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Biochemical and Biophysical Research Communications 342 (2006) 342–348
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The biochemical effect of Ser167 phosphorylation on *Chlamydomonas reinhardtii* centrin ☆,☆☆

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Received 23 January 2006
Available online 7 February 2006

Abstract

Centrin is an EF-hand calcium-binding protein found in microtubule organizing centers of organisms ranging from algae and yeast to man. Phosphorylation in the centrin C-terminal domain occurs in mitosis and is associated with alterations in contractile fibers. To obtain insight into the structural basis for the functional effect of phosphorylation, *Chlamydomonas reinhardtii* centrin C-terminal domain phosphorylated at Ser167 (pCRC-C) has been produced and characterized. The structure of pCRC-C was compared to the unmodified protein by NMR spectroscopy. The effect of phosphorylation on target binding was examined for the complex of pCRC-C and a 19 residue centrin-binding fragment of Kar1. Remarkably, the efficient and selective phosphorylation by PKA was suppressed in the complex. Moreover, comparisons of NMR chemical shift differences induced by phosphorylation reveal a greater effect from phosphorylation in the context of the Kar1 complex than for the free protein. These results directly demonstrate that phosphorylation modulates the structure and biochemical activities of centrin.

Keywords: Centrin; Caltractin; Calmodulin; Calcium sensor; Kar1; EF-hand calcium-binding protein; Energetic coupling; Conformational exchange

The microtubule organizing center (MTOC) is a complex and dynamic cellular organelle required for cytoskeletal integrity. It is fundamental to many cellular processes, including chromosomal segregation, cytokinesis, fertilization, cellular morphogenesis, cell motility, and intracellular trafficking [1]. Even though their basic functions and numerous protein components are conserved in all eukaryotes, MTOCs exhibit diverse morphologies in structures ranging from the basal body in algae to the spindle pole body (SPB) in yeast to the centrosome in humans [2]. Many MTOC-associated proteins are phosphorylated in vivo [3], and phosphorylation of MTOC components plays an important role in regulating MTOC function [1,4].

Centrin (Cen; also known as caltractin) is one of the conserved components of the MTOC [5] and is found in organisms as diverse as plants, protozoa, yeast, and humans [6]. Genetic studies have shown that Cen is

☆ This work was supported by the National Institutes of Health Grants RO1 GM-40120 and RO1 GM 65484 (to W.J.C.), NIH-SCORE Grant 5-S06GM08103 (to B.P.-R.), and R25 GM67072 (to Vanderbilt Summer Research Experiences for Undergraduates), with facilities supported by the Vanderbilt Center in Molecular Toxicology Grant (P50 ES00267) and Vanderbilt Ingram Cancer Center (P30 CA68485).

☆☆ *Abbreviations:* MTOC, microtubule organizing center; SPB, spindle pole body; Cen, centrin; pCen, phosphorylated centrin; CRC-C, 77-residue C-terminal fragment of *Chlamydomonas reinhardtii* centrin; CaM, calmodulin; Cdc31, yeast centrin; K_{19}, 19-residue Cdc31-binding domain of Kar1; PKA, protein kinase A; HSQC, heteronuclear single quantum coherence.

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.01.155

required for cell cycle-dependent duplication and segregation of the MTOC [7]. This function involves interaction with several centrosomal components, including Kar1 [8–10], Mps3 [11], and Sfi1 [12]. In *Chlamydomonas reinhardtii*, centrin mediates fiber-contraction, which is crucial to microtubule severing and subsequent flagellar excision [13]. Other cellular functions characterized include: mediation of cell integrity/morphogenesis via interaction with the Kic1 protein kinase in yeast [10]; global genome excision repair through interaction with XPC and Rad23B in humans [14]; modulation of homologous recombination and nucleotide excision repair in *Arabidopsis* [15]; and participation in nuclear mRNA export in yeast [16,17].

Centrin is an EF-hand calcium-binding protein closely related to the ubiquitous calcium sensor calmodulin (CaM). Cen and CaM share approximately 50% sequence identity. Both proteins are composed of two independent globular domains tethered by a flexible linker with each domain comprised of two helix-loop-helix calcium-binding motifs known as EF-hands. Studies of the interactions of CaM with kinase targets revealed binding through a wrap-around mode where the two domains of CaM act in concert to bind the target molecule [18]. As new targets have been discovered, new mechanisms of binding have been observed including extended and dimerization modes (reviewed in [19]). The two domains of Cen are structurally and functionally independent and mounting evidence suggests that Cen most commonly interacts with its targets in the extended binding mode [8,20–22]. The structure of $(Ca^{2+})_2$-CRC-C in complex with a peptide fragment of Kar1 has been determined [23] and reveals that CRC-C occupies an open conformation similar to that adopted by CaM domains in their Ca^{2+}-activated state. $(Ca^{2+})_2$-CRC-N also adopts an open conformation [24], whereas the apo state adopts a closed conformation similar to apo CaM [25].

Cen and CaM have distinctly different calcium-binding properties. CaM has four high affinity calcium-binding sites with dissociation constants (K_d) ranging from 1 to 10 μM under physiological salt concentrations [26], well within the range for effective response to intracellular Ca^{2+} signals. In contrast, most centrins have one or more non-functional calcium-binding sites. For example, the macroscopic binding affinity of the C-terminal domain of *C. reinhardtii* centrin (CRC-C) is significantly weaker than that observed for typical EF-hand calcium sensors such as CaM [20,23]. Despite its weak in vitro macroscopic calcium binding affinity, it is CRC-C not CRC-N that mediates the interaction with Kar1 and other target proteins [9,21]. This arises because the microscopic-binding constants in the two sites of CRC-C are nearly three orders of magnitude different such that site IV is an effective calcium sensor with a K_d of ~1 μM [21].

Cen is known to be phosphorylated in vivo and the phosphorylated protein is associated with contractile fibers found in the basal bodies of *Tetraselmis striata* [27]. The phosphorylation state of Cen is very sensitive to changes in the cellular environment; elevated calcium levels or heat stress can lead to dephosphorylation, while abrupt changes in pH or exposure to toxic compounds can induce phosphorylation [28]. Cen has been shown to be phosphorylated during mitosis by an as yet undetermined kinase. Interestingly, the same studies noted that in breast adenocarcinoma cells, phosphocentrin (pCen) is present at inappropriate times during the cell cycle and accumulates in tumor cell centrosomes, indicating a malfunction in cell cycle regulation [29].

Here, we report structural studies of PKA phosphorylated CRC-C. These represent the first steps toward characterizing the mechanism by which phosphorylation of Cen may serve to regulate interactions between it and other MTOC protein components, and in turn regulate the cell cycle.

Materials and methods

Protein and peptide preparation. Recombinant *C. reinhardtii* centrin C-terminal domain was expressed and purified as described elsewhere [20]. The 77-residue construct used in this study consists of residues Gly95 through Phe169, with an additional Gly-Ser sequence at the N-terminus left after cleavage of the $(His)_6$ tag.

K_{19}, the 19-residue peptide (KKRELIESKWHRLLFHDKK) comprising the essential yeast centrin-binding site in Kar1 (239–257) [8], was synthesized and purified as previously described [21].

Protein phosphorylation and purification. CRC-C was phosphorylated at 30 °C in vitro using PKA (Sigma–Aldrich, St. Louis, MO) in 25 mM KH_2PO_4, 50 mM $MgCl_2$, and 5 mM $CaCl_2$, pH 6.7. Seventy-five microliters of PKA (1 U/μl), 40 μl of 4 mM DTT, and 25 μl of 5 mM ATP were mixed with 360 μl of CRC-C (1 mg/ml). The reaction was stopped by flash-freezing. Phosphorylated CRC-C (pCRC-C) was purified from the reaction mixture using an anion exchange column (SourceQ, GE Healthcare, Piscataway NJ) equilibrated in buffer A (25 mM Tris–HCl, 25 mM NaCl, and 5 mM EDTA at pH 8.0). Elution was accomplished over eight column volumes by gradient addition of buffer B (25 mM Tris–HCl, 1 M NaCl). The purity of the protein fractions was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 4–12% gradient polyacrylamide gels (Invitrogen, Carlsbad, CA).

Fluorescence spectroscopy. All fluorescence experiments were performed on a Spex Fluorolog 1681 fluorometer (Spex Industries Inc., Edison, NJ) at 25 °C. The excitation wavelength was 285 nm, with slit width set to 2.0 mm. Small aliquots of appropriate dilutions of a 1 mM pCRC-C stock solution in a solution containing 150 mM KCl, 25 mM Tris, and 10 mM $CaCl_2$ at pH 7.1, were added to a 5 μM (initial concentration) of K_{19} peptide solution under identical conditions. Corrections for background fluorescence were made by subtracting the spectra from identical solutions without peptide.

Mass spectrometry. CRC-C, pCRC-C, and molecular weight calibration standards were each crystallized in a matrix of 3,5-dimethoxy-4-hydroxy-cinnamic acid. The data were acquired by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry using a PerSeptive Biosystems Voyager Elite MALDI-TOF Mass Spectrometer (Applied Biosystems, Foster City, CA). The instrument was equipped with a 337 nm nitrogen laser, a 25 kV ion source with delayed extraction (DE) coupled to 2.0 m linear flight tube, and a 3.0 m effective length in reflector mode.

NMR spectroscopy. All NMR data were recorded at 30 °C on a Bruker DRX 500 MHz spectrometer equipped with a 5 mm TXI cryoprobe and single axis pulsed field gradient accessories. Standard ^{15}N–^{1}H HSQC spectra were acquired on samples of U-^{15}N-CRC-C and U-^{15}N-pCRC-C, and for solutions containing a 1.5-fold excess of K_{19} peptide. Each sample had a protein concentration of ~100 μM in a buffer containing 25 mM Tris-d_{11}, 150 mM KCl, 10 mM $CaCl_2$, and 10% $^{2}H_2O$ with a pH of 7.3. Data were processed in XWIN-NMR (v2.6; Bruker, Rheinstetten, Germany) and chemical shift data analyzed using XEASY [30].

Structure analysis. Graphical analysis of 3D structures was carried out using the program MOLMOL [31]. Initial model building for phosphor-

ylated CRC-C was accomplished using the program PSSHOW [32]. The structure was energy minimized and further refined using the AMBER suite of molecular simulation applications [33].

Results

Production of pCen

CRC-C was phosphorylated in vitro at Ser167 by PKA. The protein was first expressed and purified as described previously [20]. An optimized yield of phosphorylated protein (approaching 80%) was obtained by adding ATP at regular intervals during the reaction and using a total incubation time of 30 h. Incubation beyond 30 h increased the presence of secondary phosphorylation without substantially increasing yield of the desired product.

Anion exchange chromatography of the resulting mixture provided complete separation of the unphosphorylated and phosphorylated proteins into two peaks which are pure as verified by SDS–PAGE (Fig. 1A). Each fraction was further characterized by MALDI-TOF mass spectrometry, which showed the appropriate mass ions for CRC-C before and after addition of the 80 Da phosphate group (Fig. 1B). Analysis by SDS–PAGE and MALDI-TOF MS of pCRC-C following NMR and fluorescence-binding experiments (*vide infra*) shows that the protein remains stably phosphorylated for 2 days at 25 °C, for 4 weeks at 0 °C, and indefinitely at −30 °C. Remarkably, attempts at phosphorylating the CRC-C/ K_{19} complex were all unsuccessful, even after multiple additions of ATP and 30 h of incubation (data not shown).

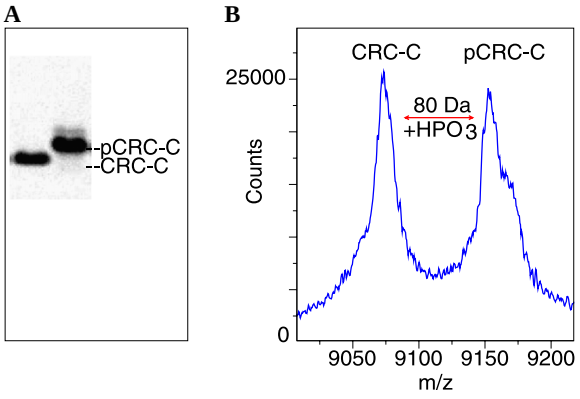

Fig. 1. PKA phosphorylation of CRC-C. (A) SDS–PAGE of CRC-C before and after phosphorylation by PKA. (B) MALDI-TOF mass spectrum of CRC-C and pCRC-C. Higher molecular weight of the mass ion by 80 U is consistent with the addition of one phosphate group to the protein.

Structural characterization of pCen

NMR was used to determine if changes in the structure of CRC-C occur following phosphorylation at Ser167 by PKA. The ^{15}N–^{1}H HSQC spectrum of pCRC-C (Fig. 2A) exhibits good dispersion of resonances consistent with a

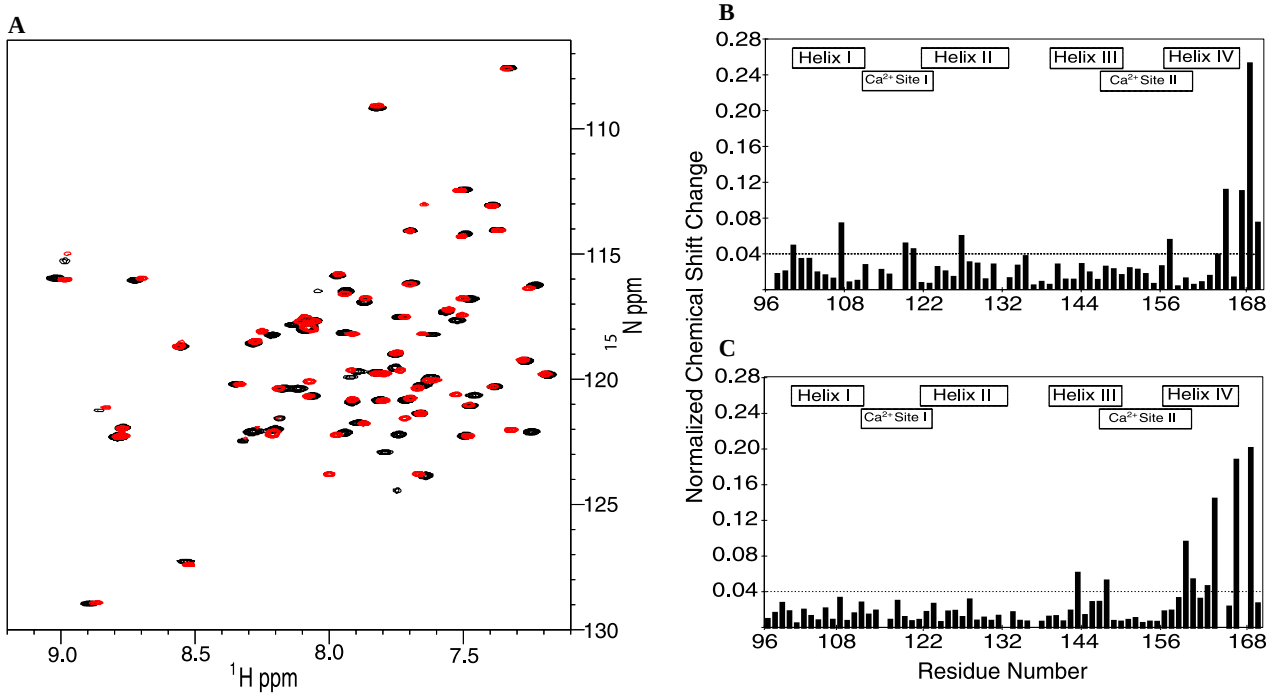

Fig. 2. Structural characterization of the complex of pCRC-C and the complexes with the Kar1 peptide. (A) Superposition of 500 MHz ^{15}N–^{1}H HSQC NMR spectra acquired at 30 °C for CRC-C (black) and pCRC-C (red). The buffer was 25 mM Tris, 150 mM KCl, and 10 mM $CaCl_2$ at pH 7.3. Resonances for N60, I62, G116, and high-field side chain amide groups appear outside the region shown. (B) Plot of normalized ^{15}N and ^{1}H chemical shift differences between CRC-C and pCRC-C. (C) Plot of normalized ^{15}N and ^{1}H chemical shift differences between CRC-C/K_{19} and pCRC-C/K_{19} complexes. Dashed lines in (B) and (C) are drawn at one standard deviation from the mean. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

folded protein. The protein construct used for these experiments consists of 77 residues, including an N-terminal Gly-Ser remaining following thrombin cleavage of the $(His)_6$ tag. Sixty resonances of pCRC-C can be assigned to residues by comparison to the previously assigned resonances of CRC-C [21]. A detailed comparison of the residue-specific changes in CRC-C upon phosphorylation was made as summarized in Fig. 2B. The normalized chemical shift perturbation plot of the ^{15}N–^{1}H HSQC spectrum of the phosphorylated protein highlights the residues most affected by phosphorylation; these include Glu101, Phe107, Thr119, Ile120, Val126, Glu156, Met163, Thr166, Leu168, and Phe169. Ser167 is not resolved in either the CRC-C spectrum [21] or in the pCRC-C spectrum. Thus, the effect of phosphorylation is seen primarily in Helix IV, but extends through tertiary contacts to other regions of the protein.

Effect of phosphorylation on target binding

In order to demonstrate that CRC-C retains function following phosphorylation, we examined the binding of the K_{19} peptide to pCRC-C. K_{19} contains a tryptophan residue that serves as a convenient fluorescent probe for monitoring binding to pCRC-C. As shown in Fig. 3, the Trp emission spectrum of K_{19} in the presence of pCRC-C displays a substantial increase in intensity and a large blue shift (approximately 30 nm at λ_{max}). These observations indicate specific binding of pCRC-C to K_{19}. The results are very similar to those obtained for the non-phosphorylated protein [21].

An NMR chemical shift perturbation analysis was performed for the pCRC-C complex with K_{19}, similar to that presented above for the free protein. The normalized chemical shift perturbation plot for the ^{15}N–^{1}H HSQC spectrum of the phosphorylated protein–peptide complex is shown in Fig. 2C. Significant chemical shift differences are observed for Ile143, Asp147, Phe159, Ile160, Ile162, Met163, Thr166, and Leu168. With the exception of Asp147 and Ile162, these residues constitute a significant portion of the hydrophobic CRC-C-binding interface with K_{19}. Of these residues, Met163, Thr166, and Leu168 are also directly affected by phosphorylation of CRC-C, and all three are located in the vicinity of Ser167. All of these residues participate in important hydrophobic interactions with the K_{19} centrin-binding motif [23], particularly with Trp10 of K_{19}.

In order to obtain deeper insight into the effect of Ser167 phosphorylation on the structure of CRC-C and the ability to bind targets, a model of the pCRC-C complex with K_{19} was generated. The high-resolution NMR structure of CRC-C in complex with K_{19} is available [23] and was used as a template. The primary effect of the addition of the phosphate group to Ser167 is alteration of the electrostatic surface of CRC-C. This is reflected in an increased negative charge distributed across the surface of the fourth helix and into the K_{19}-binding pocket (Fig. 4).

NMR chemical shift analysis implies the structure of the binding site for K_{19} is perturbed, which has implications for both the affinity and stability of binding. Fig. 5 shows a detailed view of the model of the complex at the binding interface. The presence of the phosphate group on Ser167 is seen to exert steric effects on Met163, Thr166, and Leu168, fully consistent with the NMR analysis. Perturbations of these residues in direct contact with K_{19} are also presum-

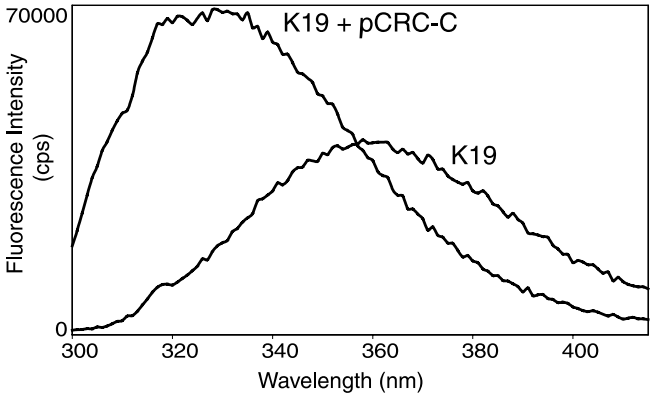

Fig. 3. Binding of pCRC-C to the Kar1 peptide. The intrinsic Trp fluorescence of the K_{19} peptide is changed upon addition of calcium-loaded pCRC-C.

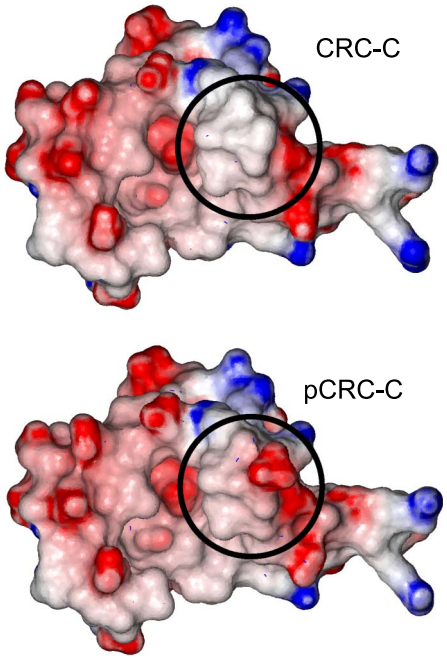

Fig. 4. Comparison of the electrostatic surfaces of CRC-C and pCRC-C. The distribution in the electrostatic charge potential is depicted as color gradients with negative charge in red and positive charge in blue. The coordinates used for CRC-C are from PDB identification code 1OQP, which was also used to build the homology model of pCRC-C. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

ably transferred further along Helix IV, resulting in the effects on Phe159 and subtle deformations in the target-binding pocket, e.g., so that Ile143 is perturbed as well. In summary, relative to the free pCRC-C, the structure of the protein is more significantly affected by phosphorylation in the context of the Kar1 complex.

Discussion

In vitro phosphorylation of CRC-C by PKA, although it does not disrupt binding between CRC-C and K_{19}, has implications for the interaction between centrin and Kar1. Fig. 5 highlights residue contacts between pCRC-C and K_{19}. Met163, Thr166, and Leu168 are slightly perturbed following phosphorylation, but the effect on these residues is intensified by K_{19} binding, which further perturbs the hydrophobic-binding interface of pCRC-C inducing significant shifts of Ile143 and Phe159. All of these residues in CRC-C participate in a key hydrophobic interaction with Trp10 of the K_{19} peptide (Phe159 also interacts with Leu14 on K_{19}), and these essential hydrophobic contacts are preserved in the interaction between pCRC-C and K_{19}.

Analysis of the electrostatic surface of the phosphorylated protein provides further insight into the binding of pCRC-C to K_{19} (Fig. 4). The addition of the phosphate group to CRC-C results in a new area of negative charge around Ser167, but also brings about a more general distribution of negative charge over the fourth helix of pCRC-C. This region of the protein is a key binding surface for the K_{19} peptide and these electrostatic changes may be largely responsible for modulating K_{19} binding to pCRC-C.

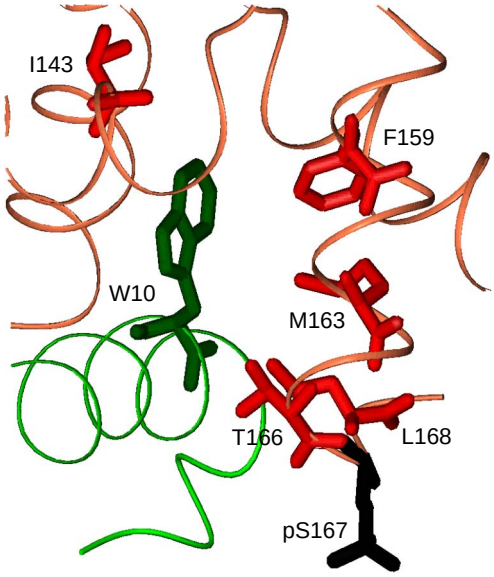

Fig. 5. Close up view of the model of the K_{19}-pCRC-C complex highlighting the binding interface. The pCRC-C backbone is colored salmon, phospo-Ser167 is black, the K_{19} backbone is light green, and the key Trp10 side chain is dark green. Side chains are drawn in dark red for key pCRC-C residues that exhibit significant NMR chemical shift perturbations upon binding of K_{19}. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Phosphorylation of Cen in vivo occurs at the G_2/M transition in the centrosomal cycle, after duplication but before separation of centrosomes [34]. Both the yeast centrin Cdc31 and its binding partner Kar1 are required for the initial stage of the SPB duplication process during early G1 [1], and both proteins also continue to be associated with the half-bridge throughout the cell cycle [11,12,35,36]. It would therefore seem logical to hypothesize that phosphorylation functions to disrupt the Kar1/Cen interaction, releasing Cen from the SPB complex so that it can participate in other functional complexes, e.g., those involved in DNA nucleotide excision repair. Our data, however, show that CRC-C in complex with K_{19} cannot be phosphorylated. This important observation provides an exciting perspective on the role of phosphorylation in the interaction of Kar1 and Cen. It would appear that dissociation of the Kar1 complex is required before Cen can be phosphorylated at the G_2/M transition. One possible albeit partial explanation of these data is that in addition to localizing centrin to the SPB early in the cell cycle, Kar1 functions to protect centrin from phosphorylation as a mechanism for keeping centrin anchored to the half-bridge through centrosome separation.

Kar1 may actually recruit centrin that has been phosphorylated as a consequence of some other cellular process for localization to the half-bridge. Such a hypothesis is supported by our results demonstrating that pCRC-C binds K_{19} peptide. Genetic studies suggest that Pkc1 kinase activity is required for proper Cdc31 function in vivo [37]. Although there is no direct evidence that Pkc1 phosphorylates Cdc31, other studies suggest a role for Pkc1 in regulating microtubule function by phosphorylation [38]. In one scenario, it may be that centrin is phosphorylated by Pkc1 at the MTOC in order to promote interaction of centrin with Kar1 and initiate chromosome duplication. This would not necessarily imply increased affinity between Kar1 and phosphocentrin; rather, assuming that phosphorylation of centrin disrupts other centrin-binding partner interactions, phosphocentrin would merely be more available to Kar1 binding. It would be interesting to determine if pCRC-C could be *dephosphorylated* while in complex with K_{19}.

Cdc31 is also known to interact with another kinase, Kic1, to promote cell integrity and morphogenesis. It is not known whether Kic1 phosphorylates Cdc31, but it has been shown that Kic1 kinase activity is dependent on Cdc31 [10]. Kic1 participates in numerous interactions involved in RAM regulation of cellular morphogensesis [39]. One hypothesis is that centrin modulates Kic1 function and that ultimately phosphorylation of centrin "switches off" Kic1 and releases Cdc31 for recruitment by Kar1 to the half-bridge.

Concluding remarks

The results presented here support the hypothesis that centrin function is modulated by phosphorylation. In particular, we demonstrated that phosphorylation of the C-terminal domain of centrin has implications for binding of centrin to Kar1. Further, the observation that Kar1 apparently protects centrin from phosphorylation provides insight into the nature of the SPB protein complex at the G_2/M transition of the cell cycle. More work is required to determine whether PKA is the only kinase to phosphorylate centrin in vivo. Experiments are also needed to determine whether phosphorylation by PKA or other kinases affects other Cen-binding partners. Along with further biophysical and structural analyses, these studies will provide new insights into the sequence of events involving centrin action in the centrosome.

Acknowledgments

We thank Susan DeSensi for assistance with the modeling and graphics, and Jonathan Sheehan for helpful discussions.

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