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TRIM36 interacts with the kinetochore protein CENP-H and delays cell cycle progression

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ABSTRACT

The tripartite motif-containing protein (TRIM) family is defined by the presence of a common domain structure composed of a RING finger, a B-box, and a coiled-coil motif. TRIM family proteins are involved in a broad range of biological processes and, consistently, their alterations result in diverse pathological conditions such as genetic diseases, viral infection, and cancer development. In this study, we found by using yeast two-hybrid screening that TRIM36 has a ubiquitin ligase activity and interacts with centromere protein-H, one of the kinetochore proteins. We also found by immunofluorescence analysis that TRIM36 colocalizes with α-tubulin, one of the microtubule proteins. Moreover, we found that overexpression of TRIM36 decelerates the cell cycle and attenuates cell growth. These results indicate that TRIM36 is potentially associated with chromosome segregation and that an excess of TRIM36 may cause chromosomal instability.

Introduction

Ubiquitination is a versatile posttranslational modification mechanism used by eukaryotic cells. The ubiquitin–proteasome pathway involves ubiquitin modification of substrates and sequential degradation by the proteasome [1]. Ubiquitin conjugation is catalyzed by ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) [2]. E3 is a scaffold protein that mediates between the ubiquitin-linked E2 and the substrate. The resulting covalent ubiquitin ligations form polyubiquitinated conjugates that are rapidly detected and degraded by 26S proteasome [3]. E3 is thought to be most directly responsible for substrate recognition. E3 ubiquitin ligases so far identified include members of the HECT (*h*omologous to *E*6-AP *c*arboxyl *t*erminus), RING finger, and U-box protein families [4–6].

TRIM36 is a member of the tripartite motif-containing protein (TRIM) family defined by the presence of a common domain structure composed of a RING finger, a B-box, and a coiled-coil motif [7]. In addition to these motifs, TRIM36 possesses carboxy-terminal fibronectin type III and SPRY (Spla kinase and ryanodine receptor) domains [8]. TRIM family proteins are involved in a broad range of biological processes and, consistently, their alterations result in diverse pathological conditions such as genetic diseases, transcriptional regulation, and cancer development [9–11]. It has been reported that TRIM36 is involved in acrosome reaction, fertilization, and embryogenesis and that TRIM36 is upregulated in the vast majority of prostate cancers and thought to be involved in the carcinogenesis [12–14]. However, the molecular function of TRIM36 has not been elucidated.

Chromosomal instability has been observed in a wide variety of human cancers and is caused by continuous chromosome missegregation during mitosis [15]. Proper chromosome segregation requires a physical connection between spindle microtubules and centromeric DNA and this attachment occurs at proteinaceous structures called kinetochores. The kinetochore is a large complex protein structure that assembles in the centromeric region of each sister chromatid and is responsible for establishing and maintaining the connection with the mitotic spindle [16]. Centromere protein-H (CENP-H) has been identified as a constitutive component of the kinetochore [17]. Recently, overexpression of CENP-H has been reported in human colorectal cancer tissues as well as cancer cell lines with chromosomal instability. Aberrant expression of CENP-H causes chromosome missegregation and plays an important role in the chromosomal instability frequently observed in colorectal cancers [18].

In this study, to elucidate the molecular function of TRIM36, we identified CENP-H as a novel TRIM36-binding protein by using yeast two-hybrid screening. We also found that TRIM36 colocalizes with α-tubulin, one of the microtubule components. Furthermore, we found that overexpression of TRIM36 decelerates the cell cycle and attenuates cell growth. These results indicate that TRIM36 is potentially associated with chromosome segregation in collaboration with CENP-H and that overexpression of TRIM36 may have an effect on chromosomal instability.

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Materials and methods

Cell culture. HEK293T and HeLa cell lines were maintained under an atmosphere of 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (Gibco BRL). NIH3T3 cells were cultured under the same conditions in DMEM with 10% calf serum (CS, Camblex).

Cloning of cDNAs and plasmid construction. Mouse TRIM36 and centromere protein-H (CENP-H) cDNAs were amplified from mouse testis cDNA by polymerase chain reaction (PCR) with Blend-Taq (Takara) using the following primers: 5′-ACCATGTCGGAATCA GAGGAGATA-3′ (TRIM36-sense), 5′-AAGCTACACATCCTCTTCATAT TC-3′ (TRIM36-antisense), 5′-GCAATGGAGGAGCAGCCGCGGGAG-3′ (CENP-H-sense), and 5′-TTCTCATAGCGTGTTGAGGTCCTT-3′ (CENP-H-antisense). The amplified fragments were subcloned into pBluescript II SK+ (Stratagene) and sequenced. TRIM36 and CENP-H cDNAs were then subcloned into pGEX-6P1 vector (Amersham Bioscience) for the production of glutathione *S*-transferase (GST)-tagged fusion protein, pBTM116 and pACT2 vector (Clontech) for a yeast two-hybrid system, pCR vector (Invitrogen) with FLAG-tag and pCGN vector with HA-tag for expression in eukaryotic cells.

Yeast two-hybrid screening. Complementary DNA encoding amino acids 343–730 of TRIM36 was fused in-frame to the nucleotide sequence for the LexA domain (BD) in the yeast two-hybrid vector pBTM116. To screen for proteins that interact with TRIM36, we transfected yeast strain L40 (Invitrogen) stably expressing the corresponding pBTM116 vector with Matchmaker HeLa cDNA library (Clontech) by the lithium acetate method.

Recombinant protein and antibodies. GST-tagged TRIM36 protein was expressed in XL-1 Blue cells and then purified by reduced glutathione-sepharose beads (Roche). Antibodies used were as follows: mouse monoclonal anti-HA (HA.11/16B12, Covance Research Products), mouse monoclonal anti-FLAG (M5, Sigma), mouse monoclonal anti-ubiquitin (P4D1, Santa Cruz Biotechnology), and mouse monoclonal anti-alpha-tubulin (TU-01, Zymed Laboratories).

Ubiquitination assay. Reaction mixtures containing 4 μg of the recombinant TRIM36 with 0.1 μg recombinant E1 (Boston Biomedica), 1 μg recombinant E2s, 0.5 U phosphocreatine kinase, 1 μg ubiquitin (Sigma), 25 mM Tris–HCl (pH 7.5), 120 mM NaCl, 2 mM ATP, 1 mM $MgCl_2$, 0.3 mM dithiothreitol, and 1 mM creatine phosphate were incubated for 3 h at 30 °C. The reaction was terminated by the addition of SDS sample buffer containing 4% β-mercaptoethanol and heating at 95 °C for 5 min. Samples were subjected to immunoblotting with anti-ubiquitin antibody.

Transfection, immunoprecipitation, and immunoblot analysis. HEK293T cells were transfected by the calcium phosphate method. After 48 h, the cells were lysed in a solution containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, leupeptin (10 μg/ml), 1 mM phenylmethylsulfonyl fluoride, 400 μM Na_3VO_4, 400 μM EDTA, 10 mM NaF, and 10 mM sodium pyrophosphate. The cell lysates were centrifuged at 16,000 *g* for 10 min at 4 °C, and the resulting supernatant was incubated with antibodies for 2 h at 4 °C. Protein A-Sepharose (Amersham Pharmacia) that had been equilibrated with the same solution was added to the mixture and then the mixture was rotated for 1 h at 4 °C. The resin was separated by centrifugation, washed five times with ice-cold lysis buffer, and then boiled in SDS sample buffer. Immunoblot analysis was performed with primary antibodies, horseradish peroxidase-conjugated antibodies to mouse or rabbit immunoglobulin G (1:10,000 dilution, Promega) and an enhanced chemiluminescence system (ECL, Amersham Pharmacia).

Establishment of stable transfectants by using a retrovirus expression system. TRIM36 cDNA was subcloned into pMX-puro (kindly provided by T. Kitamura, Tokyo University), and the resulting vector was used to transfect Plat-E packaging cells and then recombinant retroviruses were generated. NIH3T3 cells were infected with the recombinant retroviruses and selected in medium containing puromycin (2 μg/ml, Sigma).

Cell proliferation assay. NIH3T3 cells in which TRIM36 or an empty vector (Mock) was stably expressed by using a retroviral expression system were seeded in 10-cm dishes and harvested for determination of cell number at indicated times.

Cell cycle analysis. NIH3T3 cells in which TRIM36 or an empty vector (Mock) was stably expressed by using a retroviral expression system were incubated in DMEM with 0.1% CS for 24 h for serum starvation. The cells released from serum starvation were harvested at indicated times and suspended in a solution containing 20 mM Hepes, 160 mM NaCl, 1 mM EGTA, and 0.04% digitonin. The cells were incubated at 37 °C for 1 h in a solution with RNase A (100 μg/ml; Novagen) and propidium iodide (20 μg/ml) and then analyzed with a FACSCalibur flow cytometer and Cell Quest software (Becton Dickinson).

Immunofluorescence staining. HeLa cells expressing HA-TRIM36, FLAG-TRIM36, or GFP-cytokeratin 8 grown on a glass cover were fixed for 20 min at room temperature with 4% formaldehyde in PBS and then incubated for 1 h at room temperature with primary antibodies to HA, FLAG, or α-tubulin in PBS containing 0.1% bovine serum albumin and 0.1% saponin. They were then incubated with Alexa546-labeled goat polyclonal antibodies to mouse immunoglobulin or Alexa488-labeled goat polyclonal antibodies to rabbit immunoglobulin (Molecular Probes) at a dilution of 1:2000. The cells were covered with a drop of GEL/MOUNT (Biomeda) and then photographed with a CCD camera (DP71, Olympus) attached to an Olympus BX51 microscope.

Results

TRIM36 has a ubiquitin ligase activity

TRIM36 has a RING finger domain at its N-terminus and belongs to the TRIM family of proteins, some of which have been reported to be E3 ubiquitin ligases [7]. To determine whether TRIM36 actually has a ubiquitin ligase activity, we generated recombinant GST-TRIM36 protein and performed an *in vitro* ubiquitination assay. First, we examined the preference of TRIM36 for 10 different E2 enzymes in the presence of E1. Immunoblot analysis using anti-ubiquitin antibody revealed that TRIM36 acquires ubiquitination activity when it collaborates with Ubc4 (Fig. 1A). Next, to examine the requirement of ATP, ubiquitin, E1, and E2 for the ubiquitination reaction, we performed *in vitro* ubiquitination assays with various combinations in which one component (ATP, ubiquitin, E1, or E2) was missing from the reaction mixture. Immunoblot analysis using anti-ubiquitin antibody revealed that TRIM36 exhibits ubiquitination activity only in the presence of E1, E2 (Ubc4), ubiquitin, ATP, and TRIM36 (Fig. 1B). The lack of any of these components prevented ubiquitination activity of TRIM36. These findings indicate that TRIM36 is a bona fide E3 ligase.

TRIM36 interacts with CENP-H

To examine the molecular function of TRIM36, we screened TRIM36-interacting proteins in a HeLa cDNA library by using a yeast two-hybrid system. We obtained 48 positive clones from 1.0×10^6 transformants. Ten of the positive clones had sequence identities with CENP-H cDNA, which is one of the kinetochore proteins and plays an important role in chromosome segregation. Interaction between TRIM36 and CENP-H was again confirmed by a β-galactosidase assay (Fig. 2A). To examine whether TRIM36 physically interacts with CENP-H in mammalian cells, we per-