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Communication

Molecules and Cells

Cancer-Upregulated Gene 2 (CUG2), a New Component of Centromere Complex, Is Required for Kinetochore Function

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We previously identified cancer-upregulated gene 2 (CUG2) as a commonly up-regulated gene in various human cancer tissues, especially in ovary, liver, and lung (Lee et al., 2007a). CUG2 was determined to be a nuclear protein that exhibited high proto-oncogenic activities when overexpressed in NIH3T3 mouse fibroblast cells. To identify other cellular functions of CUG2, we performed yeast two-hybrid screening and identified CENP-T, a component of CENP-A nucleosome complex in the centromere, as an interacting partner of CUG2. Moreover, CENP-A, the principle centromeric determinant, was also found in complex with CENP-T/CUG2. Immunofluorescent staining revealed the co-localization of CUG2 with human centromeric markers. Inhibition of CUG2 expression drastically affected cell viability by inducing aberrant cell division. We propose that CUG2 is a new component of the human centromeric complex that is required for proper chromosome segregation during mitosis.

INTRODUCTION

The centromere is a specialized chromatin structure that acts as a platform on which kinetochore formation occurs during mitosis and meiosis (Mellone et al., 2006). The kinetochore serves as the attachment site of spindle microtubules. Multiple proteins and subcomplexes that reside in the kinetochore accomplish various roles including regulation of cell division via activation of the mitotic checkpoint. Although centromeric DNA is highly variable between species, the histone H3 variant CENP-A is the key determinant of centromere formation and kinetochore assembly (Howman et al., 2000). Besides the essential constitutive centromeric components such as CENP-A, CENP-C, CENP-I, CENP-H, and Mis12, which are required to recruit other centromeric components, approximately 40 proteins have so far been shown to be associated with CENP-A nucleosome (Black et al., 2004; Blower et al., 2002). Recently, two independent research groups described CENP-T as a new component of the CENP-A nucleosome-associated complex (Foltz et al., 2006; Izuta et al., 2006). CENP-T localizes proximally with CENP-A nucleosome and depletion of CENP-T profoundly affects cell division and cell viability.

Previously, we analyzed an oncology database containing expression profiles of numerous tumor and normal samples to discover novel cancer-causing genes that might be useful as anticancer drug targets. We identified a novel gene, cancer-upregulated gene 2 (CUG2), which is commonly up-regulated in many human cancer tissues, and demonstrated that overexpression of CUG2 can confer prominent *in vitro* and *in vivo* proto-oncogenic activities to NIH3T3 mouse fibroblast cells (Lee et al., 2007a).

In this study, we report that CUG2 specifically interacts with centromeric CENP-T, and CUG2 itself is a new member of a centromeric multiprotein complex associated with CENP-A. Our data suggests that CUG2 may be involved in the formation of a functional kinetochore complex and that appreciable oncogeneity can be achieved by the influence on the mitotic cell division process.

MATERIALS AND METHODS

Cell culture and transfection

A549, MCF-7, and HeLa cells were purchased from American Type Culture Collection (USA) and grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum. For stable cell lines, cells were transfected with pcDNA-3Flag-CUG2 using Lipofectamine (Invitrogen, USA) and grown in the presence of 800 μg/ml G418 (Invitrogen). Transient transfection of 293T cells was performed using either Effectene™ (Qiagen, USA) or polyethylenimine reagent (PEI; Sigma-Aldrich, USA) according to the vendor's instructions. For small interfering RNA (siRNA) transfection, three unique siRNAs against CUG2 mRNA were purchased from Bioneer (Korea). Cells were treated with a mixture of three duplex siRNAs using Lipofectamine (Invitrogen) at a concentration of 100 nM.

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Plasmids

To generate bait plasmid for the yeast two-hybrid screening system, full-length CUG2 cDNA was cloned in-frame into the yeast pGBKT7 vector (Clontech, USA). The construction of plasmid for the enhanced green fluorescent protein (EGFP)-CUG2 fusion protein was described previously (Lee et al., 2007a). The full-length cDNA gene of CENP-T was isolated with polymerase chain reaction (PCR)-based amplification using a human stomach cancer library (Invitrogen). To obtain Flag- or Myc-tagged proteins, the coding region of CUG2 or CENP-T was inserted into pcDNA3-3Flag or pcDNA3-6Myc constructs (kindly provided by Dr. Changhoon Kim, Korea Basic Science Institute, Korea). For the glutathione-S-transferase (GST) fusion proteins, the open reading frame of CUG2 or CENP-A was ligated to pEBG (a gift from Dr. Jaerang Rho, Chungnam National University, Korea) after digestion with *Kpn*I and *Not*I.

Yeast two-hybrid screening

Screening was performed based on the Matchmaker™ two-hybrid System (Clontech) following the user's manual. For the bait transformation, the pGBKT7-CUG2 encoding CUG2 fused to the DNA-binding domain of GAL4 was transformed into strain AH109. Cells expressing bait protein were mated with strain Y187 pre-transformed with human fetal testis libraries. The selected clones were retested under a high stringency condition and the sequences of the positive clones were analyzed.

Immunoprecipitation and GST pulldown

After transient transfection, cells were lysed in a protein extraction buffer (50 mM Tris pH 7.5, 150 mm NaCl, 0.5% NP-40, 0.5% Triton-100) containing 1 mM phenylmethylsulphonyl fluoride. For immunoprecipation, the cell lysate was incubated with 1 μg of anti-Flag (Sigma-Aldrich), anti-GFP (Sigma-Aldrich), or mouse IgG (Santa Cruz Biotechnology, USA) followed by protein A agarose (Amersham, USA) treatment. After three washes, the bound proteins were eluted by boiling in sodium dodecyl sulfate (SDS) sample buffer and were then subjected to immunoblot analysis (Lee et al., 2007b).

For the GST pulldown, cell lysate containing GST-fusion protein was incubated with 20 μl of a 50% slurry of glutathion agarose beads (Peptron, Korea) for 2 h at 4°C with gentle rotation. After washing, 20 μl SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer was added and the mixture was heated 5 min at 100°C before 12% SDS-PAGE. Standard protocols for Western blots were performed after transferred to a polyvinylidene fluoride membrane (Pall, USA). Anti-GST and anti-Myc antibodies were purchased from Sigma-Aldrich and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology. Human anti-centromere antibody (ACA) was purchased from Cortex Biochem (USA). Anti-Flag, anti-GFP, anti-GST, and anti-Myc (all from Sigma-Aldrich) were used for the detection of the relevant target protein.

Immunofluorescence microscopy

To immunostain cells, cells grown on poly-L-lysine-treated cover slips were fixed with 4% paraformaldehyde for 15 min and incubated with 0.2% bovine serum albumin in phosphate buffered saline for 30 min. Cells were incubated with monoclonal anti-Flag for the detection of Flag-CUG2 in stable cells or anti-α-tubulin (Sigma-Adrich) for spindle microtubules. If needed, cells were co-incubated with ACA serum to mark centromeres. Cover slips were then treated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse antibody and Texas red-conjugated anti-human secondary antibody (Vector Laboratories, USA). After slides were mounted with Vectashield mounting medium (Vector Laboratories) containing 4′,6-diamidino-2-phenylindole (Sigma-Aldrich), imaging was done either using an Olympus IX70 fluorescence microscope (Olympus, Japan) or a LSM5-Pascal confocal imaging system (Zeiss, Germany).

RESULTS

Identification of the interaction between CUG2 and CENP-T

In an attempt to discover unidentified cellular functions of CUG2 and elucidate the basis of its proto-oncogenic activities, we used a yeast-two hybrid method to search specific CUG2-interacting partners. Screening was performed with full-length CUG2 as bait against a human testis cDNA library. Among the selected positive clones, three contained the cDNA sequence of the recently identified centromeric component, CENP-T.

To confirm the specific interaction between CUG2 and CENP-T, we conducted co-immunoprecipitation experiments in 293T cells. As the first attempt, we tested for an interaction between the two proteins using GFP-fused CUG2 and Flag-tagged CENP-T constructs. Immunoprecipitation with anti-GFP antibody co-recovered Flag-CENP-T and GFP-CUG2 protein (Fig. 1A). Reciprocally, the presence of GFP-CUG2 was detected in the complex with Flag-CENP-T (Fig. 1B) when immunoprecipation was performed with anti-Flag antibody.

Since GFP control protein was not clearly monitored due to the background signals by the antibodies used, we next adopted a GST pull-down assay to clarify the interaction between CUG2 and CENP-T. Flag-tagged version of CENP-T along with GST-CUG2 was generated and used for the GST pull-down experiment. As shown in Fig. 1C, Flag-CENP-T was clearly present in the GST-CUG2 associated complex, whereas no CENP-T protein was found in the complex with GST alone.

Association of CUG2 with CENP-A

CENP-A is a highly conserved centromeric histone H3 variant protein that is thought to be an essential structural and functional determinant of centromeric chromatin. CENP-T has been previously identified as a component of the CENP-A-associated multiprotein complex by two independent approaches: multiple tandem affinity purification (Foltz et al., 2006) and native chromatin immunoprecipitation with anti-CENP-A antibody (Izuta et al., 2006).

Since CUG2 is associated with CENP-T, and since CENP-T is localized proximally with CENP-A, we assessed whether CUG2 could interact with CENP-A. A GST pull-down assay revealed co-fractionation of CUG2 with GST-fused CENP-A (Fig. 2A). No band was detected in the GST control sample, indicative of a specific interaction of CUG2 with CENP-A. We then queried if the triple complex containing CUG2, CENP-T, and CENP-A could form in human cells. Cells were transfected with Myc-CUG2, Flag-CENP-T, and GST-CENP-A, and the lysate was used for co-immunoprecipitation with anti-Flag antibody along with normal mouse IgG control. CUG2 or CENP-A did not co-immunoprecipitated with normal mouse IgG, but these two proteins were clearly observed in the anti-Flag precipitate that contained Flag-CENP-T (Fig. 2B). Collectively, our results support the suggestion that CUG2, CENP-T, and CENP-A can form a functional complex.

Localization of CUG2 in human centromere

To verify the centromeric localization of CUG2 in cultured cells, we generated stable cells expressing Flag-CUG2 in various cell lines such as A549, HeLa, MCF-7, and Madin Darby canine kidney (MDCK) cells. The primary staining of these cells with

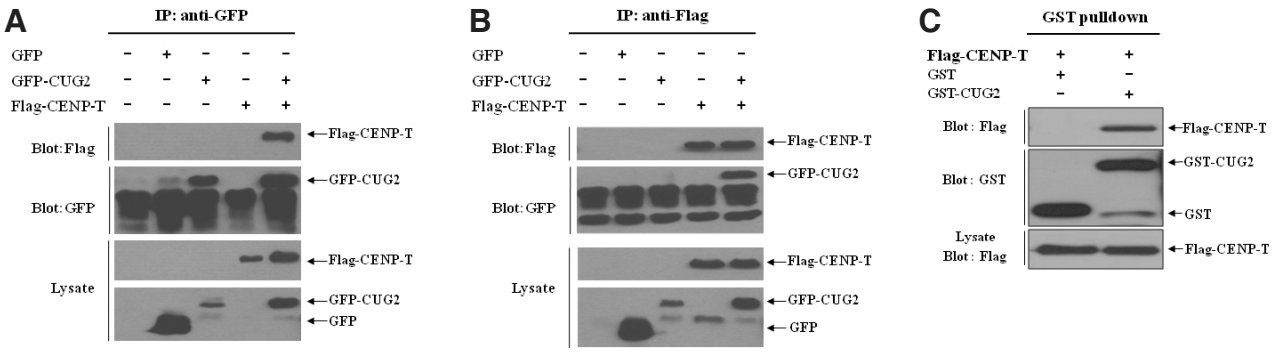

Fig. 1. Interaction between CUG2 and CENP-T. (A) Co-immunoprecipitation using anti-GFP antibody. 293T cells transfected with GFP-CUG2 and Flag-CENP-T were lysed and subjected to immunoprecipitation with anti-GFP antibody. Co-immunoprecipiated CENP-T was detected with anti-Flag antibody in a Western blot. (B) Co-immunoprecipitation with Flag-CENP-T using anti-Flag antibody. Reciprocal immunoprecipitation were conducted with the same lysates from (A) with anti-Flag antibody. The co-eluted products were identified with anti-GFP antibody. (C) Results of a GST pull-down experiment. The cell lysates of Flag-CENP-T co-expressed with either GST or GST-CUG2 were incubated with glutathion agarose beads. The presence of target proteins in the eluant was monitored with anti-Flag and anti-GST antibodies in a Western blot.

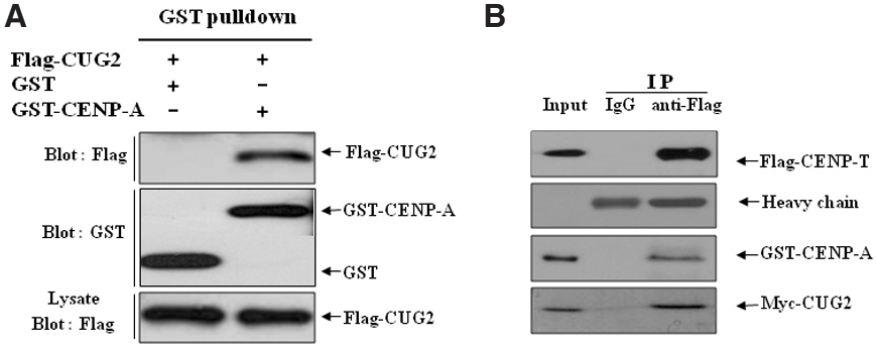

Fig. 2. Association of CUG2 with CENP-A. (A) Results of a GST pull-down assay using GST-CENP-A. Flag-tagged CUG2 was co-transfected into 293T cells with GST-CENP-A or GST control, and glutathione beads were used for trapping the GST-fusion proteins. Immunoblotting with anti-Flag antibody was conducted to detect the co-eluted Flag-CUG2. (B) Co-immunoprecipitation of CENP-A and CUG2 with CENP-T. After 293T cells were transfected with GST-CENP-A, Myc-CUG2 and Flag-CENP-T, cells were lysed and subjected to immunoprecipitation with either anti-Flag antibody or normal mouse IgG. The presence of CENP-A and CUG2 were detected by immunostaining with antibodies that recognized the conjugated tags.

anti-Flag antibody revealed the speckled appearing distribution of CUG2 corresponding to centromere pattern (Fig. 3A). A549 stable cells were then co-immunostained with ACA that recognizes human centromeric proteins including CENP-A and CENP-B, along with anti-Flag antibody. CUG2 clearly co-localized with centromeric marker proteins, consistent with localization of CUG2 in the centromere both in the interphase and during mitosis (Fig. 3B). We also tested if the distribution of CUG2 changed during the cell cycle using thymidine-blocked synchronized cells; most CUG2 proteins were retained in the centromeric region throughout the cell cycle (data not shown).

CUG2 siRNA induces aberrant cell division

To investigate the functional implication of CUG2 in cellular activities, we designed siRNAs for CUG2 knock-down in the cells. Flag-CUG2 HeLa stable cells were treated with a pooled mixture of three duplex siRNA oligonucleotides and Western analysis was carried out to test the efficiency of these siRNAs. Clearly reduced CUG2 bands were detected upon siRNA expression from the third day after transfection (Fig. 4A). During the process, massive death of HeLa cell populations upon transfection with CUG2 siRNA was apparent. The percentage of dead cells increased beginning on the day after transfection, reaching about 50% after four days (Fig. 4B), whereas no significant change was observed in the cells transfected with control siRNA (data not shown).

To understand the basis of CUG2 function related to the reduced cell survival, we immunostained CUG2 depleted HeLa cells and compared them with normal untreated cells. While normal dipolar cell division was visualized in most untreated cells, abnormal mitotic events such as multipolar spindle formation and misalignment of chromosome were frequently observed in CUG2 depleted cells. These aberrant mitosis may provoke cellular apoptotic apparatus, and might be the primary reason of the massive cell death.

DISCUSSION

Proper chromosome segregation into daughter cells during mitosis is critical to maintain the euploidy of cells and normal cell physiology (Kops et al., 2005). Aneuploidy resulting from errors in chromosome segregation is one of the common characteristics of tumors, and the onset of mitotic checkpoint from improper chromosome segregation is frequently compromised in human malignancies (Tomonaga and Nomura, 2007).

The kinetochore is a large complex of centromeric DNA and associated proteins, and is a chromosomal compartment where spindle microtubules attach during meiosis and mitosis. Therefore, it plays a fundamental role in accurate segregation of sister chromatids during cell division and acts as a monitor of the proper association between the centromere and spindles, signaling a halt to further cell division when errors are detected (Kops et al., 2005). Continuous identification of kinetochore proteins and subsequent cellular analysis also revealed that the

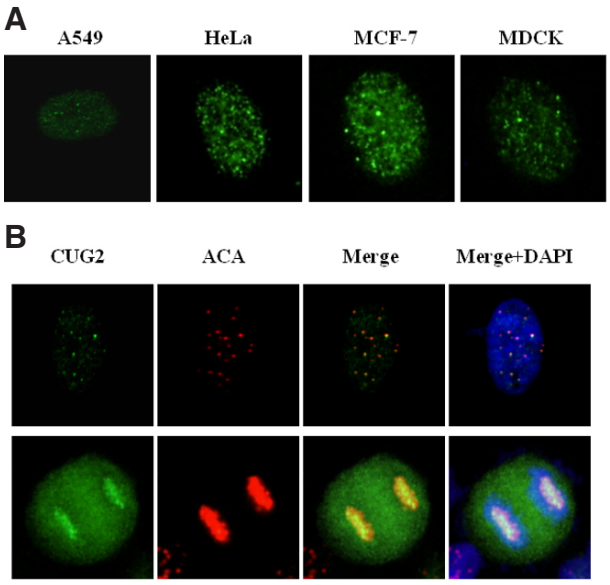

Fig. 3. Centromeric localization of CUG2 in various stable cells. (A) Distribution of Flag-CUG2 in various stable cells. A549, HeLa, MCF-7, and MDCK cells expressing Flag-CUG2 were immunostained with anti-Flag antibody. After secondary antibody treatment with FITC-conjugated anti-mouse antibody, cells were imaged by fluorescence or confocal microscopy. (B) Flag-CUG2 A549 stable cells were incubated with anti-Flag antibody in combination with ACA antiserum as a probe for centromere marker. Cells were then treated with FITC-conjugated anti-mouse and Texas red-conjugated anti-human secondary antibodies for the two-color fluorescence images.

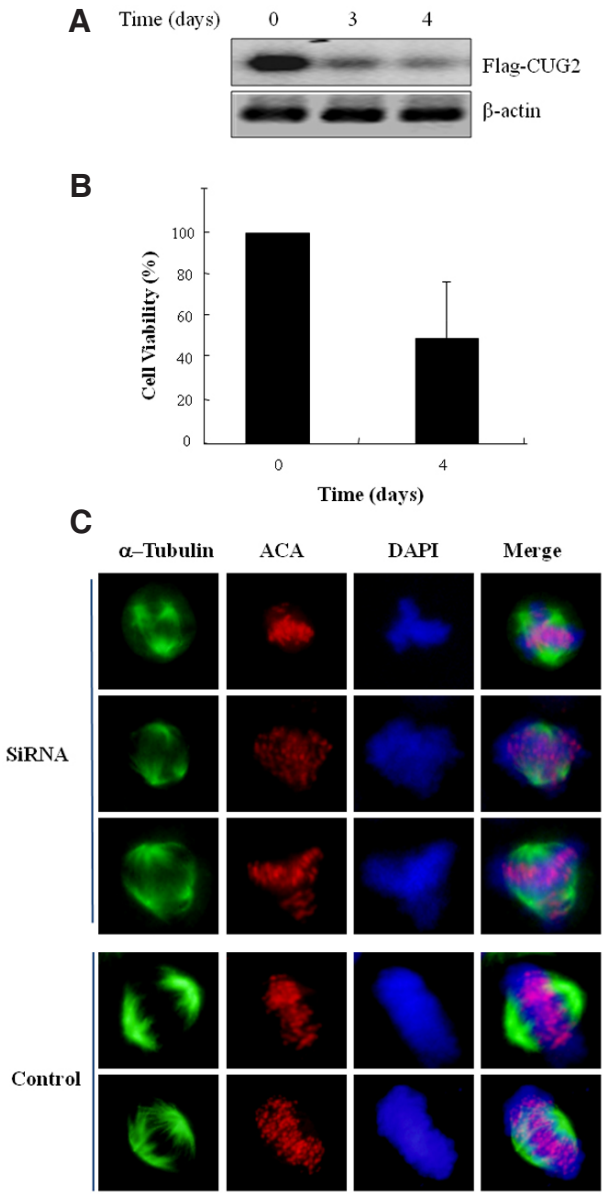

Fig. 4. CUG2 knockdown with siRNA. (A) Protein expression after siRNA treatment. After transfection of a pooled siRNA mixture to Flag-CUG2 expressing HeLa stable cells, Western blotting was performed with anti-Flag antibody. (B) Cell viability test. The percentage of dead cells was assayed using hemocytometer at 4 days after transfection with cells transfected with CUG2 siRNAs. The error bar in the figure represents ± S.D. (C) Aberrant cell division upon knockdown of CUG2. HeLa cells transfected with CUG2 siRNAs were immunostained to monitor the cellular mitosis event. Anti-α-tubulin and ACA serum were used for detection of the spindle microtubules and human centromere, respectively.

frequent mutations or disregulations of kinetochore proteins are often associated with aneuploidy and human cancers (Yuen et al., 2005). For example, CENP-A and CENP-H are amplified in human colorectal cancers, and CENP-F is amplified in head and neck squamous cell carcinomas and salivary gland tumors (Yuen et al., 2005).

We previously identified the gene encoding CUG2 using a genome-wide analysis of expression profiles of human cancer samples (Lee et al., 2007a). The gene is up-regulated in many human cancers originating from different tissues and is highly tumorigenic when expressed in mouse fibroblast cells (Lee et al., 2007a). Although localization analysis of EGFP-fused CUG2, based on the presence of nuclear localization signal sequence at the N-terminus of CUG2, revealed that CUG2 is a nuclear protein, the exact function of CUG2 in nucleus remained unknown.

In this report, we demonstrate that CUG2 localizes in the centromere, interacting with CENP-T and CENP-A, and that the depletion of this protein induces aberrant cell division with abnormal localization of spindle apparatus. These results are consistent with a critical role for CUG2 during mitotic cell division, possibly through mediation of the attachment of the chromosome and spindle microtubules. Considering the previously shown proto-oncogenic activities in NIH3T3 cells as well as its over-expression in various cancers (Lee et al., 2007a), it can be appropriately speculated that overexpression of CUG2 may promote cell division and continuous growth by incapacitating or bypassing the normal mitotic checkpoint.

During the preparation of this manuscript, a report was published describing co-immunoprecipitation of proteins with Flag-tagged CENP-T that implicated CUG2 in a centromere-associated network protein (Hori et al., 2008). The researchers generated a loss-of-function mutant of CUG2 using DT-40 chicken cells and showed that CUG2-depletion results in abnormal mitosis with a hypercondensed chromosome. On the basis of these results, the researchers renamed CUG2 as CENP-W. They further reported that CENP-T/CENP-W functions upstream of other components in the assembly of an

outer kinetochore complex. While this experimental approach was opposite to ours, the previous and present results are complimentary, reinforcing the validity of the findings.

The cellular expression of CUG2 is likely regulated at a very low level since we could barely observe the CUG2 mRNA signal in Northern blots using a commercial mRNA blot. Moreover, CUG2 is a small protein (approximately 10 kDa), which limited its detection using specific antibody presently and previously (Hori et al., 2008). Further studies to elucidate other, as yet-unknown, cellular functions of CUG2 could be highly dependent on the availability of specific CUG2 antibody sensitive enough to detect endogenous CUG2 proteins.

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