A human homolog of *Drosophila* warts tumor suppressor, h-warts, localized to mitotic apparatus and specifically phosphorylated during mitosis

Yasuyuki Nishiyama[a,b], Toru Hirota[a], Tetsuro Morisaki[a,b], Toshihiro Hara[a], Tomotoshi Marumoto[a], Shin-ichi Iida[a], Keishi Makino[a], Hideyuki Yamamoto[c], Takehisa Hiraoka[b], Nobuo Kitamura[b], Hideyuki Saya[a,*]

[a] *Department of Tumor Genetics and Biology, Kumamoto University School of Medicine, 2-2-1 Honjo, Kumamoto 860-0811, Japan*
[b] *The 1st Department of Surgery, Kumamoto University School of Medicine, 2-2-1 Honjo, Kumamoto 860-0811, Japan*
[c] *Department of Pharmacology, Kumamoto University School of Medicine, 2-2-1 Honjo, Kumamoto 860-0811, Japan*

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Abstract We identified a human homolog of *Drosophila warts* tumor suppressor gene, termed *h-warts*, which was mapped at chromosome 6q24-25.1. The h-warts protein has a serine/threonine kinase domain and is localized to centrosomes in interphase cells. However, it becomes localized to the mitotic apparatus, including spindle pole bodies, mitotic spindle, and midbody, in a highly dynamic manner during mitosis. Furthermore, h-warts is specifically phosphorylated in cells at mitotic phase, most likely by Cdc2 kinase. These findings suggest that h-warts functions as a component of the mitotic apparatus and is involved in proper progression of mitosis.

Key words: Centrosome; Mitotic spindle; Cdc2; Midbody; Nocodazole; Chromosome 6q

1. Introduction

Mitosis is characterized by dramatic morphological changes which occur in a strictly sequential order, including cytoskeletal disassembly, breakdown of the nuclear envelope, chromosome condensation, segregation and, finally, daughter cell separation. Multiple lines of evidence suggest that the molecular mechanisms of such events during mitosis are driven by two fundamental chemical reactions, phosphorylation and degradation of diverse proteins [1]. Identification of the kinases involved and determination of how they are coordinated are required for understanding how mitosis is regulated.

Recent studies have demonstrated that several serine/threonine kinases, which are highly conserved in amino acid sequence from yeast to human, are located on the mitotic apparatus during mitosis. These include yeast Cdc5p/*Drosophila* polo/human Plk1 [2–4], yeast Ipl1/*Drosophila* aurora/human Aik/human Aim [5–8], and *Aspergillus nidulans* NIMA/human Neks [9,10]. *Drosophila* and yeast mutants of these kinase genes exhibit abnormal regulation of mitosis, such as monopolar spindle, unequally separated bipolar spindle and cytokinesis failure. Therefore, identification of mammalian homologs of the mitosis-related kinases could provide a clue to the mechanisms regulating normal spindle assembly and chromosomal segregation in mammalian cells.

*Corresponding author. Fax: +81 (96) 373-5120.
E-mail: hsaya@gpo.kumamoto-u.ac.jp

The *warts* (also referred to as *lats*) gene was identified based on its ability to act as a tumor suppressor in *Drosophila* [11]. Deletion of this gene leads to the formation of cell clones that are rounded and greatly overgrown, and literally generate 'warts' on their legs and body. Thus, the *warts* gene is considered to be important in controlling cellular morphogenesis and proliferation. The protein encoded by the *warts* gene has a serine/threonine kinase domain which is highly homologous to the human myotonic dystrophy protein kinase (DM-PK) [12], the mammalian Rho-associated kinases [13] and yeast Dbf2 and Dbf20 kinases [14]. Many of the DM-PK family kinases have been reported to be involved in progression of the cell cycle, especially in mitosis. In the budding yeast *Saccharomyces cerevisiae*, temperature-sensitive mutants of *Dbf2* arrest in telophase with a terminal 'dumbbell' phenotype in which they display an elongated spindle and divided chromatin. The kinase activity of Dbf2 protein is cell cycle regulated with a peak in late mitosis, suggesting that Dbf2 is considered to play a role in late mitotic events [15].

In this paper we report the isolation of a putative human protein kinase, termed h-warts, which is highly related to the *Drosophila* warts protein and describes its possible involvement in cell cycle regulation. Immunolocalization experiments show that the h-warts protein is found at centrosome in interphase cells while it becomes localized to the mitotic apparatus in a highly dynamic manner during mitosis. Furthermore, although expression levels of h-warts protein are constant and do not fluctuate throughout the cell cycle, h-warts is phosphorylated in the mitotic phase and hyperphosphorylated when the cell cycle is arrested by disruption of the spindle with nocodazole. Thus, these results suggest that h-warts functions as a component of the mitotic apparatus and is modulated during mitosis.

2. Materials and methods

2.1. EST database screening and PCR-based full-length cDNA cloning

We searched for human homologs of the *Drosophila warts* gene by scanning a database of human genes identified by the expressed sequence tag (EST) method using tblastn. The EST clone with the accession number Z13665 has a significant sequence similarity. Based on the sequence information of the Z13665 clone, we performed PCR-based full-length cDNA cloning, as described previously [16,17]. For amplification of the 5′ region, the first PCR was performed using only P1 primer (5′-CTTGTTTTTCCTAACAGTAATAGG-3′) to amplify single-strand cDNA from a human fetal brain cDNA library (Clontech, Marathon-Ready cDNA) in a 15 μl reaction volume for 50 cycles. The first PCR product was used as a template in the second

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run, where P2 (5′-TGAAGTTGTAATCTGTTTCTTTTC-3′) and AP2 (5′-ACTCACTATAGGGCTCGAGCGGC-3′) were utilized as primers to amplify the 5′ region of cDNA. For the 3′ region amplification, P6 (5′-TATTCTCCTCAAGCATTTAAATTC-3′) was used for the first PCR, and P7 (5′-TTTATGGAGCAACATGTAGAAAAT-3′) and AP2 were used for the second PCR. The PCR fragments were ligated into a pCR2 TA cloning vector and sequenced.

2.2. Radiation hybrid mapping of h-warts gene

PCR was performed to detect *h-warts* sequences in the Genebridge 4 Radiation Hybrid Screening Panel (Research Genetics, Inc.) using a set of primers (S-1: 5′-ACACAGGCTCAGAGATTAAAA-3′ and AS-1: 5′-CCTCGAATTCCAGGCCCTTTTACAA-3′) which were designed based on a partial genomic sequence of the *h-warts* gene. The S-1 and AS-1 primers were expected to amplify a 97-bp PCR product from human genomic DNA as a template. PCR was carried out as previously described [16]. The PCR results of the Radiation Hybrid Panel were sent to the Whitehead Institute/MIT Center for Genome Research via WWW (http://www-genome.wi.mit.edu/) for mapping of the genes relative to the radiation hybrid map of the human genome.

2.3. Northern blot analysis

Northern blot derived from multiple human tissues containing 2 μg of poly(A)$^+$ RNA per lane were obtained from Clontech. The membranes were hybridized with an 1178-bp cDNA fragment of *h-warts* that had been labeled with [α-^{32}P]dCTP by random primed labeling method. The membranes were exposed to X-ray film with an intensifying screen for 3 days at −70°C.

2.4. Expression of GST fusion proteins

The cDNA fragments coding three deletion mutants of h-warts, WP (amino acid nos. 136–403), WN (nos. 395–700) and WK (nos. 682–1130), were amplified by PCR using the following sets of primers: WP, 5′-CAATTGAATTCATTAGTAAAATG-3′ and 5′-CTGAGGAATTCTTCCATTTGTAT-3′; WN, 5′-CAGGGGAATTCGCTGCTCCTTCGTCATATAC-3′ and 5′-CAAACATAGAATTCTCCATTTTAGCCCTTTTA-3′; WK, 5′-CAGGATGAATTCAGAAAGATGCTTTGCC-3′ and 5′-CATTTAGAATTCAGTGTGTTAAAC-3′, and subcloned into a pGEX-2TH bacterial expression vector. The expression and purification of the GST fusion proteins were described previously [16].

2.5. Production of anti-h-warts polyclonal antibodies and Western blot analysis

Two polyclonal antibodies against h-warts protein, termed C-2 and G-3, were raised by subcutaneous immunization of rabbit and rat with a synthetic peptide (KSAKHIKEKEQRDPIYLRDKVTQRH) and the bacterially expressed GST-WP protein, respectively.

For the Western blot analysis, samples containing equal amounts of protein (20 μg) from lysates of cultured cells were separated on a 6% polyacrylamide gel and transferred to a nitrocellulose filter with a constant current of 180 mÅ for 2 h. The filters were probed with the C-2 antibody or G-3 using the method previously described [16].

2.6. Construction of the h-warts expression plasmid

The full-length ORF of the *h-warts* cDNA was amplified by PCR from the Marathon-Ready cDNA (Clontech) by a set of primers, wts-sense (5′-GATTCTAGAATGAAGAGGAGTGAAAAGCCAG-3′) and wts-antisense (5′-CCTTCTAGAAATCCTCATTACATT-3′) containing *Xba*I site (underlines). The PCR fragments were digested with *Xba*I, and ligated into pCGN expression vector. The nucleotide sequence of the insert was confirmed. To express HA-tagged h-warts, the pCGN-h-warts was transfected into COS7 cells by the liposome-mediated gene transfer method.

2.7. Immunofluorescence staining and confocal laser scanning microscopic analysis

U2OS cells seeded on Labtek chamber slides (Nunc) were pretreated with a microtubule stabilizing buffer (80 mM potassium PIPES, pH 6.8, 5 mM EGTA, 1 mM $MgCl_2$) containing 0.5% Triton X-100 and then fixed with methanol for 10 min at −20°C [8,18]. Fixed cells were washed with a solution of 0.1 M PIPES, pH 7.2, 1 mM $MgSO_4$, 1 mM EGTA, 1.83% L-lysine, 1% BSA and 0.1% sodium azide and were subsequently incubated for 1 h with anti-h-warts (G-3), anti-α-tubulin and anti-γ-tubulin antibodies (Sigma). This was followed by incubation with FITC-conjugated and Cy3-conjugated secondary antibodies (Biosource). DNA was stained by adding propidium iodide (Sigma) to a final concentration of 1 μg/ml. After being washed with PBS, samples were mounted in 50% glycerol and visualized with a confocal laser microscope (Fluoview, Olympus).

2.8. Double thymidine cell cycle synchronization at G1/S phase

HeLa cells were treated with 1 mM thymidine for 24 h, followed by a 8 h release in fresh Dulbecco's modified Eagle's minimum essential medium (D-MEM) with 5% fetal calf serum and successive retreatment with thymidine for 14 h, and released to enter the cell cycle [19].

2.9. Alkaline phosphatase treatment

Nocodazole-treated HeLa cell lysate was extracted with digestion buffer (100 mM Tris [pH 8.0], 40 mM NaCl, 1 mM $MgCl_2$, and 0.1% SDS), followed by centrifugation at 12 000×*g* for 15 min. The lysate was treated with 30 U of alkaline phosphatase (TAKARA) for 2 h at 37°C in the presence or absence of phosphatase inhibitors (2.5 mM Na_3VO_4, 50 mM NaF, 100 mM β-glycerophosphate and 2.5 mM EDTA) and then immunoblotted with C-2 antibody.

2.10. In vitro kinase assay

HeLa cells were lysed with IP buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 1% NP-40, 10% glycerol, 5 mM EDTA, 0.5 mM EGTA, 20 mM β-glycerophosphate, 50 mM NaF, 0.5 mM ABSF, 1 μM leupeptin, 1 mM Na_3VO_4 and 1 μM microcystin LR) for 20 min. Lysates were centrifuged at 14 000×*g* for 20 min, and the supernatant was used for the kinase assay. The kinase assays were conducted at 25°C for 30 min in a final volume of 50 μl containing 20 mM Tris (pH 7.4), 10 mM $MgCl_2$, 10 μCi of [γ-^{32}P]ATP (3000 Cu/mmol), 1 μM microcystin, 8 μg of cell lysates, and 10 μg of GST-h-warts. To terminate the reaction and separate the phosphorylated GST fusion proteins, each reaction mixture was immediately chilled and mixed with 50 μl of GSH-agarose beads and 0.5 ml of ice-cold TNE buffer containing 20 mM Tris [pH 7.4], 100 mM NaCl, 1% NP-40, 1 mM EDTA, 10 mM β-glycerophosphate, and 1 mM Na_3VO_4, followed by rocking for 30 min at 4°C. The GSH-agarose beads were then washed three times with TNE buffer, and boiled in 30 μl of Laemmli sample buffer to elute GST fusion proteins. The samples were separated on 8% SDS-PAGE and visualized by autoradiography.

3. Results

3.1. Identification of the human gene homologous to Drosophila warts

To identify the human homolog of the *Drosophila warts* (*d-warts*), we performed sequence database searching using the Blast algorithm. A tblastn search of the GenBank database using the non-catalytic domain of *Drosophila* warts peptide sequences revealed that one EST clone, with the accession number Z13665, has high sequence similarity, and it was not found to be identical to any previously identified genes. Based on the sequence of Z13665, we performed a two-step PCR to clone the full-length cDNA. We identified a 3390-bp cDNA which contains a single large open reading frame encoding a polypeptide of 1130 amino acids (GenBank accession no. AF164041). We refer to this gene as *h-warts* (*human warts*). The deduced amino acid sequences of h-warts aligned with the *Drosophila* warts are shown in Fig. 1. The h-warts protein has a putative kinase domain in the C-terminal portion and contains all of the 12 protein kinase catalytic subdomains characteristic of a serine/threonine kinase [20]. The GXGXXG ATP binding consensus motif characteristic of many protein kinases is found in subdomain 1. An invariant lysine at position 734 of the protein, necessary for the ATP binding, is also present. Inspection of the h-warts amino acid sequence revealed that it contains several conserved clusters of basic amino acids (607–623, 682–698, 659–662 and 723–739)

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h-warts -----MKRSEKP--- ------EGYRQMRPK TFPASNYTVSS-RQM LQEIRESLRNLSKPS DAAKAEHNMSKMSTE DPR-QVRNPPK---F    71
d-warts MHPAGEKRGGRPNDK YTAEALESIKQDLTR FEVQNNHRNNQNYTP LRYTATNGRNDALTP DYHHAKQPMEPPPSA SPAPDVVIPPPPAIV    90

h-warts GTHHKALQEIRNSLL PFANETNSSRSTSEV NPQMLQDLQAAGFDE DMVIQALQKTNNRSI EAAIEFISKMSYQDP RREQMAAAAARP-IN   160
d-warts GQPGAGSISVSGVGV GVVGVANGRVPKMMT ALMPNKLIRKPSIER DTASSHYLRCSPALD SGAGSSRSDSPHSHH THQPSSRTVGNPGGN   180

h-warts ASMKPG-----NVQQ SVNRKQSWKGSKESL VPQRHGPPLGESVAY HSESPNSQ-TDVGRP LSGSGISAFVQAHPS NGQR--------VNP   236
d-warts GGFSPSPSGFSEVAP PAPPPRNPTASSAAT PPPPVPPTSQAYVKR RSPALNNRPPAIAPP TQRGNSPVITQNGLK NPQQQLTQQLKSLNL   270

h-warts PPPPQVRSVTPPPPP RGQTPPPRGTTPPPP --SWEPNSQTKRYSG NMEYVISRISPVP-- PGAWQEGYPPPPLNT SPMNPPNQGQRGISS   322
d-warts YPGGGSGAVVEPPPP YLIQGGAGGAAPPPP PPSYTASMQSRQSPT QSQQSDYRKSPSSGI YSATSAGSPSPITVS LPPAPLAKPQPRVYQ   360

h-warts VPVGRQPIIMQSSSK FNFPSGRPGMQNGTG -QTDFMIHQNVVPAG TVNRQPPPPYPLTAA NGQSPSALQTGGSAA PSSYTNGSIPQSMMV   411
d-warts ARS-QQPIIMQSVKS TQVQ--KPVLQTAVA PQSPSSASASNSPVH VL--AAPPSYPQKSA AVVQQQQQAAAAAHQ QQHQHQQSKPATPTT   445

h-warts PNRNSHNMELYNISV PGLQTNWPQSSSAPA QSSPSSGHEIPTWQP NIPVRSNSFNNPLGN RASHSANSQPSATTV TAITPAPIQQPVKSM   501
d-warts PPLVGLNSKPNCLEP PSYAKSMQAKAATVV QQQ-QQQ------QQ QQQVQQQ-----Q-- -VQQQQQQQQQQLQA LRVLQAQAQRERDQR   520

h-warts RVLKPELQTALAPTH PSWIPQPIQTVQPSP FPEGTASNVTVMPPV AEAPNYQGPPPPYPK HLLHQNPSVPPYESI SKPSKEDQPSLPKED   591
d-warts --ERERDQQKLANGN P----G-RQMLPPPP YQSNNNNNSEIKPPS CNNNNIQIS-----N SNLATTPPIPPAKYN NNSSNTGANSSGGSN   598

h-warts ESEKSYENVDSGDKE KKQITTSPITVRK-- -NKKDEERRESRIQS YSPQAFKFFMEQHVE NVLKSHQQRLHRKKQ LENEMMRVGLSQDAQ   678
d-warts GS--TGTTASSSTSC KKIKHASPIPERKKI SKEKEEERKEFRIRQ YSPQAFKFFMEQHIE NVIKSYRQRTYRKNQ LEKEMHKVGLPDQTQ   686
                                    * * *                 ◇
h-warts DQMRKMLCQKESNYI RLKRAKMDKSMFVKI KTLGIGAFGEVCLAR KVDTK-ALYATKTLR KKDVLLRNQVAHVKA ERDILAEADNEWVVR   767
d-warts IEMRKMLNQKESNYI RLKRAKMDKSMFVKL KPIGVGAFGEVTLVS KIDTSNHLYAMKTLR KADVLKRNQVAHVKA ERDILAEADNNWVVK   776

h-warts LYYSFQDKDNLYFVM DYIPGGDMMSLLIRM GIFPESLARFYIAEL TCAVESVHKMGFIHR DIKPDNISIDRDGHI KLTDFGLCTGFRWTH   857
d-warts LYYSFQDKDNLYFVM DYIPGGDLMSLLIKL GIFEEELARFYIAEV TCAVDSVHKMGFIHR DIKPDNILIDRDGHI KLTDFGLCTGFRWTH   866

h-warts DSKYYQ-SGDHPRQD SMDFSNEWGDPSSCR CGDRLKPLERRAARQ HQRCLAHSLVGTPNY IAPEVLLRTGYTQLC DWWSVGVILFEMLVG   946
d-warts NSKYYQENGNHSRQD SMEPWEEYSEN---- -GPKPTVLERRRMRD HQRVLAHSLVGTPNY IAPEVLERSGYTQLC DYWSVGVILYEMLVG   951

h-warts QPPFLAQTPLETQMK VINWQTSLHIPPQAK LSPEASDLIIKLCRG PEDRLGKNGADEIKA HPFFKTIDFSSDLRQ QSASYIPKITHPTDT  1036
d-warts QPPFLANSPLETQQK VINWEKTLHIPPQAE LSREATDLIRRLCAS ADKRLGKS-VDEVKS HDFFKGIDFA-DMRK QKAPYIPEIKHPTDT  1039

h-warts SNFDPVDPDKLWSDD NEEENVNDTLNGWYI NGKHPEHAFYEFTFR RFFDDNGYPYNYPKP IEYEYINSQGSEQQS DEDDQNTGSEIKNRD  1126
d-warts SNFDPVDPEKLRSND STMSSGDDVDQ---- N-DRTFHGFFEFTFR RFFDDK-QPPDMTDD QAPVYV--------- ---------------  1099

h-warts LVYV  1130
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Fig. 1. Comparison of sequence identities in human (h-warts) and *Drosophila* warts (d-warts) protein. The protein sequences were compared by Multiple Sequence Alignments at BCM Search Launcher (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html). Amino acid residues on h-warts identical to d-warts protein in the same position are indicated by shading. The putative protein kinase catalytic domain (DM-PK domain) is indicated by underline. The GXGXXG ATP binding consensus motif and an invariant lysine residue are indicated by asterisks and an open diamond, respectively. Proline rich sequences are boxed.

and six proline rich sequences (236–237, 247–251, 256–258, 263–266, 301–304 and 372–375).

A Blastp search of the non-redundant protein database revealed that the overall structure of the h-warts protein shows the highest similarity to that of d-warts. Particularly, the kinase domain of h-warts has 76% identity to that of the d-warts protein [11], 35% to the DM-PK protein [12], 46% to the PK428 protein [21], and 37% to the *S. cerevisiae* Dbf2 protein [14]. During preparation of this manuscript, a human kinase, LATS1 [22], which has high homology to d-warts has been reported. We believe that *h-warts* and *LATS1* genes are identical.

To identify the chromosomal localization of the *h-warts* gene, we performed PCR mapping using somatic cell hybrid analysis. PCR products expected to be 97 bp in size were amplified using A1 and A2 primers from 20 of 93 hybrid cell lines in the Genebridge 4 radiation hybrid panel. Linkage analysis based on comparison with the human chromosomal constructs of all hybrids, as determined by the manufacturer and the Whitehead Institute/MIT Center for Genome Research, localized the *h-warts* gene to chromosome 6q24-25.1 and placed it 3.05 cR from D6S311 (Fig. 2a).

To examine the tissue expression of *h-warts* mRNA, Northern blot analysis of different human tissues was performed (Fig. 2b). A 9-kb mRNA was detected in most of the human adult tissues except for lung and kidney.

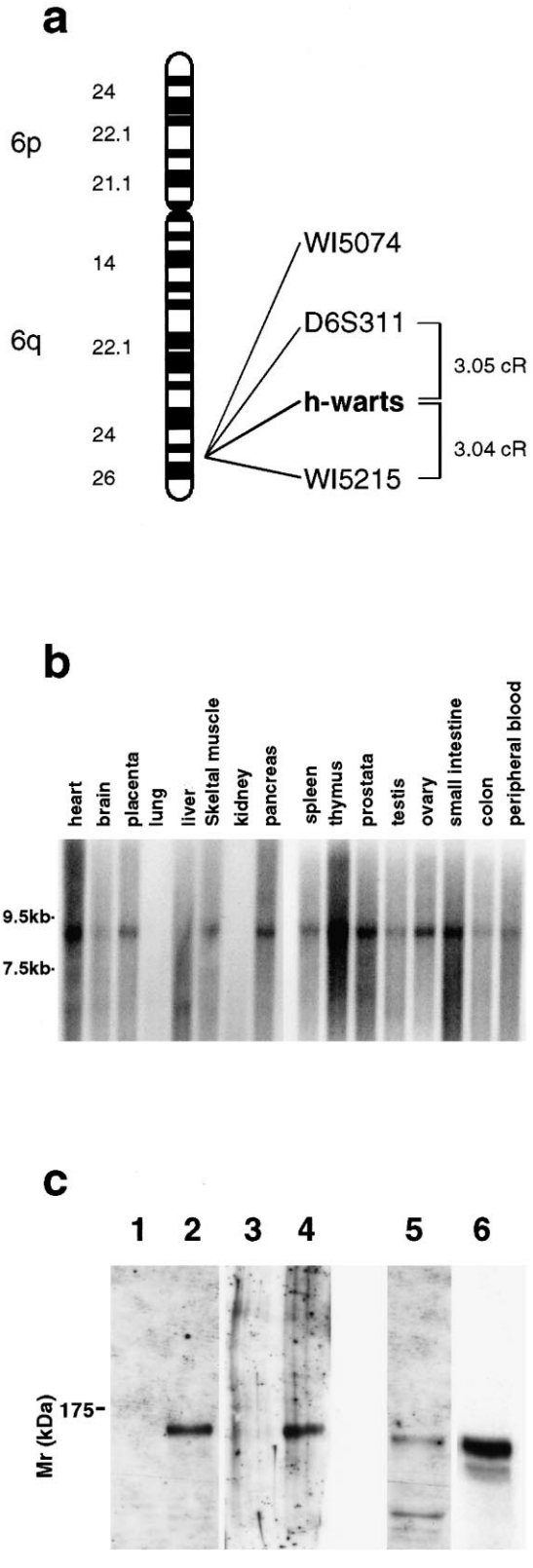
3.2. Detection of the h-warts protein

To verify the expression of h-warts protein in cultured cells, two polyclonal antibodies, termed C-2 and G-3, were generated against a synthetic peptide derived from a C-terminal region of h-warts proteins and a bacterially expressed GST protein fused with N-terminal portion of h-warts protein (WP), respectively. Western blot analysis revealed that C-2 and G-3 antibodies detected a band of about 165 kDa in lysates from COS7 cells transfected with the HA-tagged h-warts plasmid and HeLa cells (Fig. 2c). These results indicate that C-2 and G-3 antibodies recognize both the endogenous and the ectopically expressed h-warts.

3.3. Subcellular localization of h-warts during cell cycle

To examine the subcellular localization of endogenous h-warts, U2OS cells were immunocytochemically stained with the G-3 antibody. In various stages of the cell cycle, h-warts varied its localization. In interphase cells, G-3 antibody detected one or two closely spaced dots which were located close

to the nuclear envelope (Fig. 3a). These G-3 positive dots lay at the center of the interphase microtubule array (Fig. 3b, c) and were also co-stained with anti-γ-tubulin antibody (Fig. 3d, e, f), suggesting that they represent centrosomes. In prophase, it localized with spindle pole bodies and then with the entire mitotic spindle through metaphase into the early stage of anaphase (Fig. 3g, h, i). In mid-anaphase, the h-warts translocated toward the anaphase spindle midzone (Fig. 3j, k, l). As the cells progress into telophase, h-warts became concentrated to the midbody that connects daughter cells (Fig. 3m, n, o). These dynamics of h-warts protein during mitosis suggests that h-warts plays a role in the mitotic machinery during cell cycle progression.



3.4. Phosphorylation of h-warts in the mitotic checkpoint

The *Drosophila warts* gene is known to be required for the control of cell proliferation. Furthermore, h-warts has a significant homology with budding yeast cell cycle regulated kinases, DBF2 and DBF20. This evidence, together with our immunolocalization study, evoked our great interest to examine the modulation of h-warts at the different stages of the cell cycle. We first asked whether h-warts protein level is altered during the cell cycle. HeLa cells were synchronized at the G1/S boundary by a double thymidine block method. At different times after release from the block, cells were harvested and analyzed for h-warts expression by Western blotting with C-2 antibody. Total expression levels of h-warts did not change significantly throughout the cell cycle. However, a slower mobility of the h-warts band was observed in cells at 7–9 h after release from G1/S boundary, where most of the cells were in mitosis (Fig. 4a, lanes 4–6). The band shift became more obvious when cells were arrested at mitosis with nocodazole treatment (Fig. 4a, lane 9). Furthermore, in the early G1 cells, which were obtained by plating the mitotic arrested cells in nocodazole-free media for another 5 h, the lower band became predominant while the upper band was disappeared (Fig. 4a, lane 10). To examine the nature of the electrophoretic band shift of h-warts, the nocodazole-treated HeLa cell lysates were incubated with alkaline phosphatase. Alkaline phosphatase eliminated the gel shift of h-warts seen in nocodazole-treated cells, and the phosphatase-mediated conversion was inhibited by phosphatase inhibitors (Fig. 4b). These findings indicate that h-warts is specifically phosphorylated in cells at mitosis and in the cells arrested at the mitotic checkpoint by nocodazole treatment.

To determine whether the nocodazole-induced phosphorylation of h-warts occurred as a result of a spindle assembly defect or non-specifically irrespective of the cell cycle, we treated HeLa cells in various phases of the cell cycle with nocodazole and analyzed their electrophoretic mobility. Cells were synchronized at the G1/S boundary by double thymidine block and then induced to enter the cell cycle. Electrophoretic band shift caused by nocodazole treatment for 4 h was specifically observed in mitotic cells (Fig. 4c, lane 4), whereas the mobility of h-warts was not affected when cells were in the G1, S or G2 phase (Fig. 4c, lanes 1, 2, 3, 5). Thus, phospho-

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Fig. 2. Characterization of *h-warts*. a: Radiation hybrid mapping of the *h-warts* gene. The *h-warts* gene was mapped at 3.05 cR from D6S311 on chromosome 6q24-25.1. b: Expression of the *h-warts* transcript in various human tissues. The blots were hybridized with a specific cDNA probe of *h-warts*. The size marker (kb) is indicated. c: Western blot analysis of h-warts. Lysates prepared from COS7 cells transfected with the HA-tagged h-warts plasmid (pCGN-h-warts) (lanes 2, 4) and with pCGN (lanes 1, 3) were detected by C2 (lanes 1, 2) and G3 (lanes 3, 4) anti-h-warts antibodies. Lysates prepared from HeLa cells were also detected by C2 (lane 5) and G3 (lane 6) anti-h-warts antibodies.

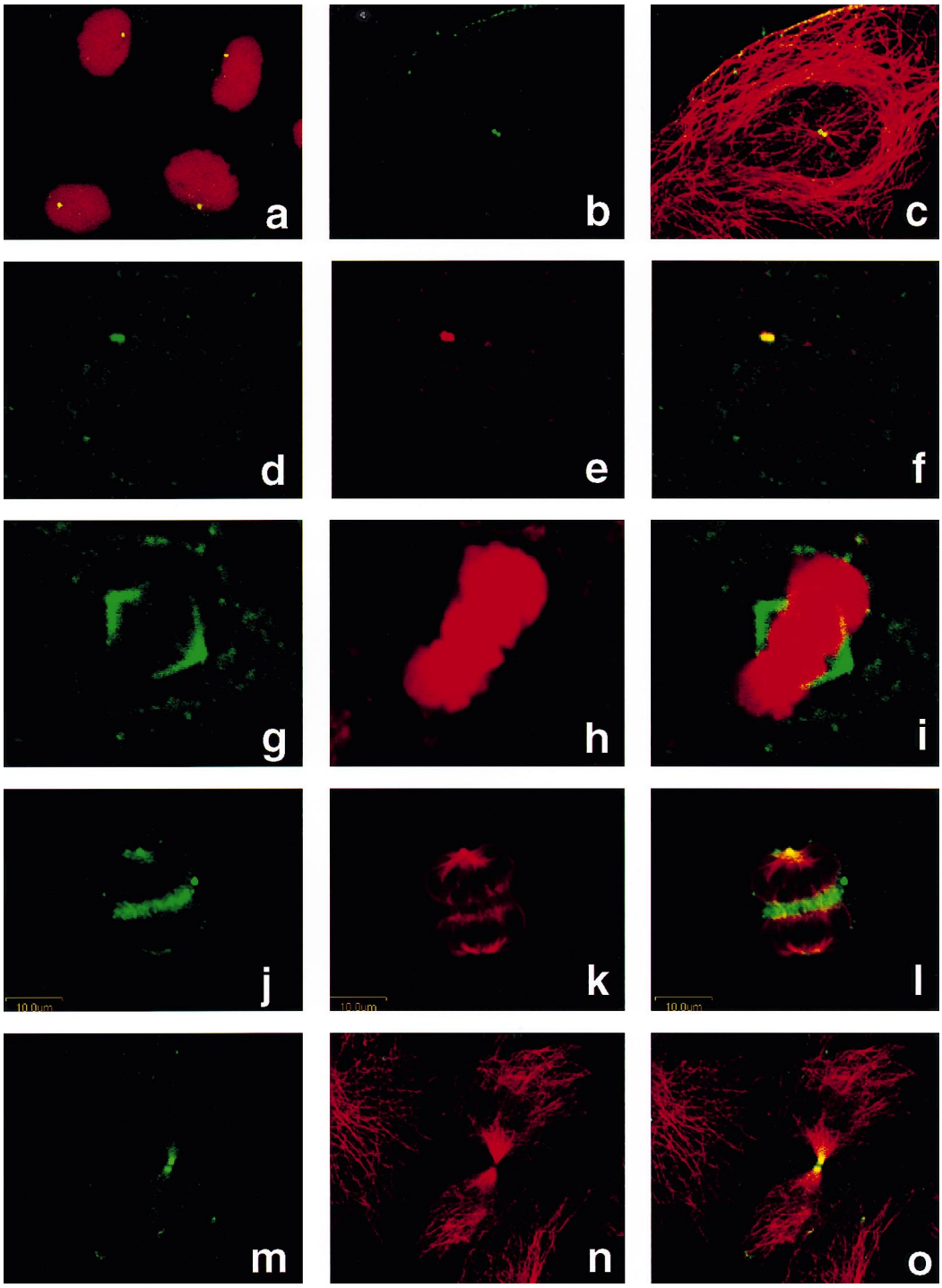

Fig. 3. Subcellular localization of h-warts in U2OS cells during the cell cycle. The cells at various stages of the cell cycle (a–f, interphase; g–i, metaphase; j–l, anaphase; m–o, telophase) were immunostained with anti-h-warts polyclonal antibody G3 visualized by FITC-conjugated secondary antibody (green) and anti-α-tubulin antibody (c, k, n) or anti-γ-tubulin antibody (e) visualized by Cy3-conjugated secondary antibody (red). DNA was stained with PI (a, h). The merged images are shown in a, c, f, i, l and o.

rylation of h-warts is closely related to disruption of mitotic spindle microtubule, which is accompanied by spindle assembly checkpoint activation.

For an alternative approach to show the specific phosphorylation of h-warts during mitosis, we performed an in vitro kinase assay using cell extracts as sources of the enzyme and three GST-h-warts deletion mutants (WP, WN and WK) as substrates. Among these deletion mutants, only WN was significantly phosphorylated by both mitotic lysates and nocodazole treated lysates (data not shown).

Among the protein kinases that have been implicated in the control of mitotic events, the role of Cdc2/cyclin B complex is pivotal and has been well characterized [23]. To examine the involvement of Cdc2/cyclin B complex in the phosphorylation of h-warts, we performed an in vitro kinase reaction of the WN domain using a mitotic lysate from which Cdc2/cyclin B

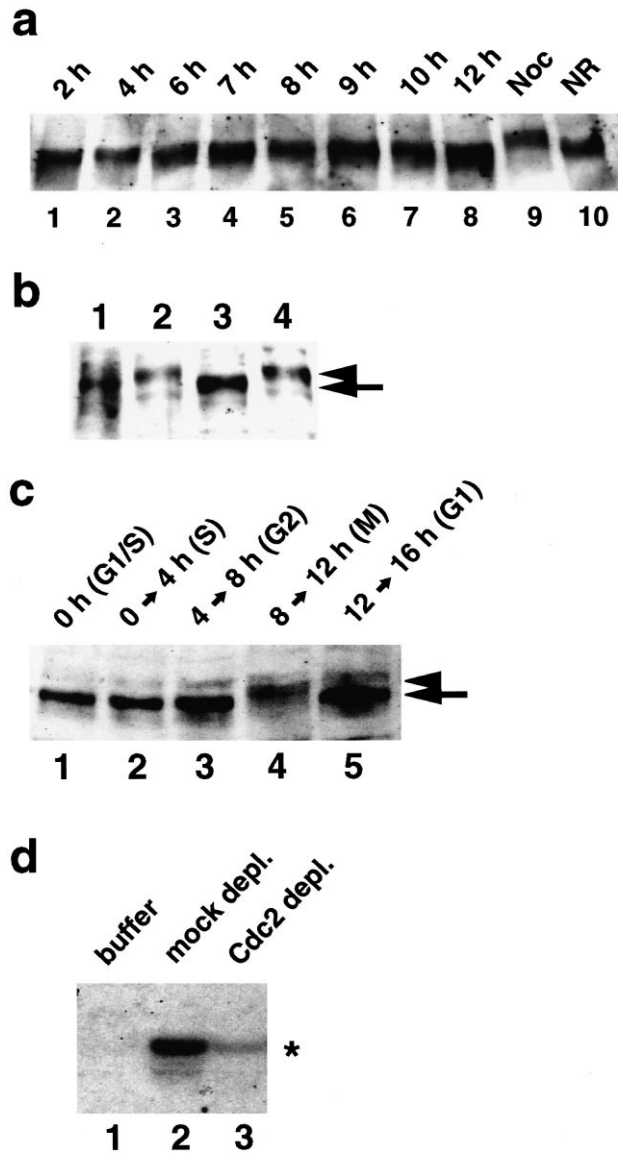

Fig. 4. Mitosis-specific phosphorylation of h-warts. a: Post-translational modification of h-warts during cell cycle. HeLa cells were synchronized at the G1/S boundary by double thymidine block. Following release from G1/S, cells were harvested at the various time points indicated (lanes 1–8). To block cells at mitosis, nocodazole (50 ng/ml) was added into cells at 5 h after release from G1/S and incubated for another 7 h (Noc: lane 9). After removal of nocodazole, cells were incubated in nocodazole-free media for another 5 h to obtain interphase cell (NR: lane 10). Samples were analyzed for h-warts protein by immunoblotting. b: Phosphatase treatment of cell extracts. Interphase (lane 1) or nocodazole-treated (lane 2) HeLa cell extract (50 μg) was immunoblotted with the C2 antibody. The nocodazole-treated extract was incubated with 100 units of calf intestinal alkaline phosphatase (CIAP) in the absence (lane 3) or presence (lane 4) of phosphatase inhibitors at 37°C for 30 min and subjected to the immunoblot analysis with the C2 antibody. Arrow and arrowhead indicate non-phosphorylated and phosphorylated form of h-warts, respectively. c: The nocodazole-induced phosphorylation of h-warts occurred as a result of a spindle assembly defect. HeLa cells were synchronized at the G1/S boundary and released into the cell cycle. The cells were then exposed to nocodazole at 0 h (lane 2, most of the cells were in G1/S phase), 4 h (lane 3, G2 phase), 8 h (lane 4, M phase) and 12 h (lane 5, G1 phase) to 4 h. The cell lysates were immunoblotted with the C2 antibody. Control lysate was obtained from the cells synchronized at the G1/S boundary without nocodazole treatment (lane 1). Arrow and arrowhead indicate non-phosphorylated and phosphorylated form of h-warts, respectively. d: Effect of Cdc2 depletion on in vitro phosphorylation of h-warts by mitotic lysate. GST-WN was incubated in the presence of [γ-^{32}P]ATP with either buffer alone (lane 1), mock-depleted mitotic cytosol (lane 2), or Cdc2-depleted mitotic cytosol (lane 3) for 30 min at 25°C. GST-WN was analyzed by SDS-PAGE and autoradiography. Phosphorylated GST-WN is indicated by asterisk. ←

was removed by p13^{suc1} beads. We found that phosphorylation of GST-WN was significantly abolished by depleting Cdc2 from mitotic lysate (Fig. 4d). These data suggest that Cdc2 or Cdc2-associated kinase(s) is responsible for the mitotic-specific phosphorylation of h-warts in vitro.

4. Discussion

In this study we have identified and characterized a human homolog of the *Drosophila* warts tumor suppressor, designated h-warts. The h-warts protein has significant homology to DM-PK family proteins. Protein kinases belonging to the DM-PK family are not only structurally, but also functionally, related to each other by an interesting coincidence. The budding yeast Dbf2 has been shown to genetically interact with Bub2p, one of the checkpoint components localizing to spindle pole bodies, and, when mutated, blocks exit from mitosis, resulting in formation of the 'dumbbell' phenotype [15,24]. The fission yeast Orb6 is reportedly required for maintenance of cell polarity during interphase and acts as an inhibitor of mitosis, suggesting that Orb6 takes part in a pathway to coordinate cell morphogenesis with progression through the cell cycle [25]. Rho-associated kinase (Rho-kinase) was shown to phosphorylate a type III intermediate filament protein exclusively at the cleavage furrow during cytokinesis, and the expression of the dominant-negative form inhibited the cytokinesis of *Xenopus* embryo and mammalian cells [26]. Citron kinase, another Rho target protein, also localizes to the cleavage furrow and midbody, and overexpression of citron mutants abrogates cytokinesis [27]. These lines of evidence indicate that many DM-PK family kinases are found in the mitotic apparatus and play an important role in progression of mitosis.

Immunocytochemical studies show that subcellular localization of h-warts alters during the cell cycle in a highly dynamic manner. During interphase, h-warts localizes to the centrosome. However, it becomes associated with the mitotic spindle during mid-mitosis and concentrated at the midbody in telophase. The dynamic localization of h-warts on the mitotic apparatus leads to our proposal that h-warts take a significant part in the cell cycle machinery. This hypothesis is supported by two biochemical characteristics of h-warts protein. First, h-warts is phosphorylated at the mitotic phase and hyperphosphorylated when the mitotic checkpoint is activated by nocodazole treatment. Second, the non-catalytic portion (WN region) of the protein is specifically phosphorylated by a kinase present in the mitotic cell lysate, which is most likely to be Cdc2/cyclin B complex. Cdc2 is known to phosphorylate a number of proteins, including nuclear lamins, nucleolin, cytoskeletal proteins, Src family protein kinases, kinesin-like motor protein HsEg5, and spindle-associated protein PRC1 [1]. The phosphorylation of these proteins is believed to alter their functions to trigger various events of mitosis [1]. Taken

together, it can be speculated that h-warts plays an important role in the control of mitotic events on the mitotic apparatus, which are functionally regulated by its phosphorylation state.

PCR mapping, using the radiation somatic cell hybrid panel, localized the *h-warts* gene to chromosome 6q, 3.05 cR apart from D6S311. The marker D6S311 was mapped at chromosome 6q24-25.1, which has been reported to be the frequent loss of heterozygosity region in human ovarian cancers and cervical cancers [28,29]. Furthermore, transfer of chromosome 6q fragments, which contains D6S311, was shown to suppress the tumorigenic phenotype of human breast cancer and ovarian cancer cell lines [30,31]. These findings suggest that the *h-warts* gene is one of the candidate tumor suppressor genes in chromosome 6q24-25. Experiments to investigate whether the *h-warts* gene is inactivated in human tumor cells are in progress.

Mutagenesis screens and the identification of spontaneously occurring mutations in *Drosophila* have led to the identification of over 50 genes in which mutations give an overgrowth phenotype, and are therefore considered tumor suppressor genes [32]. Recently, human homologs of these *Drosophila* tumor suppressor genes have been identified and shown to be implicated in various important cellular events, including growth regulation, differentiation and transformation [16,17]. Homozygous loss of *Drosophila warts* in somatic clones results in cell-autonomous formation of epithelial tumors. Thus, the *Drosophila warts* gene product is important for controlling cellular morphogenesis, as well as proliferation [11]. Recently, Tao et al. has reported a human kinase, LATS1, which has high homology to *Drosophila* warts/lats [22] and appears to be identical to h-warts. Analogous to its mutant in *Drosophila*, mice deficient of *LATS1* gene have been shown to develop soft tissue sarcomas and ovarian stromal cell tumors [33].

Failure in regulation of mitosis is well-recognized as contributing to chromosomal instability, which is a hallmark of cancers. Our present findings, together with the evidence for warts/LATS as a tumor suppressor in both *Drosophila* and mouse, suggest that h-warts is an important element for proper progression of mitosis, and that its loss of function leads to chromosomal instability, a condition that facilitates cellular transformation.

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