Control of Intrinsically Disordered Stathmin by Multisite Phosphorylation*

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Stathmin is an intrinsically disordered protein implicated in the regulation of microtubule dynamics and in the development of cancer. The microtubule destabilizing activity of stathmin is down-regulated by phosphorylation of four serine residues, Ser16, Ser25, Ser38, and Ser63. Here we have used calorimetric and spectroscopic methods, including nuclear magnetic resonance to analyze the properties of seven stathmin phosphoisoforms to bind tubulin and inhibit microtubule formation. We found that stathmin phosphorylation results in a substantial loss in hydration entropy upon tubulin-stathmin complex formation. Remarkably, a linear correlation between the free energy change of complex formation and the microtubule inhibition activities of stathmin phosphoisoforms was observed. This finding provides a biophysical basis for understanding the mechanism by which local stathmin activity gradients important for promoting localized microtubule growth are established. We further found that phosphorylation of Ser16 and Ser63 disrupts the formation of a tubulin-interacting β-hairpin and a helical segment, respectively, explaining the dominant role of these residues in regulating cell cycle progression. The insight into the tubulin-stathmin interaction offers a molecular basis for understanding the nature and the factors that control intrinsically disordered protein systems in general.

Intrinsically disordered proteins have gained enormous interest not only because they are recognized to play key roles in many central cellular processes including cell cycle control, signal transduction, and transcriptional regulation but also because of their particular importance for cancer development and protein deposition diseases (1–4). Recent genome data base searches indicated that >30% of all eukaryotic proteins may be completely or partially disordered (5). This high frequency of occurrence has provoked a change of the paradigm that stable proteins may be completely or partially disordered (5). This high frequency of occurrence has provoked a change of the paradigm that stable proteins may be completely or partially disordered (5).

In contrast, phosphorylation of Ser25 and Ser38 by an unknown kinase-phosphatase system allows creating local stathmin activity gradients, a process essential for regulating microtubule dynamics and spindle formation (30–33). Phosphorylation of all four serine residues at the G2/M transition occurs sequentially; Ser25 and Ser38 are first phosphorylated by Cdk1, with subsequent phosphorylation of Ser16 and Ser63 by unknown kinase systems (26). Phosphorylation of Ser16 and Ser63 strongly down-regulates the microtubule destabilizing activity of stathmin (26–29,34,35). In contrast, phosphorylation of Ser25 and Ser38 has only a moderate effect on down-regulation but is a prerequisite for allowing phosphorylation of Ser16 and Ser63 in vivo (26). The current knowledge of the tubulin-stathmin interaction provides a unique basis to gain detailed insight into factors regulating intrinsically disordered protein systems. To define how multiple phosphorylation sites control stathmin function, we here have explored seven stathmin phosphoisoforms by using biochemical and biophysical methods.

EXPERIMENTAL PROCEDURES

Protein Preparation—For the production of high amounts of pure and specific human stathmin phosphoisoforms, seven serine-to-alanine mutants were constructed: S25A,S38A,S63A (for p16), S16A,S25A,S38A (for p63), S25A,S38A (for p16,63), S16A,S63A (for p25,38), S63A (for p16,25,38), S16A (for p25,38,63), and S16A,S25A,S38A,S63A (4A). The microtubule-polymerization inhibition and tubulin binding activities,
The identities of stathmin proteins were assessed by mass spectral analyses. Concentrations of protein samples were determined by the Advanced Protein Assay (Cytoskeleton Inc.).

Tubulin-polymerization Assay—In vitro polymerization of tubulin was performed according to Ref. 26. Briefly, 4 μM tubulin in G buffer (80 mM PIPES-KOH, pH 6.8, 1 mM MgCl₂, 1 mM EGTA, 1 mM GTP) supplemented with 4 mM MgCl₂ was preincubated with 4 μM of stathmin (in the same buffer) for 30 min at room temperature in a total reaction volume of 100 μl. Polymerization was initiated by adding 1 μl of a 400 μM taxol stock solution and incubating at 37 °C for 1.5 h. Microtubules were separated from tubulin-stathmin oligomers by sedimentation at 300,000 × g for 15 min at 37 °C in an Optima TLX ultracentrifuge (Beckman Instruments). The protein contents of supernatants and pellets were analyzed with the bicinchoninic acid protein assay reagent (Pierce).

Biophysical Analysis—High sensitivity isothermal titration calorimetry experiments were carried out on a VP-ITC calorimeter (Microcal Inc., Northampton, MA). For each experiment, the temperature-controlled sample cell (volume 1.4 ml) was filled with either ~10 μM (for WT, 4A, p25,38, S16E, and S63E) or ~20 μM (for p16, p63, p16,63, p16,25,38, p25,38,63, and p16,25,38,63) GTP-tubulin in G buffer (80 mM PIPES-KOH, pH 6.8, 1 mM MgCl₂, 1 mM EGTA, 1 mM GTP). Either 5 μl (for WT, 4A, p25,38, S16E, and S63E) or 10 μl (for p16, p63, p16,63, p16,25,38, p25,38,63, and p16,25,38,63) of ~100 μM stathmin aliquots (present in the same buffer as tubulin) were injected into the sample cell. Binding isotherms were fitted using a nonlinear least squares minimization method assuming two independent and equal binding sites on stathmin for tubulin (8). The apparent molar heat capacity change of the binding reaction, ΔC_p, corresponds to the slope of the linear regression obtained form the fitting of the apparent ΔH values at different temperatures.

Protein samples (0.35 mg/ml) for CD were in phosphate-buffered saline (10 mM sodium phosphate, pH 7.4, 150 mM NaCl). Far-ultraviolet CD spectra and thermal unfolding profiles were recorded on a Jasco J-810 spectropolarimeter (Jasco Inc.) equipped with a temperature-controlled quartz cell of 0.1-cm path length. A ramping rate of 1 °C/min was used to record the thermal unfolding profiles.

15N,1H-HSQC NMR experiments of 19 mg/ml protein samples in G buffer were carried out at 25 °C on a Varian UnityPlus 600 spectrometer operating at 600 MHz proton frequency. For resonance assignment, three-dimensional 15N-edited TOCSY-HSQC using a clean DIPSI-2 mixing sequence and three-dimensional 15N-HSQC-TOCSY NOESY-HSQC were recorded.

Modeling—The 3.5 Å resolution x-ray crystal structure of the tubulin-RB3 stathmin-like domain (SLD) complex (PDB entry 1SAO), and the PyMol (DeLano Scientific LLC, San Carlos, CA) and Moloc (40) software packages were used for modeling studies. The accuracy of the conformations of key residue side chains was verified by inspecting the electron density map for PDB entry 1SAO. Solvent-accessible area calculations were carried out with the program NACCESS V2.1.1 with the default set of atomic radii and parameters. The stathmin residues 29–45 were taken from PDB entry 1SA0. Solvent-accessible area calculations were carried out with the program NACCESS V2.1.1 with the default set of atomic radii and parameters. The stathmin residues 29–45 were taken from PDB entry 1SA0. Solvent-accessible area calculations were carried out with the program NACCESS V2.1.1 with the default set of atomic radii and parameters. The stathmin residues 29–45 were taken from PDB entry 1SA0. Solvent-accessible area calculations were carried out with the program NACCESS V2.1.1 with the default set of atomic radii and parameters. The stathmin residues 29–45 were taken from PDB entry 1SA0. Solvent-accessible area calculations were carried out with the program NACCESS V2.1.1 with the default set of atomic radii and parameters. The stathmin residues 29–45 were taken from PDB entry 1SA0. Solvent-accessible area calculations were carried out with the program NACCESS V2.1.1 with the default set of atomic radii and parameters. The stathmin residues 29–45 were taken from PDB entry 1SA0. Solvent-accessible area calculations were carried out with the program NACCESS V2.1.1 with the default set of atomic radii and parameters. The stathmin residues 29–45 were taken from PDB entry 1SA0. Solvent-accessible area calculations were carried out with the program NACCESS V2.1.1 with the default set of atomic radii and parameters.

RESULTS AND DISCUSSION

For the following studies, milligram amounts of pure and specific single (denoted p16 and p63), double (denoted p25,38 and p16,63), triple (denoted p16,25,38 and p25,38,63), and quadruple (denoted

2 The abbreviations used are: MAPK, mitogen-activated protein kinase; PIPES, 1,4-piperazinediethanesulfonic acid; HSQC, heteronuclear single quantum correlation; SLD, stathmin-like domain.
p16,25,38,63) stathmin phosphoisoforms were produced (Fig. 2A). The activities of the proteins were assessed in vitro by a microtubule polymerization assay. Under the experimental conditions applied the efficiency to inhibit microtubule formation decreased from 90 to 0% with a differential combination of stathmin phosphorylation (Fig. 2B). These findings are consistent with stathmin sequestering tubulin dimers into assembly-incompetent complexes (9, 10), a process controlled by phosphorylation. In agreement with the microtubule polymerization data obtained in vitro (26–29), phosphorylation of Ser16 and Ser63 contributes most to stathmin inactivation.

The thermodynamics of the tubulin-stathmin interaction was assessed by isothermal titration calorimetry (supplemental Fig. 1). Between 6 and 25 °C, stathmin binds two tubulin subunits and all thermodynamic parameters are thus referred to the ternary T2S complex (supplemental Table 1). As shown in Fig. 2C, the binding reaction is predicted to be driven by both enthalpy and entropy at physiological temperatures. The large apparent negative heat capacity change of ΔCp,T2S,obs = −1504 ± 206 cal mol⁻¹ K⁻¹ suggests that the hydrophobic effect (removal of non-polar surface from water) promotes T2S complex formation (36). As a consequence, the apparent entropic and enthalpic contributions to the free energy change of complex formation vary with temperature in a linear and nearly parallel manner, changing sign at −28 and −44 °C, respectively.

Empirical studies on proteins showed that the removal of hydrophobic and polar surface from water contributes −45 and 26 cal mol⁻¹/100 Å², respectively, to ΔCp (37). We have calculated the total buried hydrophobic and polar surface areas from the 3.5 Å resolution x-ray crystal structure of the ternary complex formed between the stathmin homologue RB3 and tubulin (denoted T2R, see Ref. 11) as 5316 and 3074 Å², respectively.

Accordingly, the estimated heat capacity change ΔCp,T2S,obs amounts −1593 cal mol⁻¹ K⁻¹, in good agreement with the experimentally obtained value for the tubulin-stathmin complex (see above). These findings are consistent with a mechanism in which dehydration of the protein-protein interface is the major driving force of T2S complex formation.

Isothermal titration calorimetry showed that each stathmin phosphoisoform bound two tubulin dimers as observed for unmodified stathmin (Fig. 2D). These findings are consistent with stathmin sequestering tubulin dimers into assembly-incompetent complexes (9, 10), a process controlled by phosphorylation. In agreement with the microtubule polymerization data obtained in vitro (26–29), phosphorylation of Ser16 and Ser63 contributes most to stathmin inactivation.

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lar systems. Single phosphorylation of Ser16 or Ser63 increases $K_{D,T_2S}$ 17- and 113-fold, respectively. A strong effect was found with p16,63, which displays a 233-fold reduced binding affinity. Dual phosphorylation of Ser25 and Ser38 reduced $K_{D,T_2S}$ only 4-fold, and the down-regulating effect of the single phosphoisoforms, p16 and p63, was only marginally enhanced (on average 1.4-fold) by additional phosphorylation of Ser25 and Ser38. The isothermal titration calorimetry data of p16,25,38,63 could not be evaluated because binding was too weak. For all measured stathmin phosphoisoforms, a reduced binding entropy that is partially offset by an increased binding enthalpy is observed (Fig. 2D).

The finding that phosphorylation of Ser16 and Ser63 contributes most to the reduced binding of stathmin explains the dominant role of these residues for in vivo inactivation (26–29). The moderate effect obtained with phosphorylation of Ser25 and Ser38 correlates with their location in the proline/serine-rich loop segment of stathmin (Fig. 1), which is poorly ordered in the T2R complex (11, 12). The local perturbation caused by phosphorylated Ser25 and Ser38, however, is expected to facilitate phosphorylation of the adjacent Ser16 and Ser63 residues as suggested from in vivo data (26).

Remarkably, a linear correlation between the free energy of $T_2S$ complex formation and the tubulin polymerization inhibition activities of stathmin phosphoisoforms is observed (Fig. 3). In agreement with cell biological data (26–29), this correlation suggests that already moderate changes in the tubulin-stathmin equilibrium significantly influence microtubule dynamics and, as a consequence, microtubule function in a particular in vivo situation. This conclusion provides a biophysical basis for understanding how spatial gradients of differentially inactive stathmin molecules promote localized microtubule growth, a process essential for, e.g. mitotic spindle assembly (30–33).

The secondary structures and thermal stabilities of stathmin phosphoisoforms were probed by CD spectroscopy. CD recorded at a low temperature from unmodified stathmin revealed a spectrum with ~45% helical content (Fig. 4A). Characteristic of proteins lacking stable tertiary structure, a fully reversible, broad unfolding transition to a random coil structure was observed upon thermal denaturation (Fig. 4B). The phosphorylation of Ser63 reduces both the helical content (20–30% in the 5–30 °C temperature range) and the thermal stability of stathmin. In contrast, phosphorylation of Ser16, Ser25, and Ser38 affects only moderately its secondary structure throughout the 5–80 °C temperature range.
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Upon phosphorylation of unbound stathmin (see phosphoryl group on Ser63 introduces a kink in the helical backbone leading to the dispersion of the peptide sequence Glu55-Arg61 (23). The driving force of this distortion can be explained by the strong propensity of phosphoserine to interact with the main chain (38). Mutating Ser63 to glutamic acid (denoted S63E) only moderately affects the secondary structure, thermal stability, and tubulin binding affinity of stathmin (Table 1 and supplemental Fig. 2), underscoring the unique properties of the bulky diatomic phosphoryl group to disrupt the helical conformation of the helix nucleation site. This local effect explains the reduced tubulin binding activities of stathmin isoforms phosphorylated at Ser63 (25). The phosphoryl group hinders the alignment of residues Lys53, Leu54, and Arg57, which tightly interact with the 

The phosphoryl group is expected to result in a steric clash of its bulky phosphodiatomic group to disrupt the helical conformation, which slows down its solvent exchange rate. A close-up view of THR showing the location of the Ser16 side chain with respect to \( \alpha_1 \)-tubulin. Colors and representations are the same as in (Fig. 4C). B, superposition of \(^{15}N,^1H\) HSQC spectra of \(^{15}N\)-WT (black) and \(^{15}N\)p16 (red). Spectral changes that most likely originate from Ser16 and adjacent residues are indicated by blue arrows. The new signal at 7.55/118.2 ppm that is because of an arginine side chain forming a hydrogen bond (most likely Arg14) is indicated by a blue circle. The peak is invisible in \(^{15}N\)-WT because of fast solvent exchange, but visible in \(^{15}N\)p16, most likely because of interaction of the arginine side chain with the phosphorylated Ser16 residue, which slows down its solvent exchange rate. C, \(^{15}N,^1H\) HSQC spectra of tubulin-bound \(^{15}N\)-WT (in black with assignments) and \(^{15}N\)p16 (in red). The two resonances that have shifted upon phosphorylation of unbound stathmin (see A) and most likely originate from Ser16 and an adjacent residue are highlighted by circles.

As shown in Fig. 5A, in T2R Ser16 is located within the tight turn connecting the two \( \beta \)-strands of the \( \beta \)-hairpin. The residue is stabilized by an intermolecular hydrogen bond formed between its main chain oxygen atom and the side chain of \( \alpha_1 \)Asn356. As a consequence, Ser16 is oriented toward the \( \alpha_1 \)-tubulin surface, and an introduction of a phosphoryl group is expected to result in a steric clash of its bulky phosphorylated side chain. This prediction was tested by NMR experiments. The \(^{15}N,^1H\) HSQC measurements of \(^{15}N\)-labeled stathmin and p16 proteins revealed spectra with limited chemical shift dispersion (Fig. 5B), characteristic for intrinsically disordered proteins populating an ensemble of helical secondary structures. Comparison of these two spectra, however, reveals three prominent differences. One new peak (at 7.55/118.2 ppm), which originates from an arginine side chain forming a hydrogen bond (most probably Arg14), and two prominent N-H backbone resonance shifts were found in the \(^{15}N\)-p16 HSQC spectrum. The first peak shift (from 8.18/115.1 to 8.79/117.5 ppm) is in a spectral region typical for serine residues and is likely to originate from Ser16. The second peak shift (from 8.25/127.5 to 8.45/127.1 ppm) most probably stems from a residue close in sequence to Ser16.

Upon the addition of unlabeled tubulin to \(^{15}N\)stathmin, WT or S25A,S38A,S63A, all except the last eight C-terminal stathmin residues broaden beyond distinction in the HSQC spectrum because of the large ~200-kDa size of the complex. This finding demonstrates that most stathmin residues become tightly bound in T2S (Fig. 5C, black). In contrast, ~40 strong and several weaker resonances are visible in the spectrum of the complex formed with \(^{15}N\)p16 (Fig. 5C, red). Most of these resonances are broad showing limited chemical shift dispersion, characteristic for residues that are in rapid exchange between weakly bound and unbound random coil states. Some peaks most likely originate from the N-terminal domain of stathmin. First, both resonances shifting upon phosphorylation of unbound stathmin and which most likely stem from Ser16 and Arg14 (see above) are visible in the HSQC spectrum of the complex formed with \(^{15}N\)p16, indicating that they are not tightly bound to tubulin. Second, a stathmin fragment lacking the first 40 N-terminal residues (denoted \( \Delta N \)) showed similar tubulin-binding properties as p16 (Table 1). Third, substituting glutamic acid for Ser16 (denoted S16E) partially mimics the effect of phosphorylation on T2S complex formation (Table 1 and supplemental Fig. 2). This finding is consistent with the hypothesis that steric clash with \( \alpha_1 \)-tubulin and intramolecular interaction of the bulky phosphoryl group with the backbone (38) and/or Arg14 side chain (25) is the underlying mechanism. These data demonstrate that phosphorylation of Ser16 strongly impairs binding of the \( \beta \)-hairpin to \( \alpha_1 \)-tubulin and explain the reduced tubulin-
binding activities of stathmin isoforms phosphorylated at Ser\textsuperscript{16}. As Ser\textsuperscript{16} is conserved throughout stathmin family proteins (16), this mechanism is expected to apply to all stathmin homologues.

From a thermodynamic point of view the down-regulating effect of stathmin phosphorylation can be explained by the substantial loss in hydration entropy upon T\textsubscript{2}S complex formation, which is larger than the gain in enthalpy of the system. Consistent with this conclusion, phosphorylation of Ser\textsuperscript{16} and Ser\textsuperscript{63} disrupts the formation of the \(\beta\)-hairpin and the helix nucleation site, respectively, impairing binding of these two key secondary structure elements to \(\alpha\)-tubulin and leading to the exposure of non-polar surface to water. This finding opens an avenue to design strategies to interfere with abnormal microtubule dynamics observed in many human malignancies displaying high levels of stathmin expression (16). Targeting the \(\beta\)-hairpin and/or helix nucleation site of stathmin is expected to perturb the dynamic equilibrium of the microtubule filament system possibly inhibiting tumor invasion in vivo (21) or even inducing apoptosis of transformed cells (39).

Taken together, our findings provide new mechanistic insight into the control of stathmin function by multisite phosphorylation. They further offer a molecular basis for understanding the nature and modes of regulation of intrinsically disordered protein systems in general.

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