Phosphorylation disrupts the central helix in Op18/stathmin and suppresses binding to tubulin

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Protein phosphorylation represents a ubiquitous control mechanism in living cells. The structural prerequisites and consequences of this important post-translational modification, however, are poorly understood. Oncoprotein 18/stathmin (Op18) is a globally disordered phosphoprotein that is involved in the regulation of the microtubule (MT) filament system. Here we document that phosphorylation of Ser63, which is located within a helix initiation site in Op18, disrupts the transiently formed amphipathic helix. The phosphoryl group reduces tubulin binding 10-fold and suppresses the MT polymerization inhibition activity of Op18's C-terminal domain. Op18 represents an example where phosphorylation occurs within a regular secondary structural element. Together, our findings have implications for the prediction of phosphorylation sites and give insights into the molecular behavior of a globally disordered protein.

INTRODUCTION

Proteins are generally thought to require a well defined three-dimensional structure for function. More recently, however, many examples of native proteins and protein domains have become known that do not exhibit a stable fold under physiological conditions. The steadily increasing number of such cases has provoked a re-assessment of the protein structure–function paradigm (Wright and Dyson, 1999). A detailed molecular understanding of intrinsically flexible or partially disordered proteins is of great interest not only because they are frequently involved in a wide variety of essential regulatory functions in the cell, but also because of their particular importance for protein folding and in the development of amyloid diseases (reviewed in Wright and Dyson, 1999). Moreover, the structural consequences of reversible protein phosphorylation, a mechanism

frequently used in living cells to control the activity of disordered molecules and proteins in general, are only poorly documented.

Oncoprotein 18/stathmin (Op18), a ubiquitous cytosolic phosphoprotein that destabilizes microtubules (MTs) and forms specific complexes with tubulin dimers (Sobel, 1991; Belmont and Mitchison, 1996), represents an archetypal example of a globally disordered protein in which stable tertiary structure formation is coupled to interaction with its target protein. We recently reported that the C-terminal domain of the monomeric Op18 molecule can fold into a transient and extended helical structure, which is in rapid equilibrium with a disordered conformation (Steinmetz *et al.*, 2000). Deletion mapping of Op18 demonstrated that this 100-residue-long helix (residues Lys41 to Lys140; Figure 1) is both sufficient and necessary for stable ternary complex formation with two α/β -tubulin heterodimers (Steinmetz *et al.*, 2000). The N-terminal part of Op18 was found to contain only marginal if any secondary structure (Wallon *et al.*, 2000). However, all except the last seven Op18 residues undergo a dramatic disorder-to-order transition upon binding to tubulin (Steinmetz *et al.*, 2000). In agreement with these findings, the recently solved 4 Å resolution X-ray structure of a tubulin–stathmin-like domain complex revealed a continuous and extended 91-residue-long helix regularly bound along two head-to-tail-aligned tubulin subunits (Gigant *et al.*, 2000).

It is well known that sequential cell cycle-dependent phosphorylation on four serine residues, Ser16, 25, 38 and 63 (Figure 1) abolishes the MT-destabilizing activity of Op18, whereby phosphorylation of Ser63 within the autonomous C-terminal domain contributes substantially to Op18 inactivation (reviewed by Lawler, 1998). In order to gain a deeper insight into the

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Fig. 1. Schematic representation of human Op18 and various polypeptide chain fragments used in this study. The size of the fragments is denoted above and to the left of the bars by amino acid numbers. The N- (aa 1–40) and the C-terminal helical (aa 41–140) domains of the wild-type Op18 are indicated. Asterisks mark the positions of the four serine residues, Ser16, 25, 38 and 63, that become phosphorylated during progression through the cell cycle.

conformational behavior of human Op18, and to assess the molecular consequences of Ser63 phosphorylation, we performed a detailed study using a selection of recombinant and synthetic polypeptide chain fragments shown in Figure 1.

RESULTS

Circular dichroism (CD) spectroscopy was employed to monitor the secondary structure and thermal stability of the Op18-derived fragments (Figure 1). As illustrated in Figure 2A, at 25°C the entire C-terminal domain Op18-[41–149] and the shorter truncation mutant Op18-[41–110] revealed a spectrum characteristic of a helical conformation. In contrast, the N-terminal truncation mutant Op18-[76–149] showed only marginal secondary structure. Comparison of the spectra of Op18-[41–110] and Op18-[76–149] with that of Op18-[41–149] suggests that the segment Lys41–Lys75 is particularly important for driving helix formation in Op18. CD analysis of the first 35 (Op18-[41–75]), the middle 30 (Op18-[76–105]), and the last 35 residues (Op18-[106–140]) of the C-terminal domain supports this suggestion. As shown in Figure 2B, both Op18-[76–105] and Op18-[106–140] were only marginally helical at 5°C. Moreover, their amount of residual structure rapidly decreased with increasing temperature and the fragments were largely denatured by ~25°C (Figure 2C). In contrast, Op18-[41–75] revealed a helical content of ~60 and ~40% at 5 and 25°C, respectively, as estimated by CD. However, the broad non-cooperative thermal unfolding profile of Op18-[41–75], whose progression was very similar to the profile recorded for the full-length Op18 molecule (Steinmetz *et al.*, 2000), indicates an ensemble of rapidly fluctuating helical structures rather than the formation of a well defined helix. Together, these findings are characteristic of Lys41–Lys75 representing a helix initiation site (Baldwin and Rose, 1999) in Op18. A helical-wheel representation of the sequence is shown in Figure 2D.

To test whether phosphorylation of Ser63 affects the intrinsic helicity of the C-terminal domain of Op18, *in vitro* phosphoryl-

Fig. 2. The segment Lys41–Lys75 represents a helix initiation site. (A) Far-UV CD spectra of Op18-[41–149], Op18-[41–110] and Op18-[76–149] recorded at 25°C. (B and C) CD spectra recorded at 5°C and thermal unfolding profiles recorded at 222 nm of Op18-[41–75], Op18-[76–105] and Op18-[106–140]. (D) Helical wheel representation of the sequence Lys41–Lys75 of Op18. The starting residue, Lys41, is underlined. The curved line indicates the hydrophobic seam of the helix. Boxes mark the hydrophobic residues that were shown to be critical for the interaction of Op18 with tubulin, and Ser63 is highlighted by a circle.

ated Op18-[41–140] was produced using cyclic AMP-dependent protein kinase (PKA). Mass spectrometric peptide mapping confirmed that Ser63 was single-site phosphorylated to >95%. As illustrated in Figure 3A, at 25°C the helical content of ~50% of the phosphorylated pOp18-[41–140] molecule was globally decreased by ~25% compared with its unphosphorylated counterpart. A decrease in helicity was observed throughout the temperature range from 10 to 50°C (not shown). Together, the CD analysis was consistent with a phosphorylation-induced shift in the equilibrium from helix to random coil. The functional consequences of the single-site phosphorylation were tested by plasmon resonance competition experiments (Figure 3B) and by an MT sedimentation assay (Figure 3C). Most significantly, pOp18-[41–140] revealed a 13-fold decrease in tubulin binding affinity (apparent dissociation constant $K_d = 5400 \pm 900$ nM) compared with Op18-[41–140] ($K_d = 400 \pm 70$ nM; note that the K_d of full-length Op18 is 120 ± 50 nM). Loss of binding affinity of the molecules to tubulin also affected their ability to inhibit taxol-driven MT polymerization. As illustrated in Figure 3C, 4 and 12 μ M of full-length Op18 and Op18-[41–140], respectively, were sufficient to completely inhibit the polymerization of a 4 μ M tubulin solution. In contrast, the ability of pOp18-[41–140] to inhibit MT formation was severely suppressed by the presence of the phosphoryl group at Ser63.

To characterize functionally the critical site encompassing Ser63 in more detail, we generated a pair of 19-residue peptides,

Fig. 3. Reduced helicity in Op18 upon *in vitro* phosphorylation of Ser63 and functional consequences. (A) Far-UV CD spectra of Op18-[41–140] and pOp18-[41–140] recorded at 25°C. (B) Plasmon resonance competition experiments at 25°C with calculated mean apparent dissociation constants K_d . (C) Taxol-driven MT polymerization inhibition assay at 37°C of wild-type Op18, Op18-[41–140] and pOp18-[41–140].

Op18-[55–73] and its phosphorylated form pOp18-[55–73], and analyzed them by CD and nuclear magnetic resonance (NMR). At 5°C and pH 7.4, Op18-[55–73] exhibited a helical content of ~50% (Figure 4D). Helix formation by the peptide is a monomolecular process and is not the result of aggregation, as judged from the lack of concentration dependence of $[\Theta]_{222}$ in the range of 0.01–1 mM (not shown). NMR of Op18-[55–73] at pH 7 and 5°C indicated that this short peptide is indeed extensively helical: typical nuclear Overhauser enhancements (NOEs) expected for helical structures were observed, including $\text{H}\alpha(i)\text{--H}\beta(i+3)$ NOEs. Secondary $\text{H}\alpha$ and $\text{C}\alpha$ chemical shifts derived from a $^{13}\text{C}, ^1\text{H}$ -heteronuclear single quantum correlation (HSQC) experiment (Figure 4A) showed typical upfield shifts for $\text{H}\alpha$ and downfield shifts for $\text{C}\alpha$. The $^3\text{J}(\text{HN--H}\alpha)$ coupling constants were consistently smaller by 1–2 Hz than their random-coil values (Smith *et al.*, 1996). The $\text{C}\alpha$ secondary chemical shift as a function of sequence (Figure 4B) revealed that the helical content was highest in the center of Op18-[55–73] and weakened towards both termini, indicating that the ends are frayed (see also Figure 4C). Helix formation of the peptide was further monitored as a function of pH (Figure 4D) and salt concentration (Figure 4E) by CD at 222 nm. The pH titration profile indicates that the charged form of the five glutamates, two lysines and two arginines, and the uncharged form of His64, significantly contribute to helix stability at physiological pH. Increasing sodium chloride concentrations at pH 7.4 only moderately diminished helix formation: at 2.5 M sodium chloride the peptide still showed ~40% helicity. These findings suggest that in Op18-[55–73], a major factor in the interaction between fully charged side chains is hydrogen bonding and not merely electrostatic interaction (Smith and Scholtz, 1998). Accordingly, several putative $i, i+3$ and $i, i+4$ salt bridges (referred to as hydrogen-bonded ion pairs) between side chains most probably establish a network of interactions that accounts for the observed helix stability of Op18-[55–73].

A strong conformational impact was observed upon phosphorylation of Ser63. Compared with Op18-[55–73], at 5°C and pH 7.4, the phosphorylated peptide showed a loss in global helical structure of ~50% (Figure 4D). NMR of pOp18-[55–73]

revealed that all NOEs indicative of helical structure are significantly weaker or entirely absent. Secondary chemical shifts still indicate some helical structure, but to a lesser extent, and $^3\text{J}(\text{HN--H}\alpha)$ coupling constants showed values that are similar or slightly higher than in the random-coil state. Analysis of secondary chemical shifts along the sequence (Figure 4B) showed that the decrease in helicity is not uniform. While some helical content, although decreased by ~50%, persists in the region C-terminal to pSer63, no helicity remained in the N-terminal part (Figure 4C). Hence, it appears that the local conformational impact of the phosphoryl group on the sequence encompassing Ser63 is much more substantial compared with its global effect on the full-length C-terminal helix of Op18. The pH titration profile obtained with pOp18-[55–73] by CD at 222 nm indicates that deprotonation of the second proton of the phosphoryl group ($\text{p}K_{a2} \sim 6$) between pH 5 and 7 strongly disfavors helix formation (Figure 4D). Increasing sodium chloride concentrations essentially abolished the residual helicity of the phosphopeptide (Figure 4E), indicating that screening of the two negative charges of pSer63 did not restore helix structure. These findings are most consistent with a decrease in the intrinsic helix-forming propensity of the bulky dianionic form of pSer compared with Ser (Szilák *et al.*, 1997), and not with a major unfavorable charged–charged interaction of the covalently bound phosphoryl group with other surrounding side-chain carboxylates. In this context, phosphorylated residues have been reported to exhibit a strong propensity to hydrogen bond to the backbone, which influences the preferred conformation of peptides (Tholey *et al.*, 1999). However, the loss of helicity upon salt screening suggests that a favorable electrostatic interaction between the two negative charges of pSer63 and surrounding basic side chains and/or the helix dipole of the persisting C-terminal helix (Figure 4B) might, nevertheless, contribute to the residual helicity of pOp18-[55–73] at physiological pH and ionic strength.

DISCUSSION

It is now established that the length of the fully folded C-terminal helix allows Op18 to bind in an extended fashion along nearly

Fig. 4. Structural analysis of Op18-[55–73] and pOp18-[55–73]. (A) H α /C α region of ^{13}C , ^1H -HSQC spectra of unphosphorylated Op18-[55–73] (black) and phosphorylated pOp18-[55–73] (red) recorded at 5°C. Arrows connect signals from corresponding residues. A shift in peak position towards the upper left indicates loss of helical structure. (B) Corresponding diagrams of C α secondary chemical shifts, defined as the difference between the observed shifts and their random-coil values. Positive and negative secondary chemical shifts are characteristic of α -helical and β -sheet structure, respectively. (C) Ensembles of 20 calculated structures of Op18-[55–73] (left) and pOp18-[55–73] (right). (D and E) pH dependence of $[\Theta]_{222}$ and effect of sodium chloride on $[\Theta]_{222}$ of Op18-[55–73] (black circles) and pOp18-[55–73] (open circles) recorded at 5°C. Throughout the pH range and the salt concentrations evaluated, helix formation by the peptides remained strongly temperature dependent and occurred as a monomolecular reaction. The amino acid sequence of Op18-[55–73] is 55-EAAEERRKSHEAEVLKQLA-73. The PKA recognition sequence is underlined and Ser63 is in bold.

the entire length of two head-to-tail-aligned α/β -tubulin heterodimers (Gigant *et al.*, 2000; Steinmetz *et al.*, 2000). Due to a lack of resolution, the interaction face of the stathmin-like helix with tubulin could not be defined in the 4 Å X-ray resolution map (Gigant *et al.*, 2000). However, it is important to note that this extended and continuous helix is amphipathic, which is substantiated by the presence of heptad repeats throughout the sequence Lys41–Lys140 (Sobel, 1991). This sequence motif is well known to mediate α -helical coiled-coil interactions by a hydrophobic seam. It has been reported that both the MT-destabilizing

activity of Op18 and its capacity to sequester tubulin subunits under MT-forming conditions are especially sensitive to mutations of Leu47, Ile50 and Leu54 (Larsson *et al.*, 1999a; Figure 2D), three residues occupying hydrophobic positions of the second and third heptad repeat [i.e. as defined by the COILS algorithm using a window width of 21 (Lupus *et al.*, 1991)]. It is, therefore, reasonable to assume that the transient amphipathic helix initiated by the sequence surrounding Ser63 mediates association with tubulin subunits by its hydrophobic seam and promotes further folding of the entire Op18 molecule upon

binding. The importance of transient amphipathic helix-mediated interactions between a disordered protein and its binding target is a frequently encountered mechanism in other disordered protein systems (Wright and Dyson, 1999).

The effect of phosphorylation of a threonine or serine in a helix has been documented for a designed, non-native, dimeric bZIP coiled coil (Szilák *et al.*, 1997). The position of the residues was chosen on the solvent-exposed side of the coiled coil. Phosphorylation of these residues dramatically decreased the leucine-zipper stability, which reduced DNA binding 100-fold. The authors concluded that phosphorylation can regulate coiled-coil stability through destabilization of the helices and not by disrupting the protein–protein interface. In this context, Op18 represents a natural example where phosphorylation of a Ser residue occurs in the middle of a continuous helix. Notably, Ser63 is located on the opposite side of the hydrophobic seam formed by Op18-[41–75] (Figure 2D). According to the findings of Szilák *et al.* (1997), the decreased affinity of pOp18-[41–140] for tubulin might primarily be due to a loss of local structure. However, without an atomic resolution structure of the Op18–tubulin complex at hand, it can not be ultimately excluded that the bulky doubly charged phosphoryl group on Ser63 is also involved at the protein–protein interface. Nevertheless, it has been reported that replacement of the four serines of Op18 with glutamates (i.e. in order to mimic the negative charge imposed by phosphorylation) only modestly influences the tubulin-sequestering capacity of Op18 as compared with the fully phosphorylated inactive molecule (Curmi *et al.*, 1997; Larsson *et al.*, 1999b).

The most probable explanation for the effect of pSer63 on the preferred backbone dihedral of the surrounding residues is the disruption of the salt bridge network that drives helix formation in Op18-[55–73]. Strikingly, disruption of helix structure predominates at the N-terminal side of Ser63, towards Leu47, Ile50 and Leu54. Perturbation of this site by long-range interactions might hinder the proper alignment of these critical residues into the amphipathic helix that mediates association of Op18 with tubulin. Notably, the phosphorylation-controlled, microtubule-associated protein Tau is likewise intrinsically disordered (Schweers *et al.*, 1994). In one case, site-specific phosphorylation of Tau appears to locally influence the *cis*–*trans* prolyl isomerization equilibrium ratio of the target sequence (Daly *et al.*, 2000). The decreased rate of proline isomerization leads to decreased tubulin binding of a domain C-terminal to the phosphorylation site. Although different to the suggested molecular mechanism of pSer63 in Op18, this finding further supports the view that local phosphorylation-induced conformational perturbation can influence the binding properties of disordered proteins by long-range interactions. Finally, such a mechanism is distinct from other known mechanisms of intrinsically disordered protein assemblies where the negative charge of the phosphate moieties contributes directly to the binding free energy for complex formation (Wright and Dyson, 1999; Ostegaard *et al.*, 2000).

Residues that are targets for protein kinases are usually located within surface-exposed flexible loops or at the ends of helices and strands. Furthermore, mobility in the region around the site of phosphorylation appears to be important for kinase substrate recognition (Johnson *et al.*, 1998). Intriguingly, the intrinsic flexibility of Op18 may therefore be a necessity for the regulation

of its interaction with tubulin and MTs. Together, these considerations have important implications for the prediction of protein phosphorylation sites. In fact, a statistical analysis of protein-kinase specificity determinants based on primary structure predicts that 20–30% of the putative phosphorylation sequences may adopt surface-exposed helical or extended structures (Kreepipuu *et al.*, 1998). This finding indicates that phosphorylation of a residue that is part of a regular secondary structure element may not be so rare. However, it will be important to verify whether the predicted target sequences must have some conformational flexibility as does Op18. Disruption of local secondary structure by post-translational phosphorylation may thus represent an attractive mechanism for the fine-tuning of the association/dissociation properties of protein assemblies.

METHODS

Protein expression and production of synthetic peptides.

Protein expression in *Escherichia coli* is reported elsewhere (Steinmetz *et al.*, 2000). The N-acetylated and C-amidated Op18-[41–75], Op18-[76–105], Op18-[106–140], Op18-[55–73] and pOp18-[55–73] synthetic peptides were assembled on an automated continuous-flow synthesizer employing standard methodologies. Exact concentrations of protein and peptide solutions were determined by quantitative amino acid analysis.

In vitro phosphorylation. Op18-[41–140] was phosphorylated *in vitro* with the recombinant murine cyclic AMP-dependent protein kinase (PKA) catalytic subunit. Enzyme (250 U) and protein (2 mg/ml in 40 mM Tris–HCl buffer pH 7.4 containing 10 mM MgCl₂ and 0.2 mM ATP) were incubated overnight at 37°C. The reaction was complete as judged by the quantitative shift of the protein band to higher mobility on an 8–25% native PAGE gel. Subsequent analysis by mass spectrometry (Müller *et al.*, 1999) showed >95% single-site phosphorylation at Ser63.

CD and NMR spectroscopy. For far-ultraviolet (UV) CD spectra and thermal unfolding profiles, all protein samples except the short 19-residue peptides were analyzed in 5 mM sodium phosphate, 150 mM sodium chloride pH 7.4. Op18-[55–73] and pOp18-[55–73] were analyzed in either 1 mM sodium phosphate pH 7.4 (spectra and ionic strength dependence) or 1 mM sodium citrate, 1 mM sodium phosphate, 1 mM sodium borate buffer (pH dependence). Peptide concentrations were adjusted to 0.15 mg/ml. For NMR, peptides were studied in pure water (90% H₂O, 10% D₂O) at pH 7.0. Peptide concentrations were 4.6 mM for Op18-[55–73], and 4.2 mM and 8.8 mM for pOp18-[55–73].

Plasmon resonance experiments and microtubule sedimentation assay.

Plasmon resonance competition experiments were carried out on a BIAcore™ 2000 system. Immobilization of wild-type Op18 to the sensor chip was according to Curmi *et al.* (1997). Measurements were performed with bovine brain tubulin (Cytoskeleton Inc.) in 80 mM K-PIPES buffer pH 6.8, 1 mM EGTA, 5 mM MgCl₂. The free tubulin concentrations of pre-equilibrated mixtures of tubulin α/β -heterodimers (3 μ M) and varying concentrations of Op18 constructs were determined from the plateau levels by comparison with a standard tubulin curve, as described in the BIAcore evaluation software handbook. Dissociation constants refer to the binding of tubulin tetramers. *In vitro* polymerization of tubulin (4 μ M) in assembly buffer (50 mM MES–KOH pH 6.8, 5 mM MgCl₂, 1 mM EGTA, 1 mM GTP,

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4 μ M taxol) was assessed according to Larsson *et al.* (1997) using a standard sedimentation assay. The protein contents of supernatants and pellets were analyzed with the bicinchononic acid protein assay reagent.

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