WARTS tumor suppressor is phosphorylated by Cdc2/cyclin B at spindle poles during mitosis

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Abstract Identification of physiological substrates for Cdc2/cyclin B is crucial for understanding the functional link between mitotic events and Cdc2/cyclin B activation. A human homologue of the *Drosophila* warts tumor suppressor, termed WARTS, is a serine/threonine kinase and a dynamic component of the mitotic apparatus. We have found that Cdc2/cyclin B forms a complex with a fraction of WARTS in the centrosome and phosphorylates the Ser613 site of WARTS during mitosis. Immunocytochemical analysis has shown that the S613-phosphorylated WARTS appears in the spindle poles at prometaphase and disappears at telophase. Our findings suggest that Cdc/cyclin B regulates functions of WARTS on the mitotic apparatus.

Key words: Mitotic kinase; WARTS; LATS1; Centrosome; Cdc2; Cyclin B

1. Introduction

The activation of the Cdc2/cyclin B complex is known to play a crucial role in the onset of mitosis and the regulation of subsequent mitotic events such as centrosome separation, chromosome condensation, nuclear envelope breakdown, and microtubular reorganization by phosphorylating target proteins [1]. For instance, the Cdc2/cyclin B complex was shown to phosphorylate nuclear lamin thus leading to its disassembly, an important event in the initiation of nuclear envelope breakdown [2]. In addition, the complex associated with aster and mitotic spindles is involved in the rearrangement of the microtubule network during mitosis, by phosphorylating proteins that localize spindle pole bodies. However, there is little clear evidence to account for a functional link between mitotic events and phosphorylation of Cdc2 substrates. Therefore, identification of physiological substrates of the Cdc2/cyclin B complex and the spatial and temporal regulation of the enzyme–substrate interaction remain important goals to understanding the molecular mechanisms by which the Cdc2/cyclin B complex initiates mitosis and brings about subsequent cell cycle events [3].

The *warts* gene (also referred to as *lats*) was identified from its ability to act as a tumor suppressor in *Drosophila melanogaster* [4]. The warts/lats protein has a serine/threonine kinase domain, which is highly homologous to those of the human myotonic dystrophy protein kinase (DMPK) family proteins, and DMPK family kinases have been shown to be generally involved in various mitotic events. A human homologue of warts/lats, termed WARTS (also referred to as LATS1), has been identified recently [5,6], and mice deficient in the warts/lats homologue have been shown to develop malignant tumors analogous to its mutant in *Drosophila* [7]. Immunolocalization studies have found the human WARTS kinase at the centrosome in interphase cells, while it becomes localized to the mitotic apparatus, including spindle poles and mitotic spindles, in metaphase–anaphase, and finally, to the midbody by telophase [6]. Moreover, WARTS protein was found to be mitotically phosphorylated and to interact with zyxin, a regulator of actin filament assembly, on the mitotic apparatus during mitosis, and this mitosis-specific interaction has been demonstrated to play a critical role in controlling mitosis progression [8]. All these findings suggest that WARTS is involved in various mitotic events in mammalian cells and that inactivation of its function may result in failure of normal mitotic progression, leading to chromosomal instability.

In this study, we demonstrate that WARTS is a physiological substrate of Cdc2/cyclin B and show characteristic localization of the phosphorylated form of WARTS during mitosis.

2. Materials and methods

2.1. Synchronization

HeLa cells were synchronized at the beginning of S phase by the double thymidine block and release protocol as previously described [8]. S phase cells were collected 2 h after release from the second thymidine release. Mitotic cells were collected by mechanical shake-off from the culture plate 9.5 h after release from S phase.

2.2. In vitro kinase assay

HeLa cells (5×10^5) were collected and washed in ice-cold phosphate-buffered saline. Cell extract was prepared and subjected to kinase reaction [8]. Kinase reaction was conducted at 25°C for 30 min in a final volume of 50 μl containing 20 mM Tris, pH 7.4, 10 mM

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Abbreviations: DMPK, myotonic dystrophy protein kinase; GST, glutathione *S*-transferase; MPF, maturation promoting factor

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$MgCl_2$, 10 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; Amersham Pharmacia), 1 μg microcystin, 8 μg of cell extract or 2.5 μl of purified active Cdc2 [9], and 10 μg of glutathione *S*-transferase (GST) fusion protein. As control reactions, we used the catalytic subunit of protein kinase A (PKA) (bovine heart) (Calbiochem), the catalytic subunit of protein kinase C (PKC) (rat brain) (Calbiochem), and purified calmodulin kinase II (CaMKII) (provided by Dr. H. Yamamoto, Kumamoto University, Japan). Each reaction mixture was mixed with 30 μl of glutathione-agarose beads (50% slurry). After the incubation, the beads were washed and boiled in 30 μl of Laemmli sample buffer to elute GST fusion proteins. The sample were resolved by 12% SDS–PAGE and visualized by autoradiography.

2.3. Depletion of Cdc2

Aliquots of 150 μl of mitotic cell extracts (1 mg/ml) were incubated with 50 μl of p13-suc1 agarose beads (50% slurry; Upstate Biotechnology) at 4°C for 45 min. After centrifugation, 50 μl of fresh p13-suc1 beads was added to the supernatant and incubated for an additional 30 min.

2.4. Gel filtration chromatography

Ten dishes (10 cm diameter) of confluent nocodazole-treated HeLa cells were lysed in 1 ml of lysis buffer, subjected to three cycles of rapid freezing in liquid nitrogen and thawing at 4°C, and then centrifuged at 100 000×*g* for 30 min at 4°C. The supernatants were applied to a Superdex 200 column which was equilibrated with equilibrated buffer (20 mM Tris–HCl pH 8.0, 150 mM NaCl, 1 mM dithiothreitol). The elution was performed with the same buffer at a flow rate of 250 μl/min. An aliquot of each fraction was subjected to in vitro kinase assay and immunoblot analysis.

2.5. Peptide synthesis and production of antibodies

Phosphorylated Ser613 peptide PS613 (CKQITTpS^{613}PITVR: pS represents phosphoserine) and Ser613 (CKQITTSPITVR) were chemically synthesized by Peptide Institute (Osaka, Japan). Antibody against PS613 was prepared by injecting rabbits with PS613 coupled to keyhole limpet hemocyanin. Specific antibody against PS613, termed PS613 Ab, was purified from the obtained serum by two-step chromatography as previously described [10]: affinity chromatography on PS613-coupled Cellulofine (Seikagaku Corp.) and then absorption in S613-coupled Cellulofine.

2.6. Immunofluorescence microscopy

HeLa cells were grown on a 35 mm Petri dish to 70% confluence and pretreated with a microtubule-stabilizing buffer [11]. After being washed, cells were incubated with the following antibodies: rat anti-WARTS antibody (G3) [8], rabbit anti-pSer613 antibody (PS613 Ab), and monoclonal mouse anti-γ-tubulin (GTU-88). This was followed by incubation with FITC- or Texas red-conjugated secondary antibody. The stained cells were mounted and observed with confocal microscopy (Fluoview; Olympus).

2.7. Immunoprecipitation and immunoblots

Cells were lysed on ice for 30 min with 1% Nonidet P-40 lysis buffer [8]. Lysates were centrifuged (14 000×*g*, 20 min, 4°C). Aliquots of supernatant (0.2–2.0 mg/ml) were incubated for 2 h at 4°C with specific antibodies, and another 2 h incubation after adding 30 μl of protein A Sepharose™ 4 Fast Flow (50% slurry; Amersham Pharmacia Biotech). Immunoprecipitates were washed three times with lysis buffer before Laemmli sample buffer was added. The samples were resolved by SDS–PAGE and transferred to nitrocellulose for immunoblotting.

3. Results

3.1. Mitosis-specific phosphorylation of human WARTS by Cdc2/cyclin B

We have reported previously that WARTS is phosphorylated during mitosis [6]. To delineate the mitosis-specific phosphorylation region of WARTS, we generated three GST-WARTS truncated mutants, GST-WP, -WN and -WK (Fig. 1A) and utilized them as substrates for in vitro kinase assays. Interphase and nocodazole-treated mitotic phase lysates of HeLa cells were used as kinase fractions. Among these GST fusion proteins, GST-WN (amino acids 395–700) was phosphorylated specifically by nocodazole-treated cell lysate (Fig. 1B, lane 4).

We next examined whether the phosphorylation of GST-WN in mitotic extracts is dependent on Cdc2 kinase. GST-

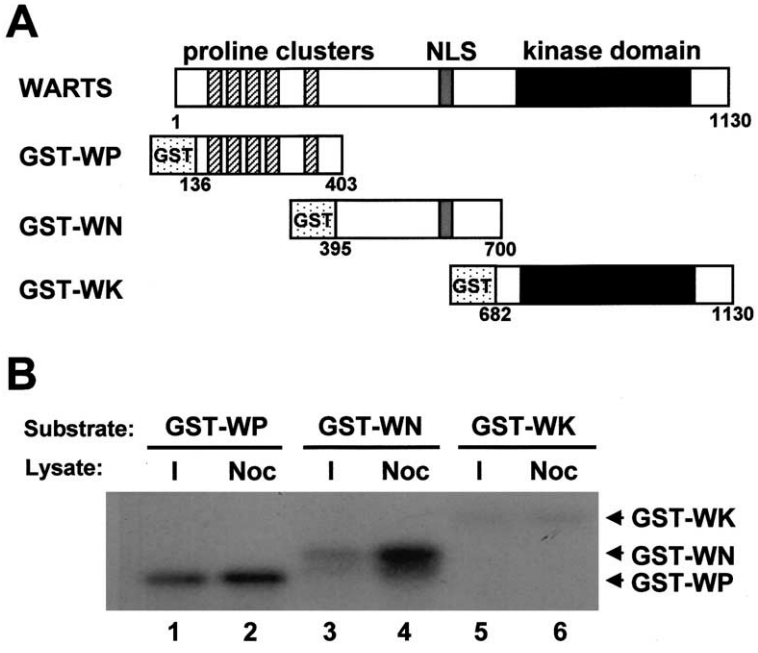

Fig. 1. Determination of a region in WARTS protein specifically phosphorylated by mitotic lysate. A: Schematic diagram of GST-WARTS deletion mutants showing their domain structures. The deletion mutants containing amino acids 136–403, 395–700 and 682–1130 of WARTS were designated GST-WP, GST-WN and GST-WK, respectively. B: In vitro phosphorylation of WARTS protein. GST-WP, GST-WN and GST-WK were incubated with either interphase (I) or nocodazole-treated (Noc) cell lysate in the presence of [γ-^{32}P]ATP. Samples were analyzed by SDS–PAGE followed by autoradiography.

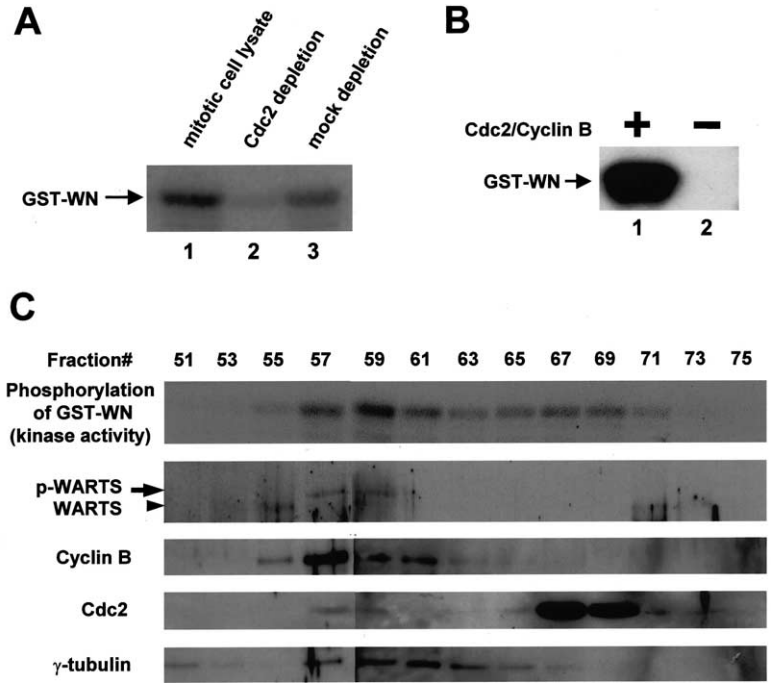

Fig. 2. Phosphorylation of WARTS by Cdc2/cyclin B complex. A: Effect of Cdc2 depletion on the in vitro phosphorylation of WARTS by mitotic lysate. GST-WN was incubated with either buffer alone (lane 1), Cdc2-depleted mitotic lysate (lane 2), or mock-depleted mitotic cell lysate (lane 3) in the presence of [γ-^{32}P]ATP. B: In vitro phosphorylation of WARTS by Cdc2. GST-WN was incubated with (+) or without the purified active Cdc2/cyclin B complex (−) in the presence of [γ-^{32}P]ATP. C: Nocodazole-treated lysates from HeLa cells were separated on a Superdex 200 column. The eluted proteins in each fraction were subjected to in vitro kinase assays (top panel) and immunoblotting with indicated antibodies. Arrow and arrowhead indicate hyperphosphorylated and hypophosphorylated WARTS proteins, respectively.

WN phosphorylation diminished significantly when Cdc2 was depleted from mitotic extracts of HeLa cells using p13^{suc1} beads (Fig. 2A, lane 2). But the mock-depleted mitotic extracts could phosphorylate GST-WN (Fig. 2A, lane 3). Furthermore, we found that the purified Cdc2/cyclin B complex directly phosphorylated GST-WN (Fig. 2B). These findings indicate that GST-WN is specifically phosphorylated by Cdc2/cyclin B, which is contained in mitotic cell lysate.

To examine whether endogenous WARTS associates with the Cdc2/cyclin B during mitosis, we separated proteins extracted from HeLa cells blocked in mitosis with nocodazole by HPLC using a Superdex 200 column (Fig. 2C). Individual fractions were assayed for GST-WN phosphorylation activity and were subjected to Western blot analysis. We found that the phosphorylated WARTS detected as a slower mobility band was cofractionated with Cdc2 and cyclin B in the fractions exhibiting GST-WN phosphorylation activity (fractions 57–59). Because γ-tubulin, a component of the centrosome,

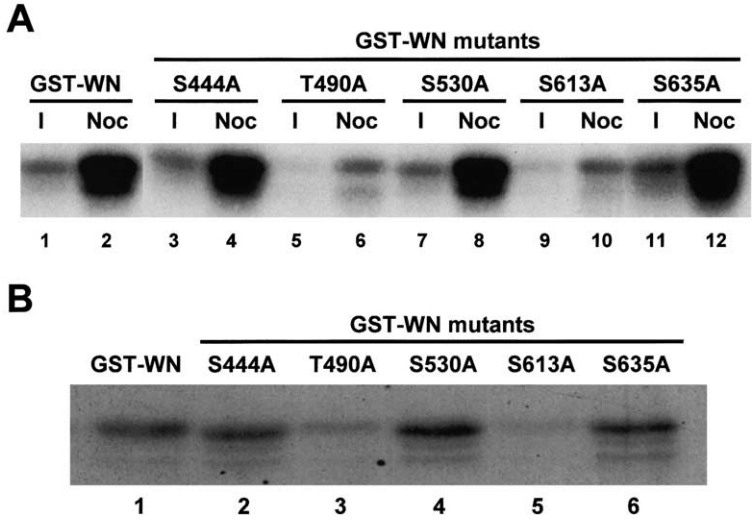

Fig. 3. Determination of Cdc2 phosphorylation sites in the WN region of WARTS. A series of GST-WN mutants were analyzed by in vitro kinase assay to determine the phosphorylation sites in the WN region. In each GST-WN mutant, one of the putative Cdc2 phosphorylation sites (Ser444, Thr490, Ser530, Ser613, or Ser635) was changed to alanine. GST-WN and the mutant proteins were incubated with mitotic lysates of HeLa cells (A) or purified active Cdc2/cyclin B1 complex (B) in the presence of [γ-^{32}P]ATP.

was also detected in those fractions, the WARTS–Cdc2–cyclin B complex appears to be localized to the centrosome during mitosis. Although fraction 55 also contained both WARTS and cyclin B, the WARTS protein detected in fraction 55 appeared to be a non-phosphorylated fast migrating form and was cofractionated with cyclin B but not with Cdc2. In addition, fractions 67–69, where a large amount of free Cdc2 exists, did not contain the WARTS protein. Thus, these findings suggest that a fraction of WARTS is associated with the Cdc2/cyclin B complex in the centrosomes and is phosphorylated by the complex during mitosis.

3.2. *Identification of the mitosis-specific phosphorylation sites in WARTS*

To determine the phosphorylation sites in the WN region of the WARTS protein, a series of GST-WN mutants were generated and subjected to an in vivo kinase assay. It was previously shown that Ser/Thr-Pro is the minimum consensus motif for Cdc2 kinase phosphorylation [10,12–14]. Therefore, we generated GST-WN mutants in which one of the putative Cdc2 phosphorylation sites (Ser444, Thr490, Ser530, Ser613, or Ser635) was replaced with alanine. While S444A, S530A and S635A mutants, as well as wild-type WN, were highly phosphorylated by mitotic lysate, the phosphorylation of T490A and S613A mutants was markedly diminished (Fig. 3A). Additionally, the T490A and S613A mutants showed significant reductions of phosphorylation by the purified active Cdc2/cyclin B complex (Fig. 3B). These results suggest that Thr490 and Ser613 in WARTS are potential phosphorylation sites for Cdc2 during mitosis.

3.3. *Production of the site- and phosphorylation state-specific antibody for WARTS*

Next, we investigated how the mitosis-specific phosphorylation of WARTS is regulated in space and time in living cells. While Thr490 is not conserved among different species, Ser613 is evolutionarily conserved in all warts/lats homologues (*Drosophila*, chicken, mouse, rat, and human) [6], suggesting that phosphorylation of the Ser613 site is biologically important. Therefore, we attempted to generate a rabbit polyclonal antibody (referred to as PS613 Ab) that specifically recognizes the phosphorylated state of WARTS at Ser613. Immunoblot analyses revealed that PS613 Ab specifically reacted with the GST-WN phosphorylated by lysates extracted from nocodazole-treated cells (Fig. 4A, lane 3) and cells at mitosis (Fig. 4B) but did not detect the GST-WN treated with interphase cell lysates. The S613A mutant of GST-WN treated with nocodazole-treated lysates was not recognized by PS613 Ab (Fig. 4A, lane 4). Furthermore, PS613 Ab reacts with the GST-WN phosphorylated by purified Cdc2/cyclin B but not with those treated with PKA, PKC and CaMKII (Fig. 4C). Taken together, PS613 Ab specifically recognizes the phosphorylation of WARTS at Ser613 by Cdc2 kinase.

3.4. *Ser613 of endogenous WARTS is phosphorylated during mitosis*

We next examined whether the Ser613 site of WARTS is phosphorylated in living cells. The endogenous WARTS was immunoprecipitated from the interphase, mitotic and nocodazole-treated mitotic lysates with anti-WARTS polyclonal antibody (C-2), which recognizes both phosphorylated and non-phosphorylated forms of WARTS, and subsequently blotted

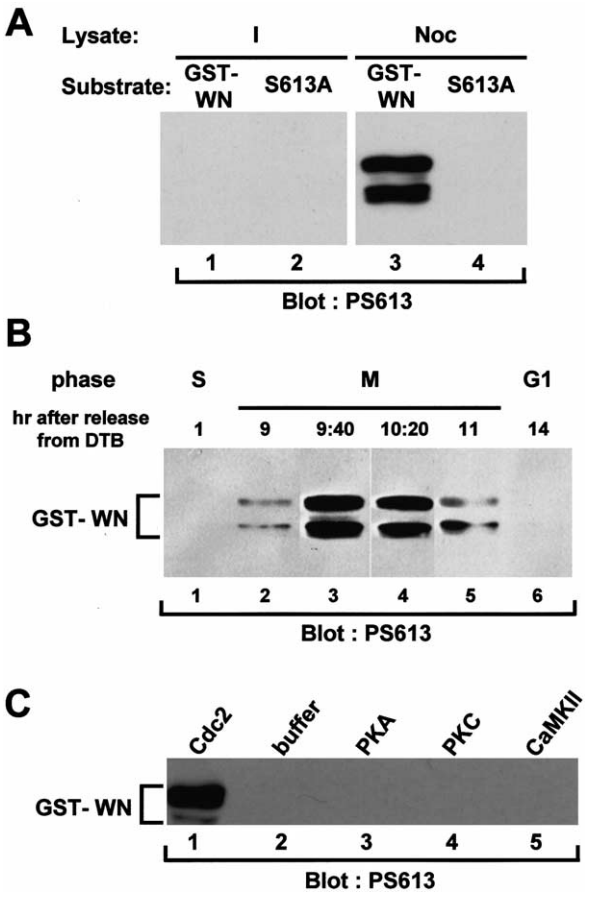

Fig. 4. Development of antibodies that recognize a mitotic specific phosphorylation site (Ser613) in WARTS. A: Specificity of phosphorylation site-specific antibodies (PS613 Ab). GST-WN and GST-WN(S613A) mutant, which were incubated with lysates prepared from interphase cells (I) and nocodazole-treated cells (Noc), were resolved by SDS–PAGE followed by immunoblotting with PS613 Ab. The lower band in lane 3 was found to be a proteolytic product of GST-WN generated during protein preparation. B: PS613 Ab reacts with Ser613 phosphorylated by mitotic lysate. HeLa cells were synchronized at the beginning of S phase. Following release from S phase, cells were harvested at the indicated time points. GST-WN, which was incubated with lysates prepared from the harvested cells, was subjected to immunoblot with PS613 Ab. C: PS613 Ab recognizes Ser613 phosphorylated by Cdc2. GST-WN, which was incubated with the purified active Cdc2/cyclin B complex, PKA, PKC, or CaMKII, was subjected to immunoblot with PS613 Ab.

with either C-2 antibody or PS613 Ab. As shown in Fig. 5A, the S613-phosphorylated WARTS, designated pSer613-WARTS, was more abundantly detected in the mitotic lysate and nocodazole-treated HeLa cells lysates than in the interphase lysates. This result suggests that the Ser613 site of endogenous WARTS is phosphorylated during mitosis.

3.5. *Subcellular localization of Ser613-phosphorylated WARTS*

Subcellular localization of pSer613-WARTS was investigated by immunocytochemical analysis using the PS613 antibody (Fig. 5B). When cells were stained with G3 antibody that recognizes both non-phosphorylated and phosphorylated WARTS, WARTS was predominantly detected at centrosomes in interphase cells and became localized to the mitotic spindles and spindle poles in a highly dynamic manner during mitosis (Fig. 5B, a–f) as we described previously [6,8]. Contrary to the G3 staining, pSer613-WARTS was not observed

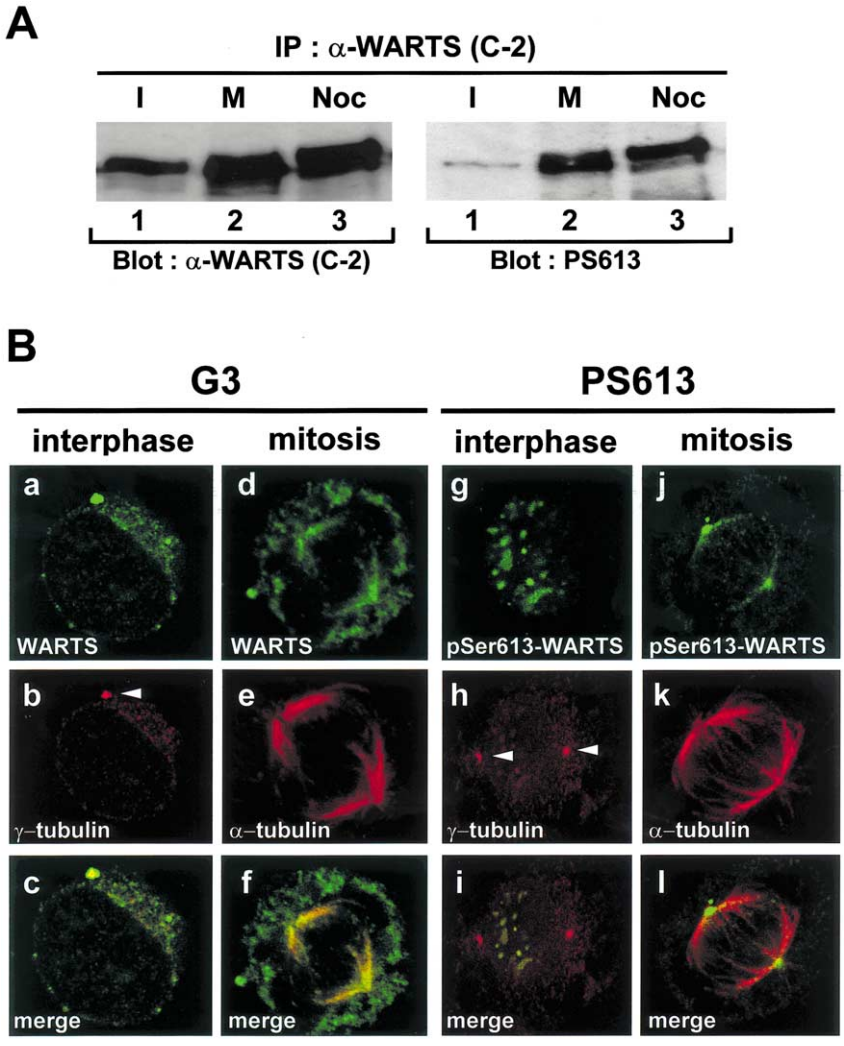

Fig. 5. Ser613-phosphorylated WARTS is localized to spindle poles during mitosis. A: Phosphorylation of endogenous WARTS during mitosis. Lysates prepared from interphase (I), mitotic (M) and nocodazole-treated (Noc) HeLa cells were immunoprecipitated with anti-WARTS antibody (C-2). The immunoprecipitates were resolved by SDS–PAGE followed by immunoblotting with anti-WARTS antibody (C-2) (left panel) or PS613 Ab (right panel). B: Subcellular localization of pSer613-WARTS. HeLa cells at interphase (a–c and g–i) and mitotic phase (d–f and j–l) were fixed and labeled with G3 antibodies which recognize both non-phosphorylated and phosphorylated WARTS (a and d) or labeled with PS613 (g and j). WARTS was visualized by FITC-conjugated secondary antibody (green). α-Tubulin (e and k) and γ-tubulin (b and h) were visualized by Texas red-conjugated secondary antibody (red). The merged images are shown in c, f, i and l. Arrowheads indicate centrosomes found in interphase cells, which were visualized by γ-tubulin antibody.

at centrosomes in interphase cells (Fig. 5B, g–i). In the cells that underwent nuclear envelope breakdown, pSer613-WARTS staining was detected at the centrosomes. At metaphase, both spindle poles were brightly labeled by PS613 and the mitotic spindles were stained weakly (Fig. 5B, j–l). The staining at the spindle poles decreased from anaphase to telophase. These findings support our biochemical data that a fraction of WARTS is phosphorylated at the Ser613 site by Cdc2 kinase at the spindle pole bodies during mitosis.

4. Discussion

In the present paper we demonstrated that the middle region of WARTS, designated the WN region, is phosphorylated during mitosis. Three lines of evidence suggest that the mitosis-specific phosphorylation of the WN region is mediated by activated Cdc2. First, the depletion of Cdc2 from mitotic cell lysates markedly abolished phosphorylation of GST-WN in vitro. Second, purified active Cdc2/cyclin B complex phosphorylated GST-WN. Third, the phosphorylated form of endogenous WARTS was eluted with the active Cdc2/cyclin B complex in the same fractions of size exclusion gel chromatography.

In vitro kinase assays using a series of GST-WN mutants revealed that Thr490 and Ser613 are sites phosphorylated by both mitotic lysate and purified active Cdc2/cyclin B. Since amino acid sequences around Ser613 site are completely conserved in warts/lats homologues identified in different species, we generated antibodies specifically recognizing the phosphorylated state of WARTS at Ser613. PS613 Ab specifically reacted with the endogenous WARTS when the cells are at mitosis or treated with nocodazole, indicating that the Ser613 site of WARTS is mitotically phosphorylated in vivo.

In the late G2 phase, maturation promoting factor (MPF), composed of cyclin B and Cdc2, appears in the centrosomes and subsequently translocates into the nucleus at prophase.

About 20 min after the nuclear translocation of MPF, nuclear envelope breakdown occurs, and then, MPF binds to the mitotic apparatus, in particular to centrosomes and mitotic spindles [15]. In addition, MPF associated with centrosomes and mitotic spindles is considered to be involved in the rearrangement of the microtubule network at mitosis by phosphorylating certain centrosomal proteins. We previously reported that a fraction of WARTS protein is localized at the centrosome in interphase and translocated dynamically toward mitotic spindles in metaphase–anaphase, and, finally, located at the midbody by telophase [6,8]. Immunofluorescence studies using PS613 Ab revealed that phosphorylation of Ser613 by WARTS occurred at centrosomes in prometaphase. This phosphorylation continued at centrosomes and mitotic spindles in anaphase, and disappeared in telophase. Localization and the timing of phosphorylation of the Ser613 site are consistent with the localization and timing of activation of the Cdc2/cyclin B complex during mitosis.

We previously showed that zyxin interacts with WARTS on the mitotic apparatus [8]. We found that the WARTS binding domain of zyxin is masked by the NH_2-terminal region in interphase cells and that the phosphorylation of the NH_2-terminal region of zyxin by Cdc2/cyclin B allows zyxin to interact with WARTS during mitosis. Those findings together with data in this study suggest that Cdc2/cyclin B is required for formation of the WARTS–zyxin complex on the mitotic apparatus to execute normal mitosis.

We have recently found that WARTS kinase activity is still low in cells arrested at prometaphase by treatment with nocodazole when Cdc2/cyclin B is highly activated. However, WARTS is significantly activated at 10 min after release from nocodazole treatment, when cells are at anaphase (Iida et al., manuscript in preparation). These findings suggest that phosphorylation of WARTS by Cdc2/cyclin B is not sufficient for activation of WARTS kinase and that an additional event(s) is required for its activation at the metaphase–anaphase transition. It can be speculated that phosphorylation of WARTS by Cdc2/cyclin B promotes a protein complex formation on the mitotic apparatus at early mitosis, which may be required for subsequent activation of WARTS kinase at the metaphase–anaphase transition.

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