Phosphorylation regulates the microtubule-destabilizing activity of stathmin and its interaction with tubulin

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Abstract Stathmin is a regulator of microtubule dynamics which undergoes extensive phosphorylation during the cell cycle as well as in response to various extracellular factors. Four serine residues are targets for protein kinases: Ser-25 and Ser-38 for proline-directed kinases such as mitogen-activated protein kinase and cyclin-dependent protein kinase, and Ser-16 and Ser-63 for cAMP-dependent protein kinase. We studied the effect of phosphorylation on the microtubule-destabilizing activity of stathmin and on its interaction with tubulin in vitro. We show that triple phosphorylation on Ser-16, Ser-25, and Ser-38 efficiently inhibits its activity and prevents its binding to tubulin.

Key words: Stathmin; Microtubule; Cytoskeleton; Phosphorylation

1. Introduction

The microtubule (MT) cytoskeleton plays a wide variety of structural and functional roles in cells, affecting their shape and motility, organizing the spatial distribution of organelles in the cytoplasm and chromosome segregation during mitosis, and integrating transmembrane signals. Thus the behavior of microtubules is influenced by many different microtubule-interacting proteins (for review see [1,2]). The characteristic property of microtubules, known as dynamic instability, involves rapid transitions between polymerization and depolymerization of α- and β-tubulin heterodimers. A large group of proteins, collectively known as microtubule-associated proteins (MAPs), favor MT polymerization thereby stabilizing them. Phosphorylation of MAPs decreases their stabilizing effect probably by reducing their direct binding to MTs [3,4]. More recently, another type of cellular factors has been identified that increase the frequency of catastrophes (transitions from growth to shrinkage of MTs) and therefore destabilize MTs in vitro and in intact cells [5–7]. These newly characterized proteins are encoded by the stathmin gene family. Stathmin is a small cytosolic protein of 19 kDa that has been independently identified by several groups as a phosphoprotein and is also called Op18, metablastin, p19, p18, and prosolin (for review see [8]). In addition to its phosphorylation induced by a diverse number of extracellular signals [8], its phosphorylation state varies during the cell cycle and peaks during mitosis [9,10]. Stathmin is mainly expressed in proliferating cells but also in developing neurons at high levels [11,12]. In PC12 cells, stathmin is a physiological substrate for mitogen-activated protein kinase (MAP kinase) upon nerve growth factor induction and inhibition of its expression with antisense oligonucleotides prevents nerve growth factor-induced differentiation [13]. These data suggested that stathmin may be involved in the regulation of the rearrangement of the microtubule cytoskeleton during morphogenesis in response to phosphorylation signals.

The known in vivo phosphorylation sites of stathmin are Ser-16 for Ca^{2+}/calmodulin-dependent kinase IV/Gr [14], Ser-16 and Ser-63 for cyclic AMP-dependent protein kinase (PKA) [15], and Ser-25 and Ser-38 for the proline-directed kinases $p34^{cdc2}$ [15,16] and MAP kinase [16,17]. Recent reports using several phosphorylation site mutants for cell transfection or microinjection have indicated that the MT depolymerizing activity of stathmin is controlled by phosphorylation [6,18,19]. However, it was not known how phosphorylation on the different sites regulates the activity of stathmin and whether the effect is direct by modulating its interaction with tubulin heterodimers [5] or indirect through its interaction with other cellular factors.

Here we have analyzed whether phosphorylation of stathmin alters its MT assembly properties using recombinant purified stathmin in an in vitro assay of MT polymerization and whether phosphorylation influences its binding to tubulin. We show that multiple phosphorylation of stathmin decreases its activity in vitro in the absence of additional factors and inhibits its interaction with tubulin.

2. Materials and methods

2.1. Preparation of recombinant stathmin and in vitro phosphorylation

Human stathmin was expressed in *Escherichia coli* and purified from the soluble cell fraction by sequential chromatography on Q-Sepharose, hydroxylapatite, phenyl-Sepharose and FPLC Mono Q. The pool from the Mono Q column was homogeneous on SDS-PAGE and over 98% pure on RP-HPLC. The purified protein was dialyzed against 20 mM Tris-HCl, pH 7.5 and stored at −80°C. The in vitro phosphorylation was performed as described earlier [20] with the following modifications. In each assay 0.3 mg of stathmin was used in a total volume of 70 μl. For phosphorylation with MAP kinase (p42, UBI), 1 μg kinase was used; for PKA (protein kinase A catalytic subunit, Boehringer Mannheim) 60 mU; for $p34^{cdc2}$ kinase (Promega) 40 U. In the phosphorylation with a combination of MAP kinase and PKA, 1 μg MAP kinase and 60 mU PKA were used. The

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Abbreviations: MAP kinase, mitogen-activated protein kinase; MAPs, microtubule-associated proteins; PKA, cAMP-dependent protein kinase; MT, microtubules; mAB, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide

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reaction mixtures were incubated at 35°C for 3 h and the reactions were stopped by freezing at −80°C. Phosphorylated stathmin was separated from unphosphorylated protein on FPLC Mono Q. A 1 ml Mono (5/5) column was equilibrated in 25 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 7.5. The samples were diluted to 1 ml in equilibration buffer and applied to the column. Stathmin was eluted from the column with a linear gradient of 10–30% of 0.5 M NaCl in equilibration buffer. Phosphorylated stathmin was detected by native PAGE, pooled, concentrated and dialyzed against 110 mM MES, 1 mM EGTA, 5 mM $MgCl_2$, pH 6.6. Protein concentrations were determined by the Lowry method [21] and the samples were stored at −80°C.

2.2. Purification of microtubules and in vitro assembly

Microtubules were prepared from porcine cerebrum by three temperature-dependent cycles of cold and warm centrifugations in assembly and disassembly buffer A (0.1 M MES, 1 mM EGTA, 0.5 mM $MgCl_2$, pH 6.4). For assembly, 1 mM GTP was added to buffer A [22]. This preparation of MTs was ca. 90% pure. For the cross-linking, MTs were further purified to obtain pure tubulin: MTs were resuspended at a concentration of 20 mg/ml in buffer A, and tubulin was separated from MAPs by ion exchange chromatography using a 5 ml P11 phosphocellulose column pre-equilibrated with buffer A. MAPs were eluted by a 15 ml linear gradient of 0–1 M NaCl in buffer A [23]. Protein concentration was determined by Bio-Rad protein assay with bovine serum albumin as standard. The assembly rate of tubulin was measured using a light scattering assay [24]. Tubulin was used at a concentration of 4 mg/ml. Absorbance was measured at 350 nm in a Camspec M350 spectrophotometer equipped with seven 50 µl cuvettes and a cooling block for temperature control.

2.3. Cross-linking of stathmin and tubulin, immunoblotting

Cross-linking experiments were performed as described previously [18] with slight modifications: stathmin (2 µM) and tubulin (10 µM) were incubated for 2 h at 4°C in 15 µl PEM buffer (80 mM PIPES (Sigma), 1 mM EDTA, 1 mM $MgCl_2$, pH 6.8). The zero-length cross-linker 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC, Sigma) was added at a concentration of 1 mM and samples were incubated at 18°C for durations indicated in the figure legend. In the experiments using phosphorylated stathmin, samples were treated for 30 min with EDC. The reaction was then stopped with an equal volume of 2× protein sample buffer and samples were analyzed by SDS-PAGE (8–16%) and immunoblotted. Immunoblots were immersed in blocking solution consisting of 5% milk powder in PBS/0.15% Tween-20. Membranes were subsequently incubated with primary antibodies diluted in 2.5% milk powder in PBS and the signals were detected using the ECL Western blotting kit (Amersham International, Buckinghamshire, UK). The rabbit antiserum against stathmin (anti-Op18:34-149) was a gift of M. Gullberg (Umeå, Sweden). Mouse monoclonal antibody against tubulin (B5-1-2) was from Sigma.

3. Results

3.1. Effect of phosphorylation of stathmin on inhibition of MT assembly

Fig. 1A illustrates that stathmin inhibited MT assembly in the in vitro polymerization assay in a dose-dependent manner. To study the effect of phosphorylation on the ability of stathmin to inhibit microtubule polymerization, the recombinant protein was phosphorylated in vitro using MAP kinase, $p34^{cdc2}$ kinase and PKA. The specific sites phosphorylated by these kinases were determined by a rapid digestion technique in combination with ESI-MS [25] (data not shown). In agreement with previous reports [15–17], the sites for MAP kinase and $p34^{cdc2}$ kinase were Ser-25 and Ser-38 and for PKA Ser-16 and Ser-63. In addition, stathmin was multiphosphorylated by the combined use of MAPK and PKA. After the phosphorylation, the samples were not homogeneous but contained unphosphorylated protein. Therefore, the phosphorylated stathmin was isolated by ion exchange chromatography on Mono Q. This purification step also removed

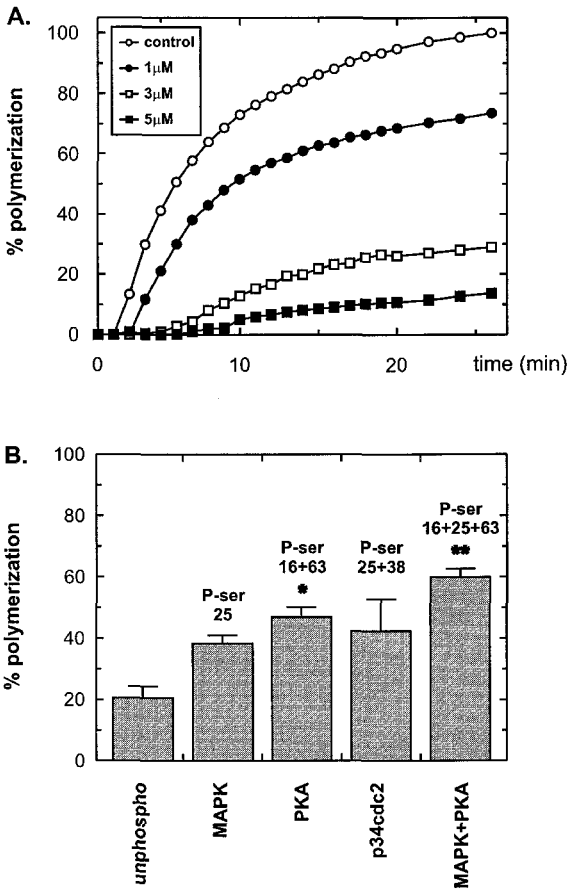

Fig. 1. Effect of stathmin phosphorylation on the assembly of MTs in vitro. A: Dose-dependent inhibition of MT assembly by unphosphorylated stathmin. Tubulin (4 mg/ml) was incubated at 4°C in the absence of stathmin (control) or in the presence of 1, 3 and 5 µM stathmin. MT polymerization was induced by changing the temperature to 37°C at time zero. Assays were performed in duplicate. 1, 3, and 5 µM stathmin resulted in inhibitions of about 27%, 71%, and 87%, respectively. B: Inhibition of MT assembly measured after 26 min polymerization in the presence of 3 µM stathmin unphosphorylated or phosphorylated by kinases as indicated. Phosphorylated residues are indicated at the top of the bars. Values are means (±S.E.M., $n=7$) with three different batches of phosphorylated samples (*$P<0.05$; **$P<0.001$). Phosphorylation by PKA alone or in combination with MAPK reduces the MT-depolymerizing activity of stathmin 2- or 3-fold, respectively.

the kinases from the protein preparations. After purification, no unphosphorylated stathmin was detectable on native PAGE. The MAP kinase phosphorylated sample was mainly mono-phosphorylated on Ser-25 with some di-phosphorylation (<25%) on Ser-25 and Ser-38, the PKA sample was di-phosphorylated on Ser-16 and Ser-63, and the $p34^{cdc2}$ kinase sample was di-phosphorylated on Ser-25 and Ser-38. The sample phosphorylated with both MAP kinase and PKA was mainly tri-phosphorylated with traces of di-phosphorylated protein (data not shown). To demonstrate the effect of the different phosphorylated forms on the inhibition of MT assembly, the proteins were used at a concentration of 3 µM at which unphosphorylated stathmin caused about 80% inhibition (Fig. 1B). The effect of phosphorylation was also tested at other concentrations (1 µM and 5 µM) at which the differences between unphosphorylated stathmin and the phosphoforms were found to be less strong (not shown). Fig. 1B shows

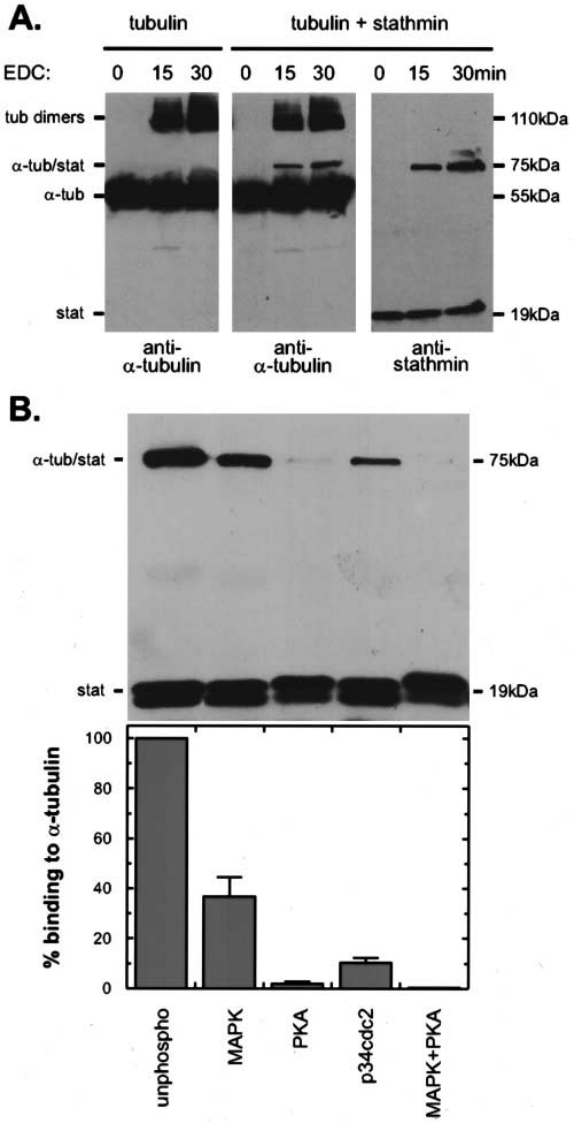

Fig. 2. Effect of stathmin phosphorylation on tubulin binding in vitro. A: Western blot analysis of stathmin-tubulin complexes following chemical cross-linking with the zero-length cross-linker EDC. Tubulin alone or in the presence of stathmin was treated with 1 mM EDC for 0, 15 and 30 min as indicated. Membranes were immunoblotted with anti-α-tubulin or anti-stathmin antibodies. B: Western blot analysis with anti-stathmin antibodies of stathmin-tubulin complexes after phosphorylation by kinases as indicated. Samples were incubated for 30 min in the presence of EDC. (The lower band of the stathmin doublet corresponds to a specific degradation product starting at amino acid 14 in the stathmin sequence [20] and was found to inhibit in vitro MT polymerization indistinguishable from the full length protein; unpublished results). A densitometric quantification of tubulin binding is shown in the histogram below. The values are expressed as percentage of unphosphorylated stathmin binding to tubulin. Values are means (±S.E.M., $n=3$).

that phosphorylation on the site for proline-directed kinases (Ser-25 and Ser-38) had no statistically significant effect on the activity of stathmin. However, phosphorylation at Ser-16 and Ser-63 significantly reduced the ability of stathmin to inhibit MT assembly. The percentage of MT polymerization increased from 20% to 46% ($P<0.05$). The most potent reduction in activity was observed when stathmin was phosphorylated on both MAP kinase (Ser-25) and PKA (Ser-16 and Ser-63) sites ($P<0.001$) demonstrating that simultaneous phosphorylation of both MAP kinase and PKA sites enhances the downregulation of stathmin activity.

3.2. *Effect of phosphorylation on stathmin-tubulin complex formation*

Stathmin has been shown to physically interact with tubulin dimers [5] and to form a complex with α-tubulin upon chemical cross-linking with the zero-length cross-linker EDC [18]. By this cross-linking method, we studied the effect of phosphorylation on the interaction of stathmin with tubulin. The results show that unphosphorylated stathmin formed an adduct of 75 kDa that reacted with both stathmin and α-tubulin antibodies (Fig. 2A) but not with β-tubulin antibodies which is consistent with previous reports by Melander Gradin et al. [18]. The size of this product corresponded approximately to the sum of stathmin (19 kDa) and α-tubulin (55 kDa). The adduct of 75 kDa did not appear in the absence of tubulin nor was a binding of stathmin to actin observed (not shown). Each phosphorylation caused a decrease in the amount of stathmin binding to tubulin (Fig. 2B). A pronounced decreased in complex formation was seen after phosphorylation at the PKA sites (Ser-16 and -63), and phosphorylation on both the MAP kinase and PKA sites (Ser-16, -25, -63) nearly abolished stathmin-tubulin complex formation.

4. Discussion

To study potential regulatory mechanisms of stathmin function, we have tested recombinant stathmin protein produced in *E. coli* and several phosphoisoforms in an in vitro MT polymerization assay. Phosphorylation on the sites for proline-directed kinases (Ser-25 and Ser-38) did not significantly reduce stathmin activity. However, stathmin containing phosphorylation on the PKA sites Ser-16 and Ser-63 was significantly less active than the unphosphorylated protein indicating that phosphorylation on Ser-16 and Ser-63 is crucial for this effect. This observation is consistent with the findings of Horwitz et al. [19] in intact cells where microinjection of recombinant protein containing a Ser to Asp mutation to mimic phosphorylation at Ser-63, but not on both Ser-25 and Ser-38, prevented the loss of the MT network induced by stathmin.

The downregulation of stathmin activity was enhanced when stathmin was simultaneously phosphorylated on the MAP kinase (Ser-25) and PKA (Ser-16 and Ser-63) sites. These data suggest that stathmin activity is regulated by phosphorylation by at least two different protein kinase signalling pathways. Interestingly, ectopic expression of stathmin mutants, substituted by alanine either at Ser-25 and Ser-38, or at Ser-16 and Ser-63, both resulted in a block in cell division [26,27] suggesting that multi-site phosphorylation of stathmin is required for normal cell division. Our observations are in agreement with findings of Horwitz et al. [19] who reported that non-phosphorylatable mutant recombinant proteins, when injected into cells, are more active than the wild-type protein in reducing the MT network. However, this study [19] using glutathione S-transferase-stathmin fusion protein, reported that Ser to Asp substitution on all four sites did not alter the activity of stathmin to block tubulin assembly in vitro. Because these mutants had nevertheless a dramatic effect in intact cells, the authors proposed the requirement of additional cellular factors for the regulation of stathmin activity.

Here we have shown that phosphorylation directly downregulates the ability of stathmin to inhibit MT polymerization. Moreover, we have demonstrated that the downregulation of the activity closely correlates with the ability of stathmin to form a complex with tubulin. While stathmin containing phosphorylation at the proline-directed kinase sites was able to bind tubulin, although at a reduced level, phosphorylation at Ser-16 and Ser-63 strongly reduced stathmin-tubulin complex formation. Phosphorylation on three sites (Ser-16, Ser-25, Ser-63) completely inhibited the tubulin binding. It is of note that the amplitude of the differences observed between the isoforms is smaller in the in vitro polymerization assay than in the binding assay which may be due to differences in the sensitivity of the assays. Nevertheless, in both assays, Ser-16 and Ser-63 appeared to be the crucial phosphorylation sites although concomitant phosphorylation of a third residue, Ser-25, further increased the effect. In summary, our data suggest that multiple phosphorylation of stathmin downregulates its activity by blocking its binding to tubulin heterodimers. The precise cellular function of stathmin in proliferating cells or during neuronal differentiation is not yet understood. However, it is likely that stathmin is involved in the reorganization of microtubules and that its microtubule-destabilizing activity is regulated by protein kinase systems in response to extracellular signals.

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