The Saccharomyces Sac3 Homologue shd1 Is Involved in Mitotic Progression in Mammalian Cells*§

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Saccharomyces Sac3 required for actin assembly was shown to be involved in DNA replication. Here, we studied the function of a mammalian homologue SHD1 in cell cycle progression. SHD1 is localized on centrosomes at interphase and at spindle poles and mitotic spindles, similar to α-tubulin, at M phase. RNA interference suppression of endogenous shd1 caused defects in centrosome duplication and spindle formation displaying cells with a single apparent centrosome and down-regulated Mad2 expression, generating increased micronuclei. Conversely, increased expression of SHD1 by DNA transfection with shd1-green fluorescent protein (gfp) vector for a fusion protein of SHD1 and GFP caused abnormalities in centrosome duplication displaying cells with multiple centrosomes and deregulated spindle assembly with up-regulated Mad2 expression until anaphase, generating polyploidy cells. These results demonstrated that shd1 is involved in cell cycle progression, in particular centrosome duplication and a spindle assembly checkpoint function.

Accurate transmission of genetic material to daughter cells in mitosis is essential. The mitosis checkpoint, also known as the spindle assembly checkpoint, is a crucial process to prevent resumption of DNA synthesis before mitosis has been completed and to ensure that chromosomes segregate evenly to daughter cells (1). The centrosome composed primarily of γ-tubulin is a major microtubule-organizing center in eukaryotic cells and plays a prominent role in mitosis, ensuring the establishment of bipolar spindles and balanced chromosome segregation. The mitotic spindle is composed primarily of microtubules, which are dynamic cytoskeletal polymers of α/β-tubulin subunits with an intrinsic structural polarity. Chromosome movement on the spindle during mitosis and meiosis is powered and regulated by the centromere of chromosomes. Nascent mitotic spindle assembly is a dynamic process with dissociating and reassociating microtubules attaching to chromosomes at their kinetochores. At the site of cell division, mitotic spindle pole bodies are localized in the cortex of the nucleus and recruit proteins involved in actomyosin ring assembly during mitotic spindle assembly (2). Cytokinesis, the final step of cell division, is affected by a wide range of chromosomal perturbations, and the spindle checkpoint is of great importance and has been intensively investigated.

The Saccharomyces suppressor of actin 3 (Sac3) gene is a suppressor of actin formation and Sac3 mutants cause a delay of the cell cycle at the G2/M transition, abnormal DNA synthesis, and different types of abnormal actin assembly (3). This suggests that mammalian homologues of Sac3 might play critical roles in cell cycle progression at the mitosis to postmitosis transition. We have identified a mouse gene homologous to Sac3, designated Sac3 homology domain 1 (shd1), and determined its sequence (GenBank™ accession number AJ131957). In mouse and human cells, two kinds of genes show sequence similarity to Saccharomyces Sac3; one is the germinal center-associated nuclear protein (ganp) gene that is up-regulated in germinal center B cells of peripheral lymphoid follicles created in response to T cell-dependent antigens (4, 5), and the other is the shd1 gene. The Sac3 homology region is 600 amino acids in mouse and is the middle peptide of the 210-kDa GANP, which also carries regions possibly involved in other function(s). The Sac3 homology region, however, is almost the entire putative SHD1 protein; amino acids 70–425 of the 425 amino acid SHD1 sequence. These two mammalian molecules with Sac3 homology might play a role in mitosis similar to Saccharomyces Sac3.

We report here studies of the expression and function of SHD1 protein. SHD1 is localized on centrosomes at interphase and at spindle poles and mitotic spindles at M phase. Using RNA interference suppression of shd1, we have investigated how SHD1 might affect the cell cycle and demonstrate that the expression of SHD1 might be necessary for centrosome duplication and proper spindle formation during cell cycle progression. This notion was confirmed in vitro by transfection with a mouse shd1 cDNA clone that encodes the Sac3 homology peptide as a fusion protein of SHD1 and green fluorescent protein (GFP). The results from both experiments suggest that SHD1 is involved in regulation of the cell cycle at M phase.\footnote{The abbreviations used are: Sac3, suppressor of actin 3; SHD1, Sac3 homology domain 1; GANP, germinal center-associated nuclear protein; GFP, green fluorescent protein; Ah, antibody; mAb, monoclonal antibody; PBS, phosphate-buffered saline; Mad2, mitotic arrest-deficient 2; DAPI, 4’,6-diamidino-2-phenylindole; PI, propidium iodide; siRNA, small interfering RNA; FACS, fluorescence-activated cell sorter.}

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EXPERIMENTAL PROCEDURES

Mice and Cell Culture—C57BL/6 mice were purchased from Charles River Japan (Yokohama, Japan). NIH3T3 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (Dainippon Pharmaceutical, Osaka, Japan), 2 mM l-glutamine (Cambrex, Baltimore, MD), 100 μg/ml streptomycin, 100 units/ml penicillin, and 50 μM 2-mercaptoethanol.

Polyclonal Antibody (Ab) and Monoclonal Ab (mAb) against Mouse SHD1—Polyclonal anti-SHD1 Ab was prepared by immunization of rabbits with glutathione S-transferase-SHD1 protein using the same shd1 cDNA fragment introduced into pGEX-4T-1 (Amersham Biosciences). Antisera were purified using protein A-Sepharose (Amersham Biosciences). A rat mAb against mouse SHD1 (clone 45-87) was prepared by the standard procedure (6).

Immunoblot Analysis—Cells were lysed with TNE buffer (10 mM Tris-HCl (pH 7.8), 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride) and cleared by centrifugation at 12,000 rpm for 20 min at 4 °C. Ten micrograms of total protein, measured by the Bio-Rad protein assay (Bio-Rad Laboratories), was analyzed by 10% SDS-PAGE and transferred to a nitrocellulose membrane. For detection of endogenous SHD1, polyclonal anti-SHD1 Ab was used in combination with horseradish peroxidase-coupled secondary Ab followed by an enhanced chemiluminescence detection kit (Amersham Biosciences). The membrane was reprobed with anti-β-actin mAb (clone AC-15; Sigma) to provide an internal standard and to evaluate the amount of cell extract in different lanes.

Immunofluorescence—Cells grown on coverslips and in Lab-Tek Chambers (Nalge Nunc International, Rochester, NY) were rinsed with PBS and fixed in PBS containing 3.7% paraformaldehyde for 5 min. For staining of SHD1, α-tubulin, pericentrin, and mitotic arrest-deficient 2 (Mad2), cells were fixed with 1% paraformaldehyde/PBS for 20 min. After washing with PBS, fixed cells were permeabilized with 0.2% Triton X-100, washed with PBS, and then blocked for 15 min with 3% bovine serum albumin in PBS. The primary Abs were as follows. Rat anti-mouse SHD1 mAb (45-87) was described above. Mouse mAbs were anti-γ-tubulin (clone GTU88, Sigma) and anti-α-tubulin (clone B-5-1-2, 2006).

FIG. 1. Expression of SHD1 proteins during cell cycling. A, detection of endogenous SHD1 protein. Western blot analysis with anti-SHD1 Ab showed a 49-kDa SHD1 band in the cell lysate of spleen cells. An asterisk marks a nonspecific band with normal rabbit serum (NRS). B, localization of SHD1 in centrosomes. NIH3T3 cells were stained by anti-SHD1 mAb and anti-γ-tubulin mAb. Counterstaining was performed with DAPI. SHD1 was colocalized with γ-tubulin at interphase and mitotic phase. C, localization of SHD1 in spindle poles, spindle, and midbody. SHD1 was colocalized with α-tubulin during M phase. Counterstaining was performed with DAPI.
Fig. 2. Impairment of mitotic progression in SHD1 knockdown cells. A, expression of shd1 transcripts and SHD1 protein in shd1-siRNA-treated cells. At 72 h posttransfection with siRNA in a 24-well plate, cells were harvested and analyzed by reverse transcriptase-PCR (left panel) or Western blot analysis (middle panel). −, non-transfection; L**, 1 μM luciferase-siRNA; S, 0.25 μM shd1-siRNA; S*, 0.5 μM shd1-siRNA; S**, 1 μM shd1-siRNA. Western blot analysis was carried out as in Fig. 1A. Relative amount of SHD1 protein was expressed as percentage in comparison...
FIG. 2—continued

with luciferase-siRNA-treated cells based on the expression of β-actin (right panel) as measured by a photoimage system. B, increased number of cells with micronuclei in shd1-siRNA-treated cells. Parent NIH3T3 cells, luciferase-siRNA-treated cells, and shd1-siRNA-treated cells were stained with DAPI. Representative data showing micronuclei (indicated by arrowheads) were shown (left panel). The percentages of micronuclei were calculated under fluorescent microscope (right panel, *, p < 0.05). The experiments were carried out three times, and each number was counted from 200 cells per group. C and D, impairment of centrosome duplication in shd1-siRNA-treated cells. luciferase- and shd1-siRNA-treated cells were stained with anti-γ-tubulin mAb or with anti-pericentrin Ab and PI. Centrosomes at the poles are indicated by arrowheads. E, monocentriole cells were observed in cells treated with shd1-siRNA by electron microscopy. Overview of the cell identified the cells at the nuclear envelope breakdown. Careful examinations of more than 200 cells revealed cells with a single centriole in the shd1-siRNA-treated cells but showed only the cells with pairs of orthogonal centrioles in the luciferase-siRNA-treated cells. Arrowheads indicate the centrioles, and the bars are the scales for the length of 1 μm. F, insufficient spindle formation in shd1-siRNA-treated cells. luciferase- and shd1-siRNA-treated cells were stained with anti-α-tubulin mAb and PI. G, decreased expression of Mad2 in shd1-siRNA-treated cells. luciferase- and shd1-siRNA-treated cells were stained with anti-Mad2 polyclonal Ab and PI. Mad2 expression was down-regulated at the kinetochore in shd1-siRNA-treated cells in comparison with in luciferase-siRNA-treated cells (as indicated by arrows).
Mitotic Regulation by Mammalian SHD1

RESULTS

Expression of SHD1 during Cell Cycling—Expression of endogenous SHD1 protein was examined by Western blot analysis. Anti-SHD1 Ab identified a 49-kDa band in spleen cells (Fig. 1A). We then examined the subcellular distribution of SHD1 by immunofluorescence staining in NIH3T3 cells with a pKJ2 vector containing a neomycin resistant gene using LipofectAMINE transfection reagent (Invitrogen) and selected with G418 (0.6 mg/ml; Promega, Madison, WI). Two weeks after selection, GFP-expressing NIH3T3 cells were transfected with pSVEGFP pA expression vector under SV40 promoter control (7). After confirming SHD1 protein expression by the fusion protein GFP signal and the relative values shown in the right panel, the expression in S** was 2% versus 3.0–3.9% of controls (Fig. 2E). These observations confirmed the abnormality of centromere duplication in shd1-gfp-treated cells.

The α-tubulin expression clearly demonstrated the insufficient spindle formation in shd1-gfp-treated cells, displaying no clear bipolar localization of spindle poles and instead the deregulated distribution of α-tubulin gathering at one of the poles with unaligned chromosomes and barely detectable spindles at the misaligned chromosomal area (Fig. 2F, b–d; more than 66% of metaphase cells). There is no clear midbody matrix in comparison with the control cells (nearly 50% of anaphase cells, representative picture in Fig. 2F, f). shd1-gfp-treated cells showed down-regulation of Mad2 expression, especially as a form associated with chromosomes (Fig. 2G; in nearly 50% of prometaphase cells as indicated by an arrow in the lower panel). Recent studies have shown that spindle assembly checkpoint proteins, Mad2 and Bub1, localize at the kinetochores of vertebrate cells during part of mitosis (12–15). As the spindle assembly checkpoint, Mad2 up-regulation delays cell cycle progression at anaphase (16). In normal cells and mock transfectants, Mad2 expression appears exclusively at prometaphase and is detected at the kinetochores (as indicated by an arrow in the upper panel). These results demonstrated that the regulation of SHD1 expression during cell cycling is important for proper centromere duplication and mitotic progression.

Effect of shd1-gfp Transfection on Cell Cycle Progression of NIH3T3 Cells in Vitro—To confirm that the abnormalities were caused functionally by suppression of shd1 transcripts, we addressed if the adverse effect was observed in the shd1 overexpressed cells. A vector that expresses a fusion protein of SHD1 amino acids 70–425 and GFP was introduced in NIH3T3 cells, and the shd1-gfp transfectants were selected by GFP-positivity after short term culture. The shd1-gfp transfectants mostly express GFP signal (82%, Supplemental Fig. 1) and showed the introduced SHD1-GFP protein in addition to the similar expression level of endogenous SHD1 protein (Fig. 3A). The same blot was confirmed by anti-GFP Ab (data not shown).
DNA profiles after PI staining and FACS analysis showed that shd1-gfp transfectants lacked G0/G1 cells containing 2n DNA and most cells had 4n DNA (Fig. 3B). However, some shd1-gfp transfectants showed greater DNA content, between 8n and 16n in DNA profiles. Microscopic analyses showed abnormalities of multi-nucleated cells, in shd1-gfp transfectants (Fig. 3C, left panel, and the calculated numbers in the right panel) in comparison with mock transfectants (data not shown). These results suggest that shd1-gfp transfection generates polyploid cells with more DNA content than the 2n of G0/G1 cells. Electron microscopic analysis of shd1-gfp transfectants found pleomorphism (Fig. 3D). Compared with mock transfectants (a), the shd1-gfp transfectants showed abnormalities in nuclear architecture (30% of cells). The shape of nuclei varied, ranging from multilobulated to multinucleated (b and c), which was apparently different from the control (a). These observations suggest that introduction of shd1-gfp interferes with chromatin segregation into daughter nuclear compartments.

Abnormality of Mitotic Progression in shd1-gfp Transfectants—To study whether the mitosis triggered in shd1-gfp transfectants is affected, we examined expression of γ-tubulin in the transfectants (Fig. 4A). In mock transfectants, γ-tubulin foci were observed in >97% of mitotic cells as clear twin spots at centrosomes at the poles in a bipolar fashion connecting to chro-
mosomes. In contrast, >30% of mitotic cells in shd1-gfp transfectants did not show a typical twin pattern but had irregular and uneven profiles and abnormal localization with more spots, suggesting that centrosome duplication was abnormal.

We examined spindle assembly in mitotic phase in shd1-gfp transfectants by α-tubulin and DNA staining (Fig. 4B). Compared with mock transfectants with clear bipolar spindles (a), shd1-gfp transfectants showed various abnormalities (i.e. tri-polar (b), tetrapolar (c), and multipolar (d) spindles; 70% of cells), which may cause asymmetrical repartitioning of chromosomes at metaphase. These chromosome segregation defects also included unaligned and unattached chromosomes (Fig. 4B, c and d, arrowheads). During anaphase, spindle poles were not clear (50% of cells, Fig. 4B, f and g) compared with control cells. More severe anaphase defects were absence of dense midbody matrix formation at the intercellular bridge region (Fig. 4B, g,

FIG. 4. Expression of mitotic checkpoint molecules in shd1-gfp transfectants. A, centrosomes in shd1-gfp transfectants. The expression of γ-tubulin was examined in comparison to DNA staining. Arrowheads indicate abnormal numbers and localization of centrosomes. B, abnormalities of spindle formation in shd1-gfp transfectants. The expression of α-tubulin was examined in comparison to DNA staining. In comparison with control cells during metaphase showing a typical appearance (a), shd1-gfp transfectants showed abnormal spindle polarization (b, tripolar; c, tetrapolar; and d, multipolar) and the defects of chromosome alignment (c and d, arrowheads). At anaphase, formation of midbody matrices and spindle poles were inhibited in shd1-gfp transfectants. In comparison to the normal formation of the midbody matrix and spindle poles in mock-transfectants (e), shd1-gfp transfectants lacked the formation of spindle poles (f and g) and/or midbody matrix (g, arrow). C, aberrant localization of Mad2 during metaphase and anaphase in shd1-gfp transfectants. Mad2 expression was examined in comparison to DNA staining. In anaphase, Mad2 was at the kinetochores in shd1-gfp transfectants.
mitotic cell cycle progression for proper cell division, especially at centrosome duplication, the spindle assembly checkpoint from metaphase to anaphase, and spindle formation. SHD1 is localized at the mitotic structures during the cell cycle. To understand the role of SHD1 in regulating the cell cycle, the level of shd1 expression in NIH3T3 cells was modulated by transfection with shd1-siRNA and shd1 cDNA treatment. Suppression of endogenous SHD1 expression by shd1-siRNA treatment clearly demonstrated the abnormality in centrosome formation. The shd1-siRNA-treated cells showed the decreased potential of centrosome duplication, displaying a single apparent centrosome at metaphase with γ-tubulin assembly. Spindle assembly of the shd1-siRNA-treated cells are irregular without any clear foci of two spindle poles at the proper sites but instead distribute the weaker α-tubulin gathering at the margin of the chromosomal area at mitosis. These results suggested that the decrease of SHD1 expression resulted in the weaker potential to create the γ-tubulin assembly to generate clear bipolar centrosomes, which is essential for the accurate separation of tetraploid chromosomes toward two daughter cells.

Contrary to the RNA interference suppression method, the shd1-gfp transfectants in the studies reported here displayed the opposite effect upon centrosome amplification, of which abnormality is similar to cells lacking p53 (17), with abnormal centrosome amplification generating multiple spindle poles in both cases. p53 has the potential to mediate its checkpoint functions as a transcription factor: p53 activates certain genes by binding to p53 response DNA elements and represses transcription of other genes lacking p53 response elements. SHD1 might act on one of the pathways critical for suppressing cytokinesis. Caffeine treatment generally rescues G2/M arrest caused by irradiation by a p53-dependent mechanism (18). However, shd1-gfp transfectants were less sensitive to caffeine rescue (data not shown). No increase of apoptotic cells in shd1-gfp transfectants were observed, suggesting that the cell cycle alteration induced by shd1 over-expression either is not directly regulated by a p53-dependent mechanism or is associated with regulation of downstream p53-dependent transcription.

A similar phenotype also resulted from impaired spindle formation, which is regulated by the spindle checkpoint. A critical requirement for spindle checkpoint function is the activation of Mad2, which prevents activation of anaphase-promoting complex-Cdc20 thereby suppressing Pds1 activation and blocking cell division (19). Mad2 expression was decreased in shd1-siRNA-treated cells and adversely increased in shd1-gfp transfectants, suggesting that shd1 affects the cell cycle via a mechanism associated with Mad2 up-regulation. We speculate that increased Mad2 is a response to accelerated cell cycle progression via anaphase-triggered SHD1 expression. However, it is possible that SHD1 has an anti-Mad2 effect on the late M phase checkpoint, and cell cycle progression might be regulated by a balance between SHD1 and Mad2. To investigate direct interactions of SHD1 and Mad2, we examined whether these molecules were associated in transfectants, but no evidence of direct interaction was found by immunoprecipitation and Western blotting (data not shown). It is now important to determine how Mad2 expression is regulated by increased SHD1 expression.

The shd1-siRNA-treated cells displayed metaphase cells with decreased number of centrosomes, and the chromosomes seem to be aligned on the metaphase plate but the alignment was apparently different from the control cells (Fig. 2C). To further examine the abnormality of the shd1-siRNA-treated cells, we carried out the electron microscopic observation, which definitely showed the cells with a single centriole in shd1-siRNA-treated cells but not in the control-treated cells. Recent report demonstrated that spindle formation and centrosome duplication might occur through various different mech-
SHD1 has no other apparent functional consensus motifs or domains associated with cell cycle regulation and gene transcription. SHD1 has 81% sequence similarity to the human shd1 gene, and both human and mouse genes are similar to Saccharomyces Sac3. We have documented a functional role for SHD1, the smallest cell cycle regulatory protein with Sac3 homology. Abnormal SHD1 expression may be related to the aneuploidy common in tumors and correlated with metastasis and poor prognosis (25, 26).

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