Zyxin, a Regulator of Actin Filament Assembly, Targets the Mitotic Apparatus by Interacting with h-warts/LATS1 Tumor Suppressor

Toru Hirota,* Tetsuro Morisaki,* Yasuyuki Nishiyama,* Tomotoshi Marumoto,* Kenji Tada,§ Toshihiro Hara,* Norio Masuko,* Masaki Inagaki,i Katsuyoshi Hatakeyama,‡ and Hideyuki Saya*

*Department of Tumor Genetics and Biology, Kumamoto University School of Medicine, 2-2-1 H onjo, Kumamoto 860-0811, Japan; ‡1st Department of Surgery, Niigata University School of Medicine, 1-757 A sairinmachi-dori, Niigata 951-8510, Japan; §Department of Neurosurgery, Kumamoto University School of Medicine, 2-2-1 H onjo, Kumamoto 860-0811, Japan; and iLaboratory of Biochemistry, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya, Aichi 464-0021, Japan

Abstract. The mitotic apparatus plays a pivotal role in dividing cells to ensure each daughter cell receives a full set of chromosomes and complement of cytoplasm during mitosis. A human homologue of the Drosophila warts tumor suppressor, h-warts/LATS1, is an evolutionarily conserved serine/threonine kinase and a dynamic component of the mitotic apparatus. We have identified an interaction of h-warts/LATS1 with zyxin, a regulator of actin filament assembly. Zyxin is a component of focal adhesion, however, during mitosis a fraction of cytoplasmic-dispersed zyxin becomes associated with h-warts/LATS1 on the mitotic apparatus. We found that zyxin is phosphorylated specifically during mitosis, most likely by Cdc2 kinase, and that the phosphorylation regulates association with h-warts/LATS1. Furthermore, microinjection of truncated h-warts/LATS1 protein, including the zyxin-binding portion, interfered with localization of zyxin to mitotic apparatus, and the duration of mitosis of these injected cells was significantly longer than that of control cells. These findings suggest that h-warts/LATS1 and zyxin play a crucial role in controlling mitosis progression by forming a regulatory complex on mitotic apparatus.

Key words: Cdc2 • interaction • mitotic spindle • phosphorylation • serine/threonine kinase

Introduction

Mitotic apparatus, which consists of a dynamic array of microtubules and associated proteins, controls mitotic events such as establishment of cell polarity, chromosomal congression and segregation, and finally, cytokinesis. Genetic analyses in yeasts and Drosophila have identified several kinases essential for normal regulation of mitosis (Glover et al., 1995; Sunkel and Glover, 1988). These mitosis-related serine/threonine kinases have been found to be highly conserved evolutionarily and localized to the mitotic apparatus (Golsteyn et al., 1995; K imura et al., 1997). Dynamic interaction of these mitosis-related kinases with proteins on the mitotic apparatus is considered to be required to achieve proper coordination of mitotic events (Bahler et al., 1998; Nigg, 1998; Bischoff and Plowman, 1999).

The warts gene (also known as lats) was identified as a tumor suppressor of Drosophila melanogaster (Justice et al., 1995; K u et al., 1995). The warts/lats encodes serine/threo-
h-warts/LATS1, has been identified and demonstrated to negatively regulate Cdc2 activity by interacting with Cdc2 in the mitotic phase (Tao et al., 1999). A homologous to its mutant in Drosophila, a component of the Drosophila mitotic spindle and the actin cytoskeleton, has been shown to develop malignant tumors (St. John et al., 1999). Moreover, h-warts/LATS1 protein was found to localize at the centrosome in interphase and to translocate dynamically toward mitotic spindles in metaphase-anaphase, and, finally, to the midbody by telophase (Nishiyama et al., 1999). Recently, the Sid2 kinase, structural homologue of DmDbf2 and a potential counterpart of h-warts/LATS1 in fission yeast, has been demonstrated to function as part of a novel signaling pathway required for onset of cytokinesis. Sid2 is a component of the spindle pole body and by virtue of its transient localization to the division site, it appears to determine the timing of ring constriction (Sparks et al., 1999). Based on these observations, h-warts/LATS1 protein is speculated to be heavily involved in mitotic events in mammalian cells and that loss of its function disrupts normal cell cycle regulation, leading to the development of tumors. Therefore, identification of cellular targets of the h-warts/LATS1 protein will provide clues to its precise cell cycle function and to its involvement in tumorigenesis.

During mitosis, adherent cells change morphology into a spherical and weakly adherent form. This morphological alteration involves rearrangement of cytoskeletal systems and dissociation of the adhesion apparatus, which are under the control of biochemical status through cell cycle progression (V erede et al., 1998). Focal adhesion plaques are an adhesion apparatus for cells to contact the extracellular matrix where the growing end of actin filament attaches to the plasma membrane. At the focal adhesion complex, a number of proteins serve as linkages between transmembrane proteins and the actin cytoskeleton, regulating actin filament dynamics (Craig and Johnson, 1996; Beckerle, 1997). A s cells proceed through mitosis, components of the focal adhesion complex are known to dissociate into the cytoplasm when bundles of actin fibers disappear. The role of these actin-regulatory proteins during mitosis, which are dispersed in the cytoplasm, remains to be established.

Zyxin is a component of the focal adhesion complex (Crawford and Beckerle, 1991) and plays a central role in actin filament polymerization in mammalian cells (reviewed in Beckerle, 1997). Several lines of evidence demonstrate that zyxin may function to recruit components required for the actin assembly machinery to specific sites in the cell and to stimulate spatially restricted actin polymerization (Crawford et al., 1992; Einhard et al., 1995; Hober et al., 1996; Prehoda et al., 1999). Interestingly, zyxin exhibits a functional nuclear export signal and has been demonstrated to shuttle between the nucleus and the sites of cell adhesion (Nix and Beckerle, 1997). These findings suggest that zyxin has an unknown second function in addition to its key role in regulating actin assembly.

In this study, we have identified the interaction of h-warts/LATS1 with zyxin on the mitotic apparatus during mitosis. The localization of zyxin on the mitotic apparatus appears to be dependent on the presence of h-warts/LATS1 protein. Furthermore, we showed that zyxin is phosphorylated specifically during mitosis, most likely by Cdc2 kinase, and that this phosphorylation controls the association of zyxin with h-warts/LATS1. The interaction between zyxin and h-warts/LATS1 on the mitotic apparatus implicates a significant role for actin regulatory proteins during mitosis.

Materials and Methods

Yeast Two-Hybrid Screening

Yeast strain L40 was used as a host for the two-hybrid screening (Vojtek et al., 1993). A yeast L40 strain carrying pBTM116HA-h-warts (amino acids 394–675) was transformed with the HeLa cDNA library constructed in pGAD-GH (Clontech) by electroporation. Transformants were screened for growth on SD plate media lacking tryptophan, leucine, and histidine prototrophy. His+ colonies were subjected to β-galactosidase assay. Plasmids harboring cDNA were recovered from positive colonies and the nucleotide sequence of plasmid DNA which conferred the LacZ* phenotype on L40 containing pBTM116HA-h-warts (396–657) were determined.

Cell Culture, Synchronization, and Transfections

HeLa, COS7, and U2OS cells were cultured in DMEM/F12 supplemented with 10% fetal calf serum without antibiotics. HeLa cells were synchronized at the beginning of M phase by double thymidine block and release protocol (first 24 h incubation with 1 mM thymidine, an interval of thymidine-free incubation for 8 h, and second thymidine incubation for 14 h). Mitotic cells were collected by mechanical shake-off from the culture plate 9.5 h after release from S phase. For transient transfection, cells in 6-well plates were transfected using FuGene6 transfection reagent following the manufacturer’s instructions (Boehringer Mannheim). To prepare for flow cytometry, cells were trypsinized, fixed with 70% methanol, and DNA were stained with propidium iodide. Cells were subjected to flow cytometry on FACScan® (Becton Dickinson). G1, S, and G2/M populations were calculated with Modfit 2.1 software (Varity).

Expression Plasmids

Mammalian expression plasmids were constructed by subcloning the PCR amplified fragment into hemagglutinin (HA)-tagged (pCGN), FLAG-tagged (pB-J-FLAG) vectors. pB-J-FLAG was constructed by inserting an aneal oligonucleotide between the XhoI and BamHI sites of pB-J-myc. All the PCR products were obtained using PyroBest DNA polymerase (Takara) and we confirmed their sequences. For glutathione-S-transferase (GST) fusion protein expression in bacteria, pGEX-2T-based plasmids were constructed as previously described (M asuko et al., 1999).

Antibody Preparation

Two polyclonal antibodies against h-warts/LATS1 were generated by injecting rabbits with two synthetic peptides, C1 (PVDPDKLWSADD-LNEEENVNDTLNG), and C2 (SDEDDQNTGSEIKNRDLVYV), coupled to keyhole limpet-hemocyanin (KLH) via the added NH2-terminal cysteine. GST–h-warts amino acids 136–700 and amino acids 136–410 were expressed in bacteria, pGEX2TH-based plasmids were constructed as previously described (M asuko et al., 1999).

Immunoprecipitation

Cells were lysed on ice for 30 min with 0.5% NP-40 lysis buffer consisting of 0.5% NP-40, 25 mM Tris-CI, pH 7.5, 137 mM NaCl, 1 mM EDTA, 1 mM EGTA, 5% glycerol, 2 μg/ml aprotinin, 20 mM β-glycerophosphate, 1 mM AP, 10 μM leupeptin, 1 μM pepstatin, and 1 mM Na3VO4. lysates were centrifuged at 14,000 g for 20 min. A aliquots of supernatant (200 μg, ~2.0 mg/ml) were incubated for 1 h at 4°C with specific antibodies, and another 1 h incubation after adding 30 μl of protein G/A agarose beads (50% slurry; Calbiochem). A filter being washed, the bound proteins were analyzed by immunoblotting.
For coimmunoprecipitation of h-warts/LATS1 and zyxin, HeLa cells were lysed on ice for 30 min with five cell volumes of the 0.1% NP-40 lysis buffer (0.1% NP-40, 100 mM NaCl, 25 mM Tris-Cl, pH 8.0, 5 mM EGTA, 1 mM MgCl₂, and 5% glycerol supplemented with 1 mM DTT, 2 μg/ml aprotinin, 20 mM β-glycerophosphate, 1 mM AβSF, 10 μM leupeptin, 1 μM pepstatin, 1 mM Na₃VO₄, 1 mM benzamidine, and 1 μM microcystin), and centrifuged at 14,000 ×g for 20 min. Precleared 40 μg quantities of cell lysate (~4 mg/ml) were mixed with 3 μl of anti-zyxin antibody for 3 h on ice, then 15 μl of protein A-Sepharose beads (Amersham Pharmacia) and conjugated anti–rabbit/mouse IgG antibody (Amersham Pharmacia), and fixed for 10 min on ice. A preextraction followed by permeabilization with 0.2% Triton X-100/PBS, otherwise with 4% paraformaldehyde/PBS, pH 7.4, for 15 min at room temperature, was removed, made to 10 mM DTT and 0.1% bromophenol blue, boiled again and then subjected to immunoblotting. Quantification analysis was performed by MacBAS V2.5 software.

**In Vitro Pull-Down Assay and Solution-Binding Assay**

For pull-down assay, 30 μg of GST fusion protein was immobilized on glutathione-agarose, and equilibrated with 0.5% NP-40 lysis buffer. A aliquots of cell lysate (200 μg, ~2.0 mg/ml) were incubated with the glutathione-agarose for 1 h. The bound proteins were analyzed by immunoblotting. In vitro solution binding assay was performed as described (Ijiri et al., 1998). In brief, 10 μg of GST fusion proteins was immobilized on the glutathione-agarose and equilibrated with buffer B (20 mM Hepes-KOH, pH 7.9, 50 mM NaCl, 1 mM MgCl₂, 17% glycerol, and 2 mM DTT). The glutathione-agarose beads were washed and incubated with 200 μl of His-h-warts in 0.1% NP-40 lysis buffer B for 1 h at 4 ℃. A feter washing with buffer B, the bound proteins were analyzed by immunoblotting.

**In Vitro Kinase Assay**

Synchronized HeLa cells were washed with ice-cold PBS and lysed on the plate with RIPA buffer as previously described (Ijawa et al., 1996). Lysates were centrifuged at 14,000 ×g for 20 min, and the supernatant was used for the kinase assays. Kinase reaction were conducted at 25℃ for 30 min in a final volume of 50 μl containing 20 mM Tris pH 7.4, 10 mM MgCl₂, 30 μl of [γ-32P]ATP (3,000 Ci/mmol; Amersham Pharmacia), 1 μM microcystin, 5 μM pepstatin, 8 μg of lysates, and 10 μg of GST fusion-protein. Each reaction mixture was then chilled and mixed with 30 μl of glutathione-agarose beads (50% slurry) and 0.5 ml of ice-cold TNE buffer, followed by rocking for 30 min at 4℃. The glutathione-agarose beads were washed and boiled in 30 μl of Laemmli sample buffer to elute GST fusion proteins. The samples were resolved by 8% SDS-PAGE and visualized by autoradiography. The result of gel analyses were quantified by MacBAS (FujiFilm).

**Depletion of Cdc2**

A aliquots of 150 μl of mitotic cell lysate (1.0 mg/ml) were incubated with 50 μl of p13-suc1 agarose beads (50% slurry; Upstate Biotechnology) at 4℃ for 45 min. After centrifugation, 50 μl of fresh p13-suc1 beads was added to the supernatant and incubated for an additional 30 min. The Cdc2 kinase assay was performed by the SignaTECT assay system (Promega) in which biotinylated peptide derived from histone H1 was used as a substrate and radiolabeled phosphorylated substrate was recovered with streptavidin matrix. The purified active Cdc2 kinase was prepared as previously described (Kusubata et al., 1992).

**Immunofluorescence Microscopy**

U2OS cells were grown on 35-mm petri dishes to 75% confluence and microinjected using semi-automated micro-manipulator/injector (Eppendorf 5171/5246). Cells in prometaphase were selected for injection on the basis of morphology by phase contrast images, and injected with a 1.0 mg/ml solution of purified GST fusion proteins and rhodamine-tubulin (Cytokeleton) in PBS, pH 6.9, supplemented with a final concentration of 0.5 mM GTP. The microscopical stage was maintained at 37℃ and the procedure was completed within 20 min to minimize pH changes. After injection, cells were incubated at 37℃ for 20 min for rhodamine-tubulin to be distributed to the mitotic spindle, followed by detergent preextraction (7.5 μg/ml digitonin in KH M buffer) and a methanol fixation protocol as described above.

Synchronized HeLa cells were injected into the cytoplasm with a combination of 1.0 mg/ml solution of purified GST fusion proteins together with 1.0 mg/ml β-galactosidase (Sigma-Aldrich). Cells were fixed with 0.5% glutaraldehyde/PBS, pH 7.2, at subsequent time points, followed by incubation with 1 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) in 150 mM NaCl, 0.01% sodium deoxycholate, 0.02% NP-40, 2 mM MgCl₂, 5 mM KFe(CN)₆, and 5 mM KFe(CN)₆ for 12 h at 37℃. A feter removing the X-gal solution, cells were overlaid with aceto-orcein (Merck) in 60% acetic acid to visualize chromatin.

**Results**

**Identification of h-warts/LATS1 Interacting Protein**

To identify proteins that physically interact with h-warts/LATS1, we conducted a HeLa cDNA library screen by the yeast two-hybrid method. We used a region of amino acids 394–675, which does not contain the kinase domain of h-warts/LATS1, as the bait for the screen (Fig. 1 A). From 2.3 × 10⁶ initial transformants, 53 clones were found to confer both the His⁺ and LacZ⁺ phenotypes on L40 containing pB TM 116HA/h-warts. These positive clones were subjected to secondary screening to eliminate false positives, and confirmed the specific interaction between h-warts/LATS1 and protein encoded by a library cDNA within the yeast cells. A mong the positive clones, we isolated four independent cDNA clones encoding partial protein fragments that derived from zyxin, a regulator of actin assembly (Fig. 1 A). The other h-warts/LATS1-interacting proteins identified in this screen will be described elsewhere.

**Interaction of Zyxin with h-warts/LATS1**

To examine whether zyxin interacts with h-warts/LATS1 in intact cells, we coexpressed zyxin with h-warts/LATS1 in COS7 cells (Fig. 1 B). Full-length zyxin and full-length h-warts/LATS1 were tagged with FLAG and HA epitopes, respectively, at their NH₂-termini. When the lysate coexpressing FLAG-zyxin with HA-h-warts/LATS1 was immunoprecipitated with the anti-FLAG antibody, HA-h-warts/LATS1 was detected in the FLAG-zyxin immune complex (Fig. 1 C, lane 1). Conversely, FLAG-zyxin was detected in the HA-h-warts/LATS1 immune complex (Fig. 1 C, lane 4). Neither FLAG-G-zyxin nor HA-h-warts/LATS1 was detected in the unrelated control IgG immunoprecipitates from lysates expressing both proteins (data not shown).
An additional experiment to confirm the interaction of zyxin with h-warts/LATS1 was performed. Two polyclonal antibodies were raised against h-warts/LATS1 by injecting two KLH-conjugated synthetic polypeptides, C1 (amino acids 1,041–1,063) and C2 (amino acids 1,111–1,130), into rabbits. Endogenous h-warts/LATS1 was immunoprecipitated by the anti-C1 antibody and was detected at immunoblotting by the anti-C2 antibody (Fig. 1 D, lane 8). When the COS7 cell lysate expressing HA-tagged zyxin (full-length) was immunoprecipitated with the anti-C1 antibody, HA-zyxin (full-length) was coprecipitated with endogenous h-warts/LATS1 (Fig. 1 E, lane 3).

Furthermore, we tested whether endogenous zyxin and h-warts/LATS1 interact in vivo. Using anti-zyxin antibody generated in rabbits, zyxin was immunoprecipitated from Hela cell lysate. The endogenous h-warts/LATS1 was detected in the immunoprecipitate (Fig. 1 F). Although comparison of the band profiles revealed that the small fraction (9%) of h-warts/LATS1 interacts with zyxin, these results indicate the association of h-warts/LATS1 and zyxin in intact cells.

Since zyxin harbors three copies of LIM domain in the COOH-terminal third of the molecule (Fig. 2 A), known as a protein–protein interacting structure, we first examined whether the LIM domain of zyxin serves as a binding interface for h-warts/LATS1 interaction. COS7 cell lysates...
expressing HA-tagged zyxin either full-length, NH₂-terminal two-thirds (Δ1) or COOH-terminal third (Δ2), which contains three LIM domains, were incubated with GST–h-warts/LATS1 (amino acids 136–700) fusion protein bound to glutathione-agarose beads (Fig. 2 B, lanes 1–3). The pull-down assay revealed that HA-zyxinΔ2 coprecipitated with GST–h-warts/LATS1 but HA-zyxinΔ1 did not (Fig. 2 B, lanes 7 and 9). Unexpectedly, the full-length HA-zyxin was hardly detectable in the GST–h-warts precipitate (Fig. 2 B, lane 5).

We next sought to delineate in detail the region(s) of zyxin important for its association with h-warts/LATS1 using GST fusion proteins containing a full-length or various truncated zyxin (Fig. 2 A). Same amounts of GST-zyxin fusion proteins bound to glutathione-agarose beads were incubated with baculoviral expressed His-tagged h-warts protein (full-length), and the retained proteins were analyzed by immunoblotting with anti-C2 antibody. While GST-zyxin-LIM1/2 (Δ3) and GST-zyxin-LIM1/2/3 (Δ2) were found to bind with purified h-warts protein, the other GST-zyxin mutants did not (Fig. 2 C). This in vitro binding experiment demonstrated that zyxin directly interacts with h-warts/LATS1 and that the region containing both LIM 1 and LIM 2 domains of zyxin is essential for their binding.

Interestingly, consistent with the findings shown in Fig. 2 A, GST-zyxin (full-length) did not interact with His-tagged h-warts/LATS1. However, their association was detected in in vivo binding assays (Fig. 1, C, E, and F). Based on these findings, we speculated that the LIM 1/2 domains are masked in full-length zyxin and that an intramolecular and/or intermolecular modification may regulate the interaction between zyxin and h-warts/LATS1.

Localization of h-warts/LATS1–Zyxin Complex to Mitotic Apparatus

Next, we compared the subcellular localization of h-warts/LATS1 and zyxin by immunocytochemical analysis. We raised antibody against zyxin (amino acids 24–35) in rabbits by the procedure described by Macalma et al. (1996). Consistent with their previous characterization, this antibody recognized a single band of ~82 kDa, which is described as the molecular mass of full-length zyxin by immunoblotting (Fig. 3 A). Indirect immunofluorescent studies revealed that the antibody specifically detected concentrated zyxin at the focal adhesion plaques, where the bundles of actin filaments end (Fig. 3 B). In the mitotic cells, the characteristic staining of zyxin was found to disappear from focal adhesions and was seen as a diffuse cytoplasmic distribution (Fig. 3 C). A similar change in subcellular distribution was observed in vinculin, another focal adhesion protein with a proline-rich stretch functionally related to zyxin, as cells retracted and rounded up during mitosis (data not shown). Notably, with careful observation of the zyxin immunostaining, a zyxin signal was barely seen at the mitotic apparatus (Fig. 3 C, b, arrows). This signal became more distinct when cells were fixed with acetone/methanol solution (Fig. 3 D).
To examine localization in detail, cells were processed in a detergent-preextraction protocol which washes out the free cytoplasmic proteins. As previously reported, h-warts/LATS1 locates to centrosomes during interphase (Fig. 4 A, a–c). When cells enter prometaphase/metaphase, h-warts/LATS1 translocates toward the mitotic spindle array as well as the spindle poles (Fig. 4 A, d–f). As the cell commits to anaphase (Fig. 4 A, g–i) and later to telophase (Fig. 4 A, j–l), h-warts/LATS1 is found progressively at the bundle of microtubules at the midzone, called the central spindle, that connects daughter cells (Fig. 4 A, m–o). The detergent-preextraction immunostaining procedure showed that a fraction of zyxin was also found to associate with mitotic apparatus during mitosis. As cells entered mitosis, when assembly of the mitotic spindle begins from spindle poles, zyxin initially located to the mitotic spindle (Fig. 4 B, a–c), and this association with the mitotic spindle became prominent by metaphase (Fig. 4 B, d–f). As cells proceeded into anaphase, zyxin was detected on the spindle between segregated sister chromatids, which are composed of bidirectional overlapping polar spindle (Fig. 4 B, g–i). In late anaphase, zyxin became detectable as a distinct wide band extending across the midzone of the central spindle (Fig. 4 B, j–l). This central spindle staining persisted into telophase when the spindle compacts into the midbody (Fig. 4 B, m–o). These specific staining patterns on the mitotic apparatus were neither detected with the preimmune IgG nor with anti-vinculin antibody (data not shown). When cells were double-stained for h-warts/LATS1 and zyxin, the yellow color produced by superim-
posing green and red demonstrated that h-warts/LATS1 and zyxin colocalize on the mitotic spindle, spindle poles and midbody of the dividing cells (Fig. 4 C). The dynamic changes in zyxin and h-warts/LATS1 localization on the mitotic apparatus is identical, suggesting that zyxin interacts with h-warts/LATS1 on the mitotic apparatus, including mitotic spindle and central spindle at midzone of the dividing cells.

Specific Phosphorylation of Zyxin during Mitosis

Since h-warts/LATS1 was reported to be posttranslationally modified in a cell cycle-specific manner and to play a critical role in cell cycle regulation (Tao et al., 1999; Nishiyama et al., 1999), we asked whether zyxin also has any cell cycle phase-specific changes concomitant with that of h-warts/LATS1 protein. For cell cycle analysis, HEK293 cells were synchronized at the beginning of G2 phase by double thymidine block method. At different times after release from block, cells were harvested and analyzed for zyxin by immunoblotting with anti-zyxin antibody (Fig. 5 A). Although levels of zyxin expression did not change throughout the cell cycle, a slow-migrating form of zyxin appeared in cells at 9 and 10 h after release from S phase, where considerable number of cells were in the mitotic phase as monitored by cyclin B protein level (Fig. 5 A, lanes 4 and 5). This slow migrating band became prominent when cells were treated with the microtubule-depolymerizing agent, nocodazole (Fig. 5 A, lane 8), whereas vinculin protein remained stable throughout cell cycle. To test whether this mitosis-specific modification of zyxin is phosphorylation, we examined the effect of phosphatase treatment on the mobility shift. The slow-migrating bands found in the mitotic and nocodazole-treated cell lysates were converted into the fast-migrating bands after zyxin was incubated with calf intestine alkaline phosphatase (Fig. 5 B, lanes 4 and 6). These results indicate that the slow-migrating form is phosphorylated zyxin, and that zyxin is specifically phosphorylated during mitosis.

For an additional approach to demonstrate the specific phosphorylation of zyxin during mitosis, in vitro kinase assay using cell lysates was employed. We used cell lysates from various phases in the cell cycle as sources of the enzyme and GST-zyxin as the substrate. GST-zyxin was phosphorylated significantly with mitotic cell lysate (Fig. 5 C, lane 3). To determine the phosphorylation region(s) in the molecule GST-zyxinΔ1 and GST-zyxinΔ2 were processed for the in vitro kinase assay. GST-zyxinΔ1, which corresponds to the NH2-terminal two thirds of zyxin, was significantly phosphorylated with mitotic cell lysate whereas no phosphorylation was detected in GST-zyxinΔ2 (Fig. 5 D).

Characterization of Zyxin-Kinase during Mitosis

To address the class to which zyxin-kinase belongs, we used various specific inhibitors that have been developed for kinases. The kinase assay was performed with mitotic cell lysates which were preincubated with various kinase inhibitors (Fig. 6 A). GST-zyxin phosphorylation activity in the cell lysate was specifically inhibited not only when the mitotic cell lysate was preincubated with the broad serine/threonine kinase inhibitor staurosporine (100 nM), but also after preincubation with olomoucine, which is a specific inhibitor of Cdc2 (Fig. 6 A). Therefore, to examine whether Cdc2 kinase is the responsible kinase for zyxin phosphorylation in the mitotic cell lysate, we prepared mitotic cell lysate depleted of Cdc2, which should contain the full complement of mitotically active kinases except for Cdc2, including kinases activated downstream of Cdc2 (Fig. 6 B). By monitoring the total protein concentration and the Cdc2 kinase activity of the depleted mitotic cell lysate (Fig. 6 C), we found that phosphorylation of GST-zyxin was significantly inhibited by depleting Cdc2 from the mitotic cell lysate (Fig. 6 D, lane 3). A addition of active Cdc2 complex to the Cdc2-depleted mitotic cell lysate restored the phosphorylation activity (Fig. 6 D, lane 4), indicating that loss of zyxin phosphorylating activity was due to removal of Cdc2 but not to other components in the lysate. Furthermore, the purified Cdc2 kinase complex was sufficient for zyxin phosphorylation without requiring any other components (Fig. 6 E). These data demonstrate that Cdc2 is a kinase responsible for the mitosis-specific phosphorylation of zyxin in vitro, even though other active kinases are present in the mitotic lysate.

Phosphorylation of Zyxin by Cdc2 Regulates Binding to h-warts/LATS1

Three lines of evidence suggest that zyxin interacts with h-warts/LATS1 during mitosis and that the interaction is regulated by the phosphorylation of zyxin. First, zyxin undergoes posttranslational modification during mitosis, which was shown to be phosphorylation. Second, h-warts/LATS1 binds to the region containing LIM1 and LIM2 domains of zyxin and an intra/inter-molecular modification of zyxin may be required for h-warts/LATS1 to access to the binding domains. Third, a fraction of zyxin is distributed to the mitotic apparatus, where it colocalizes with h-warts/LATS1 protein. Since our results demonstrated that Cdc2 is the kinase responsible for mitotic phosphorylation of zyxin, we postulated that the Cdc2-mediated phosphorylation of zyxin promotes interaction between zyxin and h-warts/LATS1. To test this possibility, GST-zyxin (full-length) was phosphorylated by active Cdc2 complex, followed by incubation with recombinant His-tagged h-warts/LATS1. His-tagged h-warts/LATS1 precipitated with phosphorylated GST-zyxin (Fig. 7 A, lane 5) while the control unphosphorylated GST-zyxin did not (Fig. 7 A, lanes 2 and 4).

Furthermore, to examine whether zyxin associates with h-warts/LATS1 specifically during mitosis in the intact cells, endogenous zyxin was immunoprecipitated from either interphase or mitotic cell lysate and probed with anti-h-warts/LATS1 antibody. The endogenous h-warts/LATS1 coprecipitated with zyxin from mitotic lysate was found to be more abundant than that from interphase cell lysate (Fig. 7 B, lane 4). These results indicate that zyxin interacts with h-warts/LATS1 during mitosis, and that the interaction is regulated by phosphorylation of zyxin. This mitosis-specific interaction explains why the proportion of the zyxin-binding h-warts/LATS1 to its entire pool was less than 10% in asynchronized cells (Fig. 1 F). It is also interesting to note that the zyxin-binding h-warts/LATS1 migrated slower than its entire pool (Fig. 1 F), suggesting that mitotic phosphorylated form of h-warts/LATS1
Figure 4 (continues on facing page).
Interacting with h-warts/LATS1

To test whether dynamic changes in zyxin localization on the mitotic apparatus is dependent on h-warts/LATS1 distribution, we attempted to disrupt endogenous zyxin localization by injecting excessive amounts of an h-warts/LATS1 fragment (amino acids 136–700) that is shown to bind preferentially to zyxin (Fig. 2 B). Prophase/prometaphase U2OS cells were microinjected with purified GST–h-warts/LATS1(136–700) fusion protein, or control GST protein. Rhodamine-labeled tubulin was injected together with the GST fusion proteins not only to distinguish injected cells but to monitor mitotic spindle organization as the cell cycle proceeds. After injection, cells were subjected to the detergent-preextraction immunostaining with anti-α-tubulin antibody. Immunofluorescence staining was performed with Texas red–conjugated anti–mouse IgG antibody (a, d, g, j and m) and FITC–conjugated anti–rabbit IgG antibody (b, e, h, k, and n). The merged pictures of the upper two panels are shown in the lower panel (c, f, i, l, and o). Representative cells in prometaphase (a–c), metaphase (d–f), anaphase (g–i), telophase (j–l), and later telophase (m–o) are shown. (C) I dentical distribution of h-warts/LATS1 and zyxin to the mitotic apparatus. U2OS cells were prepared and fixed as in (A) and processed for incubation with rabbit anti-zyxin antibody (a and d) and rat anti-h-warts antibody (b and e). Immunofluorescence staining was performed with FITC–conjugated anti–rabbit antibody and Cy3–conjugated anti–rat IgG antibody. The merged pictures of the left two panels are shown in the right panel (c and f). Bars, 10 µm.

Discussion

In searching for cellular targets of the h-warts/LATS1 tumor suppressor protein, we have identified zyxin which plays a central role in actin assembly and organization. During interphase, zyxin is found at cellular locations that are enriched in actin filaments, including the leading edge and focal adhesion plaques, whereas h-warts/LATS1 is localized to centrosome. However, during mitosis, when zyxin is dissociated from focal adhesions, a fraction of the free cytoplasmic zyxin becomes associated with mitotic spindles. We have shown that zyxin is phosphorylated during mitosis and that the mitotic phosphorylation is required for association with h-warts/LATS1 on the mitotic process. To test this possibility, we examined whether h-warts/LATS1(136–700) microinjected cells can proceed through mitosis normally. HeLa cells, synchronized at S phase, were coinjected with GST–h-warts/LATS1 (136–700) or GST–mock and β-galactosidase to detect injected cells. Cells were fixed at various time points after release from an S phase block, and the frequency of mitotic cells (mitotic index) in cells having β-galactosidase activity was determined (Fig. 9 A). While the control experiments exhibited a peak mitotic index at 10.5 h after release from S phase and a decrease after 12 h, h-warts/LATS1(136–700)–injected cells revealed a high mitotic index after 10.5 h and continued to increase until 12 h after the release (Fig. 9 B). Apparently, cells injected with h-warts/LATS1(136–700) had a prolonged mitotic phase. These observations suggest that disruption of h-warts/LATS1–zyxin complex leads to impairment of normal mitotic progression.
apparatus. These findings implicate dual functions for zyxin: not only does it play a key role in cell adhesion and cytoskeletal organization in interphase cells, but it also acts as a participant in mitotic control by forming a complex with h-warts/LATS1 on the mitotic apparatus.

**Regulation of the Interaction between h-warts/LATS1 and Zyxin**

We have shown a physical association of zyxin with h-warts/LATS1 and that a region containing the first and second LIM domains (LIM 1/2) of zyxin is responsible for the interaction. The LIM domain is a protein binding motif which is found in a wide variety of proteins involved in transcription, cell adhesion, and cytoskeletal organization (Schmeichel and Beckerle, 1994; Dawid et al., 1998). Proteins with multiple LIM domains, such as zyxin, are considered to function as scaffolds for the assembly of protein complexes. To date, only cysteine-rich protein 1 (CRP1) has been reported to associate with the LIM 1 domain of zyxin (Sadler et al., 1992). Our findings demonstrated that a combination of LIM 1 and LIM 2 domains, but not that of LIM 2 and LIM 3, is essential for zyxin to interact with h-warts/LATS1, suggesting that a specific protein recognition mechanism is used for their interaction. The association of h-warts/LATS1 with the full-length zyxin has been detected by in vivo but not by in vitro binding experiments. However, the deletion of the NH₂-terminal region of zyxin has allowed the LIM1/2 domain to interact with h-warts/LATS1 in vitro. Therefore, we speculate that the LIM1/2 domains are masked in full-length zyxin and that the posttranslational modification, such as phosphorylation and/or proteolysis, gives rise to the conformational alterations in zyxin, exposing the LIM domains on the surface of the molecule, which allows h-warts/LATS1 to approach.

Although zyxin was first described as a phosphoprotein (Crawford and Beckerle, 1991), both the mechanism and the biological significance of the phosphorylation have remained elusive. In this study, we demonstrated that the NH₂-terminal region of zyxin is mitotically phosphorylated. Three lines of evidence presented here suggest that the mitotic phosphorylation of zyxin is mediated by Cdc2, a primary kinase to drive mitosis. First, depletion of Cdc2 from mitotic cell lysates completely abolished phosphorylation of zyxin. Second, purified active Cdc2 complex phosphorylated zyxin. Third, zyxin is phosphorylated during mitosis when Cdc2 is active. A number of studies have focused on the role of Cdc2 in the coordination of cellular and biochemical events during mitosis, and identification...
of its physiological substrates continues to represent a major challenge (Nigg et al., 1996; Nurse, 2000). It cannot be completely ruled out the possibility that another kinase phosphorylates zyxin in vivo. However, the previous findings that a fraction of the cellular pool of Cdc2 is associated with the mitotic spindle, where it forms an active kinase complex with cyclin B (Bailly et al., 1989), also supports our present observations. As predicted by our hypothesis, the Cdc2-dependent phosphorylation has been shown to allow the full-length zyxin to interact with h-warts/LATS1 (Fig. 7 A). In fact, zyxin associates with h-warts/LATS1 preferentially during mitosis (Fig. 7 B), and this interaction might be significant for the subsequent cellular events in cell division. However, the binding of phosphorylated full-length zyxin to h-warts/LATS1 was not as efficient as that of the Δ2 protein (Fig. 7 A), so the possibility can not be excluded that the interaction is regulated by not only phosphorylation but other unknown modifications of full-length zyxin.

**Spatial Control of Zyxin Localization during Mitosis**

Zyxin has been shown to dissociate from focal adhesions and to distribute diffusely into the cytoplasm coincidentally with the mitotic disappearance of focal adhesions. Subsequently, a fraction of this free cytoplasmic zyxin becomes colocalized with h-warts/LATS1 on the mitotic apparatus (Figs. 3 and 4). Since zyxin plays a critical role in the actin filament assembly at focal adhesions (Beckerle, 1998, 1997), dissociation of zyxin may contribute to actin stress fiber disassembly in the mitotic cells. The other focal adhesion components, such as paxillin (Y amaguchi et al., 1997) and p130CAS (Yamakita et al., 1999), have been reported to be phosphorylated during mitosis when focal adhesions dissociate. As for zyxin, it can be speculated that the mitotic phosphorylation is a signal for triggering detachment of zyxin from adhesion sites. However, two observations presented here support the possibility that mitotic phosphorylation is required mainly to recruit zyxin to the mitotic spindle. First, although most of the zyxin became dissociated during mitosis, both phosphorylated and dephosphorylated zyxin were detected in the cells at mitosis (Fig. 5, A and B). Second, the immunolocalization study demonstrated that a subfraction of zyxin localized to the mitotic apparatus (Fig. 3, C and D), which was clearly detected by the preextraction method (Fig. 4 B).
The interaction between zyxin and h-warts/LATS1 on the mitotic apparatus implicates a significant role of actin regulatory proteins during mitosis. Zyxin serves as a scaffold for gathering actin regulatory proteins, such as Ena/VA SP-profilactin complex or vav-small GTPase complex, at focal adhesion plaques (Beckerle, 1997). In fission yeast...
and D.rosophila, defects in profilin, which is shown to be essential to form the actomyosin contractile ring, results in the failure of cytokinesis (Balasubramanian et al., 1994; Giansanti et al., 1998). Rho GTPase-mediated signal is required for the organization of cortical components to form the contractile ring in mammalian cells (O’Connell et al., 1999). Moreover, a number of genetic analyses converge on the idea that components associated with mitotic spindle may cooperate in formation of the contractile ring by mediating the biochemical signaling or the physical interaction between two structures (Williams et al., 1995; Adams et al., 1998). All these findings support the idea that zyxin also plays a role in cell division by regulating actin filament assembly at midzone of the dividing cell.

The duration of mitosis in cells injected with h-warts/LATS1(136–700) fragment, which significantly perturbed zyxin specifying to the mitotic apparatus, was significantly longer than that of control cells, mainly due to delay in exit from mitosis (Fig. 9). An intriguing explanation for the observation is that the mislocalization of zyxin, as well as zyxin-binding partners for actin polymerization, induces discoordination of contractile ring in dividing cells, and thereby delays their exit from mitosis. Alternatively, h-warts/LATS1 enzymatic activity may be modulated by zyxin, so that disruption of their interaction results in inactivation of h-warts/LATS1 itself. It can not be excluded, however, that another, as yet unknown, protein(s) is also functionally impaired by h-warts/LATS1(136–700) fragments to produce these results.

To maintain genomic stability and proper ploidy, it is crucial that cell division occurs at the end of anaphase after chromosome segregation. The molecular mechanism
through which h-warts/LAT51 serves as a tumor suppressor is unknown, but one possible explanation is that h-warts/LAT51 may play a critical role in cell division processes by forming a complex with zyxin on the mitotic apparatus. A brogation of this interaction may involve failure in normal mitotic progression, leading to chromosomal instability, which is a hallmark of malignant tumors.

We thank Drs. Q. Hu, H. Maruta, and A. Kikuchi for providing the pCGN, pGEX2TH, and pBl-Myc plasmids, respectively; Dr. K. Tanabe for critically reading the manuscript; Dr. J. Moon for editorial assistance; Drs. Y. Arima, S. Honda, M. Nitta, and H. Nakamura for valuable suggestions; Mr. K. Ida (Olympus) and Mr. K. Ueda (Uniscience) for technical assistance; and T. Aino for secretarial assistance.

This work was supported by a grant for Cancer Research from the Ministry of Education, Science and Culture of Japan (H. Saya).

Submitted: 16 November 1999
R revised: 14 April 2000
A accepted: 19 April 2000

References

A dams, R. R., A. A. Tavares, A. Salzberg, H. J. Bellen, and D. M. Glover. 1989. pavarotti encodes a kinase-like protein required to organize the central spindle and contractile ring for cytokinesis. Genes Dev. 12:1483–1494.

Bailer, J. A., B. Steever, S. Wheatley, Y. Wang, J. R. Pringle, K. L. Gould, and D. McCollum. 1998. Role of polo kinase and Mis12 in determining the site of cell division in fission yeast. J. Cell Biol. 143:1603–1616.

Bailly, E., M. Doree, P. Nurse, and M. Bornens. 1989. p34cdc2 is located in both nucleus and cytoplasm; part is centrosomally associated at G2/M and enters vesicles at anaphase. EMBO (Eur. Mol. Biol. Organ.) J. 8:3985–3994.

Bai, J., M.spectrof, T. Hiraoka, N. Kitamura, and H. Saya. 1999. A human homolog of Drosophila warts tumor suppressor, h-warts, localized to mitotic apparatus and specifically phosphorylated during mitosis. FEBS Lett. 459:159–165.

Nix, D. A., and M. C. Beckerle. 1997. Nuclear-cytoskeletal shuttling of the focal contact protein, zyxin: a potential mechanism for communication between sites of cell adhesion and the nucleus. J. Cell Biol. 138:1139–1147.

Nurse, P. 2000. A long twentieth century of the cell cycle and beyond. Cell. 100: 73–78.

O’Connell, C. B., S. P. Wheatley, S. A. Hnilde, and Y. L. Wang. 1999. The small GTP-binding protein rho regulates cortical activities in cultured cells during division. J. Cell Biol. 144:305–313.

Prehoda, K. E., D. J. Lee, and W. A. Lim. 1999. Structure of the enabled/VASP homology 1 domain-peptide complex: a key component in the spatial control of actin assembly. Cell. 97:471–480.

Reindrich, M., K. J. Juve, D. Tripier, and U. Walter. 1995. Identification, purification, and characterization of a zyxin-related protein that binds the focal adhesions and microfilament protein VASP (vasodilator-stimulated phosphoprotein). Proc. Natl. Acad. Sci. USA. 92:7956–7960.

Sadler, I. A., W. A. Crawford, J. W. M. Ichihara, and M. C. Beckerle. 1992. Zyxin and c-CRK: two interactive LIM domain proteins associated with the cytoskeleton. J. Cell Biol. 119:1373–1377.

Schmeichel, K. L., and M. C. Beckerle. 1994. The LIM domain is a modular protein-binding interface. Cell. 79:211–219.

Sparks, C. A., M. Orphee, and D. McCullum. 1999. Sid2p, a spindle pole body kinase that regulates the onset of cytokinesis. J. Cell Biol. 146:777–790.

St. John, M. A., M. Tao, X. Fei, R. Fukushima, M. L. Carcangiu, D. F. Brownstein, A. F. Parlow, J. McGarr, and T. Xu. 1999. Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunctions. Nat. Genet. 21:182–186.

Sunkel, C. E., and D. M. Glover. 1988. Polo, a mitotic cofactor protein of Drosophila displaying abnormal spindle poles. J. Cell. Sci. 99:25–38.

Tao, W., S. Zhang, G. S. Turechek, R. A. Stewart, M. A. St. John, W. Chen, and T. Xu. 1999. Human homolog of the Drosophila melanogaster lat tumor suppressor modulates CDC2 activity. Nat. Genet. 21:177–181.

Teraoka, Y., M. Tatsuka, F. Suzuki, Y. Yasuda, S. Fujita, and M. Otsu. 1998. A LIM-U-a mammalian monocot-associated protein required for cytokinesis. EMBO (Eur. Mol. Biol. Organ.) J. 17:667–676.

Toyn, J. H., and L. H. Johnston. 1994. The Dbf2 and Dbf20 kinases of budding yeast are activated after the metaphase to anaphase cell cycle transition. EMBO (Eur. Mol. Biol. Organ.) J. 13:1103–1113.

Verde, F. D., J. W. Thomas, and F. Nurse. 1996. Fission yeast orb1, a novel protein kinase related to mammalian rho kinase and myotonic dystrophy kinase, is required for maintenance of cell polarity and coordinates cell morphogenesis with the cell cycle. Proc. Natl. Acad. Sci. USA. 95:7526–7531.

Vojtek, A. B., S. M. Hellenberg, and J. A. Cooper. 1993. Mammalian R as interacts directly with the serine/threonine kinase Raf. Cell. 74:205–214.

Williams, B. C., M. F. Riedy, E. W. Williams, M. Gatti, and M. L. Goldberg. 1995. The p34cdc2 kinase cofactor CLP3A is a midbody component required for central spindle assembly and initiation of cytokinesis. J. Cell Biol. 129:709–723.

Xu, T., W. Wang, S. Zhang, R. A. Stewart, and W. Y. Yu. 1995. Identifying tumor suppressors in genetic mouse models. Drosophila latz51 gene encodes a putative protein kinase. Development. 121:1053–1063.

Yamaguchi, R., Y. Mazaki, K. Hiranaka, S. Hashimoto, and H. Sabe. 1997. Miotis specific serine phosphorylation and downregulation of one of the focal adhesion proteins, paxillin, in the Drosophila latz gene. J. Cell Biol. 138:1139–1150.

Yamakita, Y., T. Sasahara, Y. Ohba, et al. 1992. p13suc1 suppresses the catalytic function of p34cdc2 kinase for intermediate filament proteins. In vitro, J. Biol. Chem. 267:20153–20162.

Macalma, T., J. Otte, M. E. Hensler, S. M. Boekhoff, H. A. Louis, M. Kalf-Suske, K. H. G. Rzczesz, D. von der Ahe, and M. C. Beckerle. 1996. Molecular characterization of human yixin. J. Biol. Chem. 271:31470–31478.

Madanayake, P. M., E. Fujisawa, T. Tatsuno, T. Matsuoka, H. Bito, T. Ishizaki, and S. Naramiya. 1998. Role of ctnin kinase as the target of the small GTPase Rho in cytokinesis. Nature. 394:491–494.

Masuko, N., K. Minami, K. Uwahara, K. Fukunaga, T. Sudo, N. A. Raki, H. Yamada, Y. Y. Amada, Y. Miyamoto, and H. Saya. 1999. Interaction of NE-digSA P102, a neuronal and endocrine tissue-specific membrane-associated guanylate kinase protein, with calmodulin and P50-NSA P90. A possible regulatory role in molecular clustering at synaptic sites. J. Biol. Chem. 274:5792–5798.

Nigg, E. A. 1998. Polo-like kinases: positive regulators of cell division from start to finish. Curr. Opin. Cell Biol. 10:776–783.

Nigg, E. A., A. Blangy, and H. A. Lane. 1996. Dynamic changes in nuclear architecture during mitosis: the role of protein phosphorylation in spindle assembly and chromosome segregation. Exp. Cell Res. 229:174–180.

Nishiyama, Y., T. Horiya, T. Morisaki, T. Hara, T. Marumo, S. Iida, K. M. Minami, H. Yamamoto, T. Hiraoka, N. K. Itamura, and H. Saya. 1999. A human homolog of Drosophila warts tumor suppressor, h-warts, localized to mitotic apparatus and specifically phosphorylated during mitosis. FEBS Lett. 459:159–165.