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Myosin Phosphatase-Targeting Subunit 1 Regulates Mitosis by Antagonizing Polo-like Kinase 1

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SUMMARY

Myosin phosphatase-targeting subunit 1 (MYPT1) binds to the catalytic subunit of protein phosphatase 1 (PP1C). This binding is believed to target PP1C to specific substrates including myosin II, thus controlling cellular contractility. Surprisingly, we found that during mitosis, mammalian MYPT1 binds to polo-like kinase 1 (PLK1). MYPT1 is phosphorylated during mitosis by proline-directed kinases including cdc2, which generates the binding motif for the polo box domain of PLK1. Depletion of PLK1 by small interfering RNAs is known to result in loss of γ-tubulin recruitment to the centrosomes, blocking centrosome maturation and leading to mitotic arrest. We found that codepletion of MYPT1 and PLK1 reinstates γ-tubulin at the centrosomes, rescuing the mitotic arrest. MYPT1 depletion increases phosphorylation of PLK1 at its activating site (Thr210) in vivo, explaining, at least in part, the rescue phenotype by codepletion. Taken together, our results identify a previously unrecognized role for MYPT1 in regulating mitosis by antagonizing PLK1.

INTRODUCTION

Protein phosphatase 1 (PP1) is known to play critical roles in a variety of mitotic events. In *Saccharomyces pombe* (Ohkura et al., 1989), as well as in *Aspergillus* (Doonan and Morris, 1989) and *S. cerevisiae* (Bloecher and Tatchell, 1999), mutations of its catalytic subunit (PP1C) have been demonstrated to cause defects in chromosome separation. In *Drosophila*, a PP1C mutation results in multiple effects including defective spindle organization and abnormal sister chromatid segregation (Axton et al., 1990). In mammalian cells, PP1C inactivation results in failure in anaphase progression, as well as in mitotic exit (Fernandez et al., 1992) and cytokinesis (Cheng et al., 2000).

These pleiotropic effects indicate that PP1C controls the phosphorylation states of diverse substrates in different locations and at various steps of cell division. Because PP1C itself has a broad substrate specificity, one critical question is how PP1C targets specific substrates at the right time and in the right place. Recent studies emphasize the critical role of PP1C-targeting subunits in targeting PP1C to specific substrates at precise locations (Ceulemans and Bollen, 2004; Cohen, 2002). More than 50 targeting subunits have been reported to form a complex with PP1C, and this would generate many forms of the phosphatase with distinct substrate specificities and/or intracellular targeting ability. Such complex formation with various targeting subunits provides a means for the limited number of PP1C isoforms to antagonize a wide array of serine/threonine kinases with different substrate specificities. Two recent studies, for example, have demonstrated that Repo-Man, a novel PP1C-targeting protein, is essential for recruiting PP1Cγ, one of three mammalian PP1C isoforms, to chromatin during anaphase and plays a critical role in the maintenance of chromosome architecture (Trinkle-Mulcahy et al., 2006; Vagnarelli et al., 2006). Because of the complexity of temporal and spatial control of mitotic events, a multitude of PP1C-targeting subunits must be involved. Despite the wealth of knowledge regarding the regulation of mitotic kinases, little is known of how PP1C-targeting molecules control various aspects of PP1C's functions in mitotic progression, or which serine/threonine kinases they antagonize.

Myosin phosphatase-targeting subunit 1 (MYPT1; also called MBS or M130) is a known regulator of PP1C (Cohen, 2002; Hartshorne et al., 2004). MYPT1 binds to PP1Cβ and to a 20 kDa subunit of unknown function to form a heterotrimeric holoenzyme. MYPT1 also binds to certain substrates, bringing that substrate and the catalytic subunit together. The MYPT1-containing holoenzyme is often called myosin phosphatase because it effectively dephosphorylates the regulatory light chain of myosin II and controls actomyosin contractility in smooth muscle and nonmuscle cells. This holoenzyme has also been shown to dephosphorylate, at least in vitro, other substrates including adducin, moesin, tau, MAP2, and a transcriptional repressor HDAC7, suggesting that MYPT1 could have broader functions than myosin regulation (Matsumura and Hartshorne, 2008).

We previously showed that MYPT1 is specifically phosphorylated during mitosis (Totsukawa et al., 1999). Here we identify these mitosis-specific phosphorylation sites, revealing that MYPT1 is phosphorylated by proline-directed kinases including cdc2 kinase. This phosphorylation generates a binding motif for

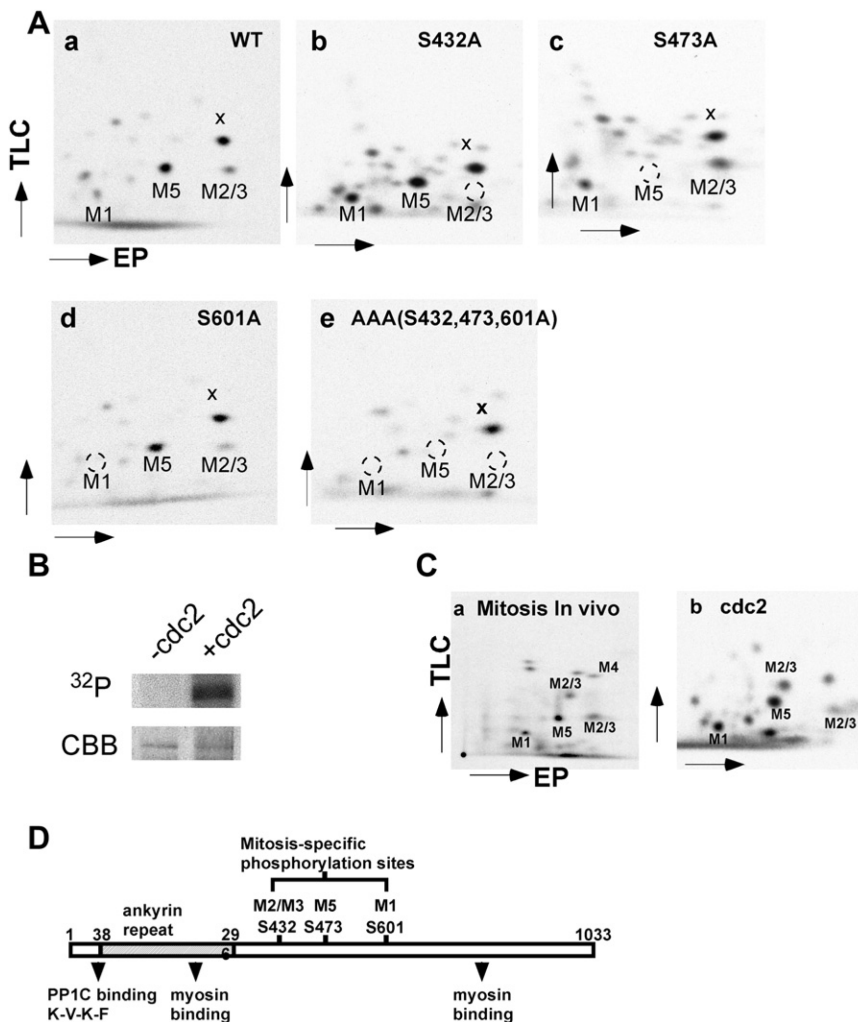

Figure 1. Identification of the Mitosis-Specific Phosphorylation Sites of MYPT1

(A) Phosphopeptide map analyses of wild-type (a), as well as single-point mutants of S432A (b), S473A (c), S601A (d), and a triple mutant (e). M2/3 spot disappeared by S432A (Ser432 replaced with Ala) mutation (b), M5 by S473A mutation (c), and M1 by S601 mutation (d). M1, M2/3, and M5 all disappeared by triple mutation (e). X is a *Xenopus*-specific spot. The directions of the first dimension (electrophoresis; EP) and the second dimension (thin layer chromatography; TLC) are indicated by arrows.

(B) In vitro phosphorylation of MYPT1 by cdc2. Upper panel, autoradiography; lower panel, Coomassie brilliant blue staining.

(C) Phosphopeptide map analyses of MYPT1 phosphorylated in vivo (a) and phosphorylated in vitro by cdc2 (b). Phosphorylation at the M2/3 site sometimes gave two spots as shown here (Totsukawa et al., 1999).

(D) Diagram of rat MYPT1 indicating locations of the mitosis-specific phosphorylation sites.

the polo box domain (PBD) of polo-like kinase 1 (PLK1). Indeed, MYPT1 directly binds to PLK1 via its PBD, coimmunoprecipitates with PLK1 as a complex, and colocalizes with PLK1 on centrosomes, as well as on kinetochores.

PLK1 is an essential mitotic kinase that is involved in a variety of mitotic events including the G2/M transition, centrosome maturation and separation, mitotic spindle formation, chromosome segregation, and cytokinesis (Barr et al., 2004; Blagden and Glover, 2003; Dai and Cogswell, 2003). As expected from PLK1's multiple functions, PLK1 phosphorylates a diverse set of substrates and changes its subcellular localization from the centrosome and kinetochore during prophase and metaphase, to the central spindle during anaphase and telophase. However, it is not well understood how the activity of PLK1 is controlled during execution of these diverse mitotic events, or what phosphatase antagonizes PLK1 in vivo.

The physical association between MYPT1 and PLK1 suggests that MYPT1/PP1C phosphatase is involved in PLK1-mediated mitotic events. In mammalian cells, PLK1 depletion by siRNA or PLK1 inactivation by antibody injection is known to block bipolar spindle assembly, resulting in mitotic arrest and apoptosis (Lane and Nigg, 1996; Liu and Erikson, 2002; Sumara et al., 2004; van Vugt et al., 2004). We found that simultaneous reduction of MYPT1 and PLK1 expression rescues these phenotypes. Importantly, MYPT1 depletion increases phosphorylation of PLK1 at Thr210, a site responsible for the activation of PLK1, and MYPT/PP1C is able to dephosphorylate PLK1 at Thr210 in vitro. Our results indicate that MYPT1/PP1C is a phosphatase that counteracts PLK1, and suggest an unexpected mode of kinase regulation by a PP1C-targeting subunit: phosphorylation of a PP1C-targeting subunit recruits a kinase to its antagonizing phosphatase, allowing regulation of the kinase's activity.

RESULTS

Cdc2 Phosphorylates Mitosis-Specific Sites in MYPT1

As a first step toward understanding the significance of the mitosis-specific phosphorylation of MYPT1, we identified its phosphorylated residues. Using a series of truncation mutants coupled with analyses by phosphopeptide mapping of point mutants at possible phosphorylation sites, we found that MYPT1 residues Ser432, Ser473, and Ser601 were phosphorylated during mitosis (Figure 1A). These residues are located in the central region of MYPT1 and had no previously assigned function (Figure 1D). Because all three phosphorylation sites are followed by proline, we tested whether a proline-directed kinase such as cdc2 can phosphorylate MYPT1 at these sites. As Figure 1B shows, cdc2 kinase stoichiometrically phosphorylated full-length MYPT1: radioisotope experiments revealed that approximately 3 moles of PO_4 per mol of full-length MYPT1 were incorporated (data not shown). Phosphopeptide mapping (Figure 1C) revealed that cdc2 kinase was able to phosphorylate

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