 10 nm okadaic acid for 24 h. Although all these micrographs were taken from K562.AS1 cells, the same types of abnormalities were seen in all cell lines. The micrographs in the left panels (A, C, E, G, and I) show anti-tubulin indirect immunofluorescence staining, and those in the right panels (B, D, F, H, and J) show DNA staining with DAPI. The spindle abnormalities (indicated by arrowheads) ranged from bipolar spindles with disorganized arrays of microtubules and dispersed chromosomes (A and B), disrupted bipolar spindles (C and D), unipolar spindles (E and F), and multipolar spindles with chromosomes aggregated in multiple metaphase plates (G and H) or randomly scattered chromosomes between spindle poles (I and J).

**Discussion**

PP1/PP2A-mediated dephosphorylation of p34^cdc2^ substrates is believed to play an important role in mitotic progression, especially in the exit from mitosis. This has been suggested by a number of studies that demonstrated a block in mitosis in diverse cell types following okadaic acid treatment (19–22). Exposure of K562 cells to okadaic acid was previously shown to result in growth inhibition that was associated with a pronounced mitotic arrest (19). In a second study, exposure of HeLa cells to okadaic acid was shown to result in defective anaphase separation (20). A third study showed that prolonged exposure of LLC-PK cells to okadaic acid results in disruption of chromosome alignment at the metaphase plate and spindle deformities associated with a high frequency of multipolar spindles (21). This study concluded that okadaic acid blocks cells at the transition from metaphase to anaphase (21). PP1 and PP2A have also been implicated to play a role in the metaphase/anaphase transition in several yeast mutants and in starfish oocytes (23–25). Although numerous studies have contributed to our current appreciation of the role of okadaic acid-sensitive protein phosphatases in the progression through mitosis, particularly at the metaphase/anaphase transition, the targets of dephosphorylation by these phosphatases or the precise signaling pathways that control the exit from mitosis remain entirely unknown.

On the basis of our previous observation of the increased stathmin phosphorylation in vivo following exposure of K562 cells to okadaic acid (10) and the known mitotic arrest that results from okadaic acid treatment of K562 cells (19), we previously proposed that dephosphorylation of stathmin by okadaic acid-sensitive protein phosphatases may be an important signaling event that is critical for the depolymerization of the microtubules that make up the mitotic spindle and for the exit from mitosis (10). When stathmin is phosphorylated by p34^cdc2^ kinase as cells enter mitosis, its microtubule-depolymerizing activity is lost, tubulin is polymerized, and the mitotic spindle is formed (1). However, to complete the division cycle and enter a new G1 phase, cells need to depolymerize their microtubules to disassemble the mitotic spindles. The importance of the phosphorylation of stathmin by p34^cdc2^ kinase for the assembly of the mitotic spindle as cells enter mitosis was previously demonstrated (1). We hypothesized that the reversal of this process by stathmin dephosphorylation might be equally important for the ability of cells to complete their division cycle.

We undertook the present study to determine whether dephosphorylation of stathmin by okadaic acid-sensitive protein phosphatases might be an important event in the postmetaphase process of microtubule depolymerization. If stathmin is important for the exit from mitosis, inhibiting its dephosphorylation should result in a more profound mitotic arrest in the presence of stathmin deficiency. Thus, we tested the hypothesis that inhibition of dephosphorylation by okadaic acid would result in a more profound mitotic block in the presence of decreased levels of stathmin. Although we previously demonstrated that exposure of K562 cells to okadaic acid results in increased phosphorylation of stathmin, the effects of okadaic acid on K562 cells is not limited to the inhibition of dephosphorylation of stathmin (10). However, the only difference that we are aware of between the control and stathmin-inhibited K562 cells that were used in this study is in the level of expression of stathmin (3). Thus, the observed differences in the effects of okadaic acid in the different cell lines may be attributed to its effect on stathmin phosphorylation. Our analyses of the effects of okadaic acid on mitotic progression demonstrated that increased inhibition of stathmin expression results in increased sensitivity to the antimitotic effects of okadaic acid. This increased sensitivity was shown by three different assays that evaluated the effects of okadaic acid on cell growth, cell cycle distribution, and the mitotic index in control and stathmin-inhibited cell lines. These experiments were performed using cell lines that consisted of pools of stably transfected cells. This should eliminate the possibility that the observed differences in okadaic acid sensitivity may be because of clonal variations in the cell lines that were selected for this study.

Our analyses of the morphology and the distribution of cells in mitosis revealed that exposure of K562 cells to okadaic acid at low concentration results in a higher proportion of cells in a metaphase-like mitotic stage and a lower proportion of cells in late mitosis (anaphases or telophases). This postmetaphase block in mitosis is more profound in the presence of stathmin inhibition. This suggests that inhibition of dephosphorylation of stathmin arrests cells late in mitosis. Furthermore, exposure to higher concentration of okadaic acid resulted in a significantly higher proportion of abnormal mitotic spindles in stathmin-inhibited cells than in the control cells. The abnormal mitotic spindles in the stathmin-inhibited cells were predominantly multipolar with scattered or aggregated chromosomes, a mitotic abnormality that is frequently associated with an aberrant anaphase (19–21). These observations suggest that the observed spindle abnormalities are probably a result of a more severe deficiency of the active (i.e. dephosphorylated) form of stathmin in the K562.AS cells. This may be responsible for the impairment of the ability of cells to undergo normal chromo-
some segregation at the onset of anaphase and prevent the exit from mitosis and entry into a new division cycle. Thus, the increased frequency of aberrant multipolar anaphases in okadaic acid-treated stathmin-inhibited K562 cells confirms that dephosphorylation of stathmin by okadaic acid-sensitive protein phosphatases is important during transition of cells from metaphase to anaphase.

Entry into and exit from mitosis involves a number of structural transitions that are mediated through changes in the dynamic equilibrium of microtubules that make up the mitotic spindle. We measured the fraction of polymerized tubulin in the different cell lines in the presence and absence of okadaic acid. We found that inhibition of stathmin in K562 cells results in an increase in the polymerization of tubulin subunits in the absence of exposure to okadaic acid. This increased polymerization of microtubules in stathmin-inhibited cells is consistent with a recent report that demonstrated a similar increase in microtubule polymer levels in newt lung cells that were depleted of stathmin by either microinjection of stathmin antibody or antisense stathmin oligonucleotides (26). However, the major aim of this study was to compare the effects of okadaic acid on tubulin polymerization in the presence or absence of stathmin inhibition. Our experiments showed that exposure of the same cells to okadaic acid results in a further increase in microtubule polymerization in K562.AS2 cells relative to the control K562.C cells. These findings are consistent with the postulated model of the microtubule regulatory activity of stathmin (1, 2). If phosphorylation of stathmin early in mitosis abrogates its microtubule-depolymerizing activity (1), inhibition of dephosphorylation of stathmin by okadaic acid should inactivate its microtubule-depolymerizing function and result in increased microtubule polymerization and overstabilization of the mitotic apparatus. This in turn would give rise to aberrant spindle morphology. The observed increase in the proportion of aberrant mitotic spindles along with the increase in the content of polymerized tubulin in okadaic acid-treated stathmin-inhibited cells provides strong support for the hypothesis that dephosphorylation of stathmin by okadaic acid-sensitive protein phosphatases plays a critical role in the regulation of exit from mitosis.

Acknowledgments—We are extremely grateful to Dr. Jonathan Licht and Dr. James Manfredi from the Derald Ruttenburg Cancer Center for reviewing this manuscript and providing helpful comments.

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TABLE III

| Abnormal spindles       | No OA | +10 nM OA (24 h) |
|-------------------------|-------|-----------------|
|                         | K562.C| K562.AS1 | K562.AS2  | K562.C | K562.AS1 | K562.AS2  |
| Unipolar                | 0     | 4.0      | 6.0        | 4.0    | 3.0      | 7.0        |
| Aberrant bipolar        | 7.0   | 9.7      | 11.0       | 23.7   | 27.7     | 36.7       |
| Multipolar (>2 poles)   | 0     | 1.0      | 0.7        | 9.0    | 41.3     | 42.0       |

FIG. 6. Effects of okadaic acid on the microtubule network in K562 cells with different degrees of stathmin inhibition. A, representative images of α-tubulin-stained microtubules in untreated K562.C, K562.AS1, and K562.AS2 cells as indicated. B, representative images of α-tubulin-stained microtubules in okadaic acid (OA)-treated K562.C, K562.AS1, and K562.AS2 cells as indicated.

FIG. 7. Effects of okadaic acid on tubulin polymerization in K562 cells with different degrees of stathmin inhibition. A, representative autoradiographs of Western blot analyses of polymerized (P) and soluble (S) forms of tubulin derived from untreated or okadaic acid (OA)-treated K562.C, K562.AS1, and K562.AS2 cells as indicated. B, the bar graphs show quantitative data of the relative ratios of polymerized/soluble (P/S) tubulin in untreated and okadaic acid-treated K562.C, K562.AS1, and K562.AS2 cells as indicated. The data presented are the mean ± S.D. of three different experiments. C, representative autoradiographs of Western blot analyses of total tubulin derived from untreated or okadaic acid-treated K562.C, K562.AS1, and K562.AS2 cells as indicated.
Potential Role for Stathmin in Mitotic Exit

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J. Biol. Chem. 2001, 276:31209-31215.
doi: 10.1074/jbc.M011654200 originally published online June 19, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M011654200

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